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The Impact of Process Parameters on 1,3-Propanediol Production and 3-Hydroxypropionaldehyde Accumulation in Fed-Batch Fermentation of Glycerol with *Citrobacter freundii* AD119

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Abstract: Microbial production of 1,3-propanediol (1,3-PD) has attracted the interest of scientists for decades. Its product offers an environmentally friendly and sustainable alternative to fossil-based raw materials for chemical synthesis. *Citrobacter freundii* is one of the natural producers of 1,3-PD known for its ability to yield it in significant titers. An efficient bioprocess requires an in-depth understanding of the factors that influence the performance of its biocatalyst. The effects of pH, temperature, stirring rate, and substrate concentration on glycerol fermentation in fed-batch cultures of *C. freundii* AD119 were investigated in this study. In addition to monitoring the kinetics of substrate utilization and the formation of the final products, the concentration of 3-hydroxypropionaldehyde (3-HPA), an inhibitory intermediate of glycerol bioconversion, was analyzed. When the optimal working conditions were used (pH 7.0, temperature 30 °C, stirring rate of 80 rpm, and glycerol concentration below 15 g/L during the fed-batch phase), 53.44 g/L of 1,3-PD were obtained. When the process was performed at temperatures of 33 °C or higher or in acidic pH (6.5), an elevated concentration of 3-HPA was observed and the process halted prematurely.

Keywords: 1,3-PD; pH-stat fed-batch fermentation; optimization of culture conditions; crude/waste glycerol; reuterin; glycerol feeding strategies

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1. Introduction

The valuable organic compound 1,3-propanediol (1,3-PD) has wide application in the production of polymers, solvents, adhesives, laminates, detergents, cosmetic and pharmaceutical products, coatings, antifreeze additives, and freshness-keeping agents [1–7]. The compound 1,3-PD can be obtained using either a chemical or a biotechnological process. The second has the advantages of requiring mild conditions and being environmentally friendly. In general, there are three types of fermentation processes: batch fermentation, fed-batch fermentation, and continuous fermentation. In batch fermentations, the total amount of the substrate is introduced to the medium at the beginning of the processes. The production of high titers of a product requires a high concentration of the substrate, which could inhibit the process. During continuous fermentation, fresh medium is continuously fed to the vessel at the same rate at which the fermentation liquid is withdrawn from it. In a fed-batch process, fresh medium or selected nutrients are introduced as the process proceeds. Thus, the fed-batch process allows obtaining a high concentration of the product and reduces the inhibition from the substrate. For this type of fermentation, maintenance of the proper substrate concentration is essential. In one of the approaches to this problem,

the pH-stat fed-batch culture, the addition of substrate is coupled to the delivery of a neutralizing agent used to maintain the pH of the broth.

Many species of bacteria have the ability to produce 1,3-PD, including those belonging to the genera of *Clostridium*, *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Lactobacillus* [8–12]. In 1,3-PD producers, the catabolism of glycerol consists of two coupled branches—one oxidative, the other reductive. In the oxidative branch, glycerol is transformed to dihydroxyacetone, which then undergoes glycolysis to form various end-products, such as lactic acid, acetic acid, ethanol, etc. In the reductive pathway, glycerol is converted to 3-hydroxypropionaldehyde (3-HPA), which is then reduced to 1,3-PD. The reductive pathway maintains the redox balance of the cell, as it uses $NADH_2$ and regenerates NAD^+ [4,13,14].

It is worth emphasizing that the intermediate of the reductive branch, 3-HPA, belongs to a multi-component antimicrobial system called reuterin. It is a dynamic system which mainly consists of 3-HPA, its dimer, hydrate, and acrolein. The health-promoting properties of reuterin have been demonstrated, which include anti-infective, anti-inflammatory, and anti-carcinogenic activities. Moreover, the strong antimicrobial activity of this system makes it suitable for preserving various types of food [15]. However, the accumulation of 3-HPA during the microbial production of 1,3-PD leads to negative consequences which include cell growth limitation and premature cessation of the process. There are numerous reports on this phenomenon [1,3,16–19]. It is known that 3-HPA induces oxidative stress in the cells, and its dimer inhibits the synthesis of DNA [18,20]. Barbirato et al. [17] noticed differences in the accumulation of 3-HPA in various microorganisms and its dependence on the concentration of glycerol. Premature cessation of fermentation was observed at elevated glycerol concentrations for *Enterobacter agglomerans*, *Klebsiella pneumoniae*, and *Citrobacter freundii* strains. No accumulation of 3-HPA, however, was found to occur in cultures of *Clostridium butyricum* [1].

The stirring rate and pH of the medium can also influence the kinetics of 1,3-PD fermentation. Zheng et al. [19] noticed that by using a higher stirring rate, a higher 1,3-PD productivity and a decreased 3-HPA concentration can be achieved in *K. pneumoniae* cultures. According to Barbirato et al. [1], when a pH value of the culture medium of 8.0 was used, the production of 3-HPA in *E. agglomerans* did not affect fermentation kinetics, as it was low and transient.

There is scarce information, however, on the impact of culture conditions on 3-HPA and 1,3-PD production with *C. freundii*. For this reason, optimization of selected fermentation parameters such as temperature, stirring rate, and pH with respect to 1,3-PD production and 3-HPA accumulation with *C. freundii* AD119 was conducted in this study. Additionally, two feeding strategies were examined in a pH-stat fed-batch culture of this microorganism performed under optimized fermentation conditions.

2. Materials and Methods

2.1. Bacterial Strain

The strain used in this study—*C. freundii* AD119 (Polish Collection of Microorganisms-PCM, accession number B/00044)—was isolated during previous screening experiments [21].

2.2. Medium

The cultures were performed in a previously optimized medium [22] consisting of biodiesel-derived waste glycerol (Archer Daniels Midland Company-AMD, Malbork, Poland) 40 g/L (calculated as pure glycerol), yeast extract 2 g/L, $(NH_4)_2SO_4$ 1.1 g/L, $MgSO_4 \cdot 7H_2O$ 0.58 g/L, $CoCl_2$ $6H_2O$ 13.55 mg/L in water. The medium used for the propagation of inoculum had the same composition, except being prepared with phosphate buffer instead of water.

2.3. Fed-Batch Fermentation

The experiments were run in a 5 L Biostat B plus fermentor (Sartorius, Göttingen, Germany). The medium volume was 1 L; no gas sparging was used. Inoculum was cultured

for 20 h prior to the start of the process, and its volume was 10% of the medium volume. The optimization studies were performed in a one-factor-at-a-time fashion, and the initial values of the tested parameters were as follows: pH value = 7.0; temperature = 30 °C, stirring rate = 80 rpm. Furthermore, the investigated factors and their ranges (in parentheses) were as follows: temperature (28–37 °C), pH (6.5–8.0), and stirring rate (40–160 rpm).

During the fermentation, glycerol was delivered continuously with the supply rate controlled by the pH regulation system (pH-stat fed-batch approach). A single solution composed of 20% KOH and 41% glycerol in water was used for the purposes of both controlling the pH of the fermentation liquid and feeding the microorganisms with the carbon source. This synchronized the rate of substrate delivery with fermentation kinetics.

2.4. Analytical Methods

The concentrations of glycerol and the metabolites (1,3-PD, lactic acid, acetic acid, succinic acid, and ethanol) in culture broth were determined with HPLC as described previously [22].

The concentration of 3-HPA was determined by a colorimetric method using acrolein as the standard [23,24]. Briefly, 30 µL of the sample was mixed with 150 µL of concentrated HCl and 50 µL of DL-tryptophan solution (DL-tryptophan 0.505% (*w*/*v*); 0.417% HCl (*v*/*v*); 0.25% toluene (*v*/*v*)). The reaction mixture was incubated for 20 min at 40 °C. After the incubation, the absorbance at 560 nm was measured with TECAN infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.5. Statistical Analysis

The results of each triplicate analysis of examined compounds and biomass of used strain during experiments performed in duplicate are expressed as mean ± standard deviation (SD). The effect of fermentation parameters, i.e., temperature, pH and stirring rate, on the final levels of examined metabolites (1,3-PD, lactic acid, acetic acid, succinic acid, and ethanol) and dry cell weight (DCW) of *C. freundii* AD119 was determined by the one-way analysis of variance (ANOVA) exploiting TIBCO Statistica data analysis software, version 13.3.0 (TIBCO Software Inc., Palo Alto, CA, USA). During the analysis, the F test and the post hoc Tukey's HSD test were used to evaluate the impact of individual factors and the significance of differences between the average concentrations of metabolites and biomass obtained under different conditions of the conducted bioprocess. A critical significance level of $\alpha = 0.05$ was adopted throughout the analysis.

3. Results and Discussion

The effect of fermentation parameters on glycerol bioconversion in fed-batch cultures of *C. freundii* AD119 was investigated. The strain and the medium were described in previous reports [21,22]. The strain was selected from a pool of 3800 isolates, which included microorganisms belonging to the genera *Klebsiella*, *Citrobacter*, *Hafnia* [21]. As no improvement was observed when glycerol was supplemented with co-substrates [22], the medium used in this study contained glycerol as the sole carbon source. The goal of this study was to establish optimal conditions for the process of 1,3-PD production from crude glycerin obtained from an industrial biodiesel production site. At the same time, the accumulation of 3-HPA, a toxic metabolic intermediate, was monitored, and the impact of its concentration on the performance of the fermentation was studied.

The choice of an industrial by-product as the carbon source was not accidental. Such a substrate contains various impurities which introduce the risk of a negative impact on the microorganisms used for fermentation, such as methanol, mineral salts, heavy metals, mono- and diglycerides of fatty acids, free fatty acids, soaps [25]. Nonetheless, it is considered an attractive substrate for bioconversion, and, as such, it was used in this study. Reports exist which indicate that the substitution of pure glycerol with crude glycerin leads to decreased yields of biomass and 1,3-PD and affects the profile of metabolites [26–28]. On the other hands, Moon et al. [29] reported improved bioconversion of glycerol to 1,3-PD

when crude glycerin was used instead of the pure substance. In the same paper, it was also shown that bacteria belonging to the genus *Klebsiella* show greater resistance to the impurities of the industrial by-product than *Clostridium* spp. Nevertheless, our previous findings indicate no interferences from the chosen substrate on glycerol bioconversion to 1,3-PD in the case of the strain used here [22]. Moreover, as no improvement was observed when glycerol was supplemented with co-substrates, the medium used in this study contained glycerol as the sole carbon source.

3.1. Impact of Temperature

To investigate the effect of temperature on the fermentation, fed-batch fermentations were performed at temperatures ranging from 27 to 36 °C. The changes in dry cell weight (DCW) and metabolite levels (1,3-PD, lactic acid, acetic acid, succinic acid, ethanol) produced as the result of glycerol bioconversion were depicted in Figure 1. The conducted experiments revealed that the temperature of the process affects the dynamics of *C. freundii* AD119 growth and the accumulation of metabolites. Table 1 presents the final concentrations of the produced metabolites and DCW and the maximum concentration of 3-HPA observed in the process.

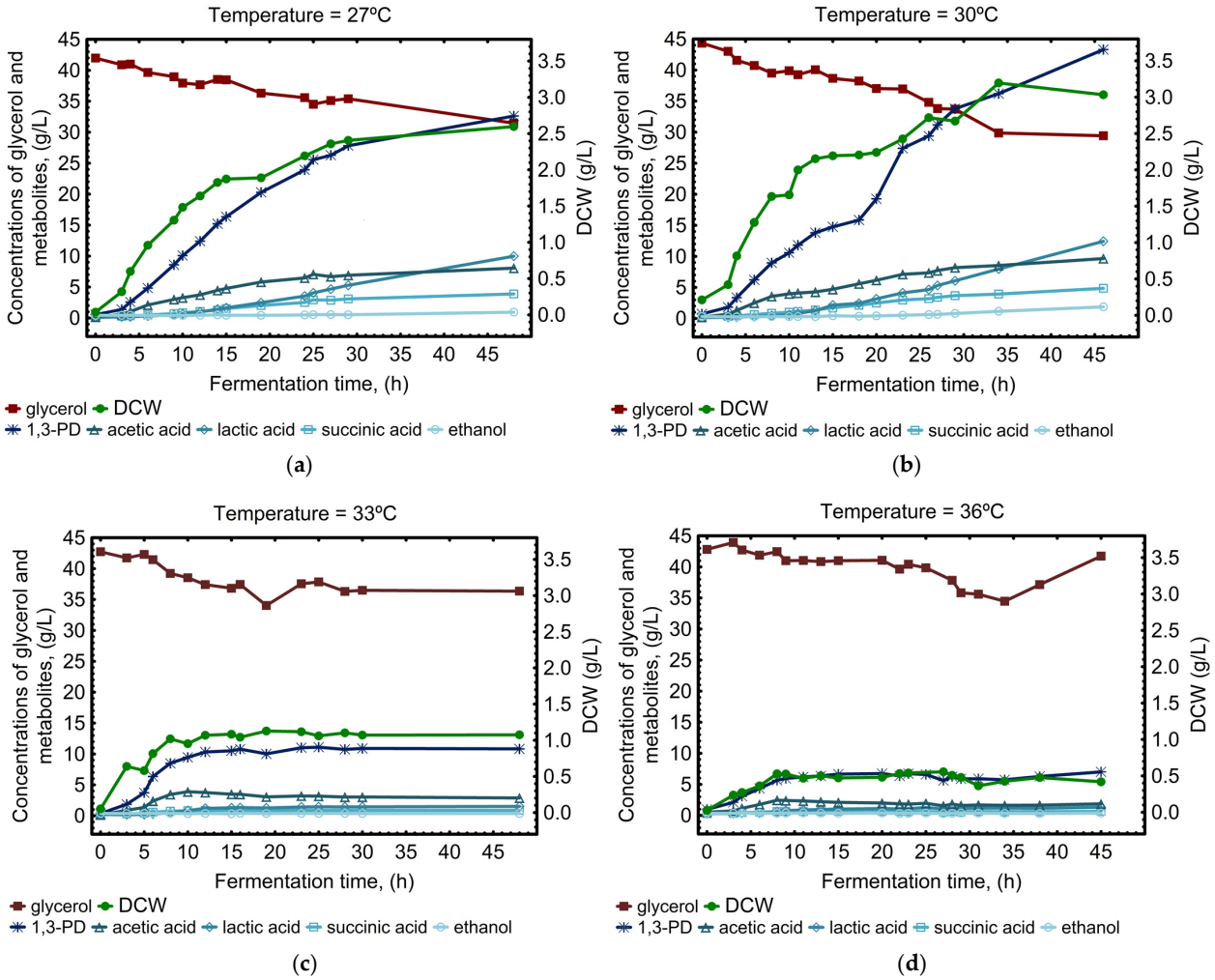


Figure 1. Time course of dry cell weight (DCW) and metabolite production (1,3-propanediol (1,3-PD), lactic acid, acetic acid, succinic acid, ethanol) with *C. freundii* AD119 in a fed-batch culture with a fixed pH value of 7.0 and stirring rate of 80 rpm at (**a**) 27 °C, (**b**) 30 °C, (**c**) 33 °C, and (**d**) 36 °C.

Table 1. Final concentrations of metabolites (1,3-propanediol (1,3-PD), lactic acid, acetic acid, succinic acid, ethanol) and dry cell weight (DCW) in a fed-batch culture of *C. freundii* AD119 at various temperatures.

Metabolites	Temperature (°C)			
	27	30	33	36
1,3-PD (g/L)	32.62 ± 0.17 [a]	43.30 ± 2.07 [b]	10.83 ± 0.44 [c]	7.00 ± 0.20 [c]
Lactic acid (g/L)	9.97 ± 1.02 [a]	10.82 ± 0.05 [a]	1.52 ± 0.04 [b]	1.33 ± 0.14 [b]
Acetic acid (g/L)	8.04 ± 0.59 [a]	9.65 ± 0.04 [b]	2.89 ± 0.08 [c]	1.85 ± 0.05 [c]
Succinic acid (g/L)	3.88 ± 0.18 [a]	4.85 ± 0.04 [b]	0.88 ± 0.01 [c]	0.66 ± 0.02 [c]
Ethanol (g/L)	0.94 ± 0.08 [a]	1.86 ± 0.09 [b]	0.35 ± 0.12 [c]	0.36 ± 0.01 [c]
DCW (g/L)	2.60 ± 0.34 [a]	3.03 ± 0.14 [a]	1.08 ± 0.30 [b]	0.42 ± 0.18 [b]
3-HPA (mmol/g DCW) *	3.68 ± 0.99 [a]	4.12 ± 0.34 [a]	8.81 ± 1.49 [b]	13.40 ± 0.12 [c]

* maximum concentration of 3-HPA achieved Means ± SD with different superscript letters in the same row differ significantly ($p < 0.05$).

The best results were obtained at 30 °C, in which case 43.30 g/L of 1,3-PD were produced. The highest concentrations of the accompanying metabolites were also observed at this temperature. Lactic (10.82 g/L) and acetic (9.65 g/L) acids were the dominating by-products. Succinic acid and ethanol were also present in the fermentation broth at the concentrations of 4.85 g/L and 1.86 g/L, respectively. Throughout the process, glycerol concentration was maintained in the range of 30–45 g/L.

Interestingly, in the experiments performed at temperatures surpassing 30 °C, cessation of product formation and cell growth was observed. At these temperatures, the final concentrations of 1,3-PD were around four times lower than in the process conducted at 30 °C. The titers of the accompanying fermentation products were lower when compared to those observed in the process performed at 30 °C, and the fed-batch mode of fermentation prevented depletion of glycerol. Thus, the inhibition could neither be attributed to an excessive concentration of fermentation by-products nor to starvation. Available data indicate that the accumulation of 3-HPA, an intermediate of the conversion pathway of glycerol to 1,3-PD, leads to such a result [2,17]. Moreover, in an experiment in which exogenous 3-HPA was added to a culture of actively growing bacteria, immediate growth cessation was observed [17]. In a report by Celińska et al. [30], a genetically modified strain of *C. freundii*, which overexpressed 1,3-PD oxidoreductase, showed superior capability for 1,3-PD production (35.6 g/L, vs. 25.5 g/L of the wild strain) accompanied by a decreased accumulation of 3-HPA. The overexpressed enzyme is responsible for catalyzing the final step of the pathway—transformation of 3-HPA to 1,3-PD. The concentration of 3-HPA was thus determined (Figure 2). In all the cultures in which cessation of fermentation was observed, the maximal concentration of this intermediate was found to be at least two-fold increased, compared to the process performed at 30 °C.

In the experiments performed at temperatures higher than 30 °C, the concentration of 3-HPA reached its maximum between 8 and 13 h of fermentation, at which time also the premature cessation of the processes was observed. Although the accumulation of 3-HPA in the medium was transient, the fermentation has not been observed to resume after the titer of the intermediate had decreased. It is noteworthy that when the strain was cultured in a medium with the glycerol having been replaced with glucose, no inhibition of the fermentation was observed even at a temperature of 37 °C (Figure 3). As expected, no 1,3-PD production was observed in this case as glycerol is the sole substrate that can be converted to the diol in microorganisms not subjected to genetic manipulation [4].

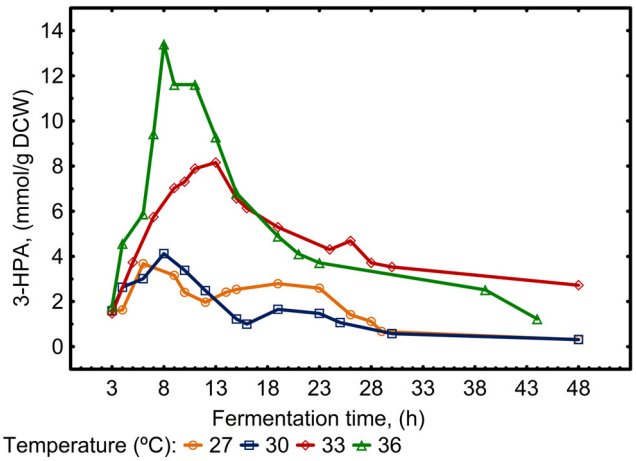
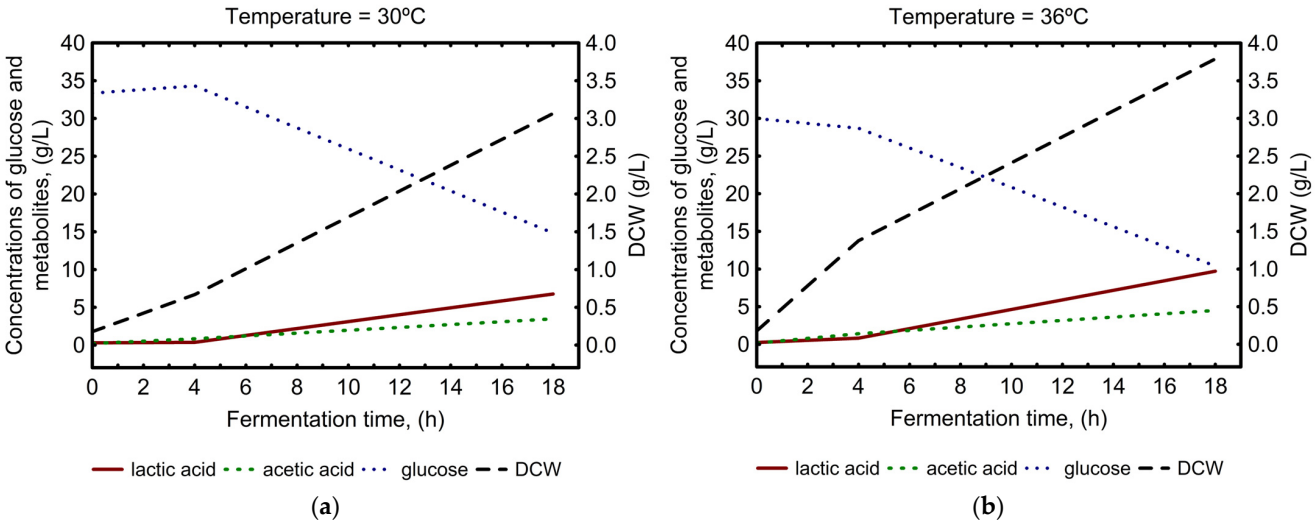


Figure 2. Production of 3-hydroxypropionaldehyde (3-HPA) with *C. freundii* AD119 in fed-batch cultures with a fixed pH value of 7.0 and stirring rate of 80 rpm at various process temperatures.



(**a**) (**b**)

Figure 3. Changes in concentrations of glucose, metabolites (lactic acid, acetic acid), and dry cell weight (DCW) of *C. freundii* AD119 in a fed-batch culture vs. time at (**a**) 30 and (**b**) 36 °C.

Cessation of the processes related to 3-HPA accumulation was studied in *E. agglomerans*, *K. pneumoniae*, and *C. freundii* [17]. Nonetheless, to the best of our knowledge, the influence of process temperature on this phenomenon in *C. freundii* has not been examined before. According to previous reports, the optimum temperature for 1,3-PD formation in *C. freundii* is 37 °C [31–33]. Our findings for *C. freundii* AD119 are strongly contrasting and indicate that strains of *C. freundii* require a cautious approach to finding the optimal process temperature. The temperature of 30 °C was selected for the subsequent stages of our research.

3.2. Impact of pH

The influence of pH (6.5, 7.0, 7.5, 8.0) on the fermentation performance was studied. The kinetics of glycerol utilization and the formation of products are depicted in Figure 4.

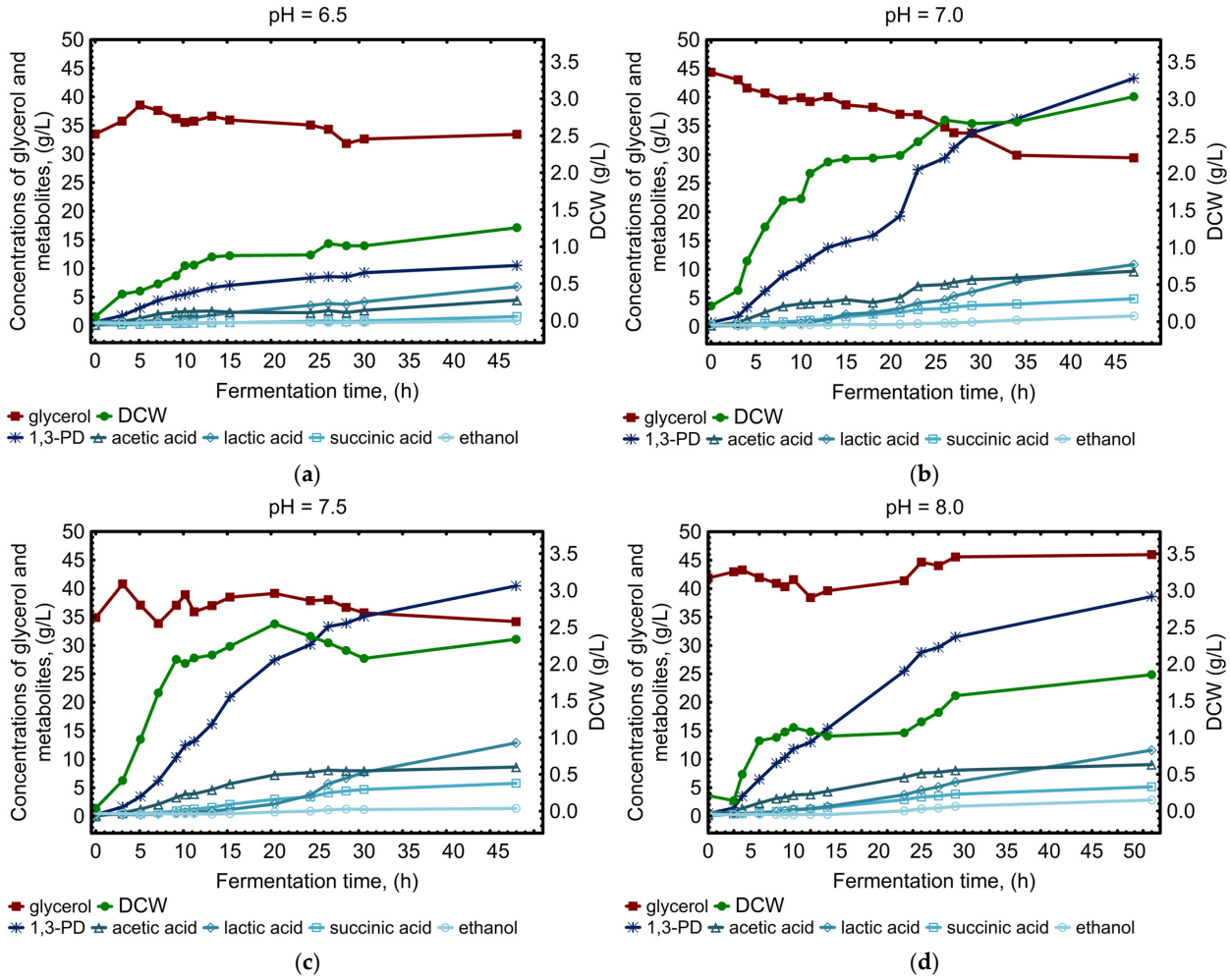


Figure 4. Time course of dry cell weight (DCW) and metabolite production (1,3-propanediol (1,3-PD), lactic acid, acetic acid, succinic acid, ethanol) with *C. freundii* AD119 in a fed-batch culture with a fixed temperature of 30 °C and stirring rate of 80 rpm at pH (**a**) 6.5, (**b**) 7.0, (**c**) 7.5, and (**d**) 8.0.

The maximal concentration of 1,3-PD of about 40 g/L was reached in 48 h in fermentations performed at a pH of 7.0–8.0, which was significantly higher in comparison to the process conducted at a pH of 6.5 (Table 2).

Table 2. Final concentrations of metabolites (1,3-propanediol (1,3-PD), lactic acid, acetic acid, succinic acid, ethanol) and dry cell weight (DCW) in fed-batch cultures of *C. freundii* AD119 at various pH values.

Metabolites	pH Value			
	6.5	7.0	7.5	8.0
1,3-PD (g/L)	10.50 ± 0.04^{a}	43.30 ± 2.07^{b}	40.46 ± 2.02^{b}	38.61 ± 2.10^{b}
Lactic acid (g/L)	6.79 ± 0.05^{a}	10.82 ± 0.05^{ab}	12.87 ± 0.27^{b}	11.58 ± 1.43^{b}
Acetic acid (g/L)	4.45 ± 0.02^{a}	9.65 ± 0.04^{b}	8.62 ± 0.45^{b}	9.05 ± 0.07^{b}
Succinic acid (g/L)	1.59 ± 0.01^{a}	4.85 ± 0.04^{b}	5.77 ± 0.13^{b}	5.15 ± 0.26^{b}
Ethanol (g/L)	0.84 ± 0.01^{a}	1.86 ± 0.09^{b}	1.35 ± 0.27^{ab}	2.82 ± 0.02^{c}
DCW (g/L)	1.26 ± 0.01^{a}	3.03 ± 0.14^{b}	2.34 ± 0.02^{c}	1.85 ± 0.19^{ac}
3-HPA (mmol/g DCW) *	22.00 ± 2.55^{a}	4.12 ± 0.34^{b}	5.00 ± 0.23^{b}	4.39 ± 0.31^{b}

* maximum concentration of 3-HPA achieved; Means ±SD with different superscript letters in the same row differ significantly ($p < 0.05$).

Batches were also performed at these pH values for 250 h which revealed that the pH of 7.0 was the optimum as it allowed obtaining the highest concentration of 1,3-PD (Figure 5).

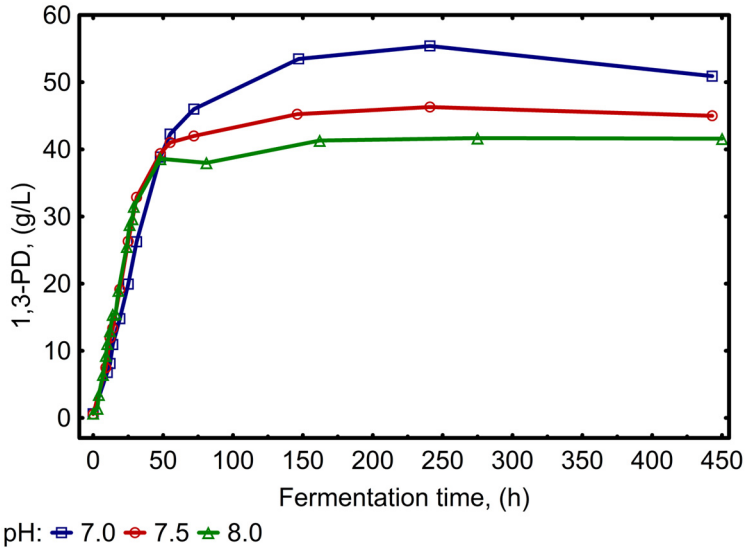


Figure 5. Effect of pH values on 1,3-propanediol (1,3-PD) production with *C. freundii* AD119 in 250 h batches.

The concentration of 1,3-PD obtained when the culture was performed at pH 6.5 was over three-fold lower. Moreover, the concentrations of by-products and biomass obtained at this pH level were also decreased. A premature reduction of the 1,3-PD production rate was observed; thus, the concentration of 3-HPA was determined (Figure 6).

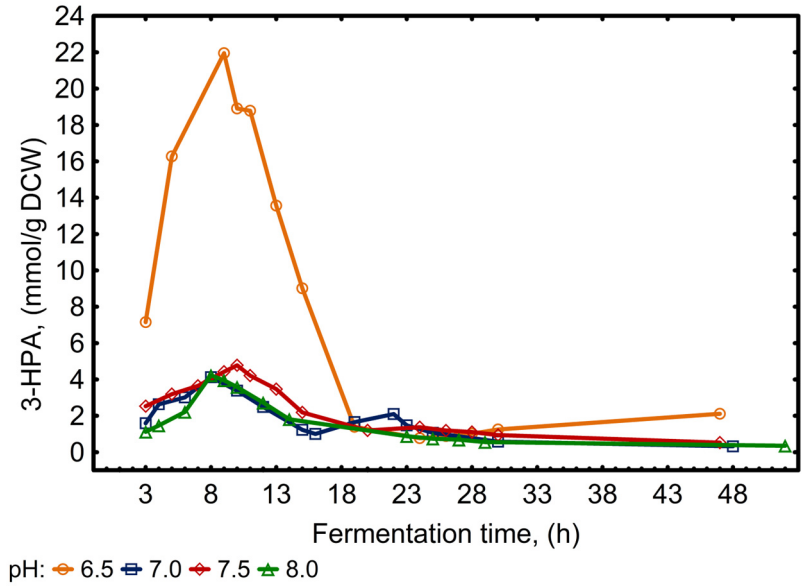


Figure 6. Production of 3-hydroxypropionaldehyde (3-HPA) with *C. freundii* AD119 in fed-batch cultures with a fixed temperature of 30 °C and stirring rate of 80 rpm at various pH values.

The accumulation of 3-HPA was observed at each pH value. Its concentration was below 5 mmol/g DCW in all the cultures except the one conducted at pH 6.5. In that case, the maximum 3-HPA concentration was reached after 9 h of fermentation and was above 20 mmol/g DCW. The coincidence of the peak in 3-HPA concentration and process cessation leads one to the assumption that the accumulation of the intermediate was

the probable reason behind the premature end of fermentation. It has been proven by Barbirato et al. [1,17] that low pH during fermentation results in 3-HPA accumulation and decreased 1,3-PD production. Kongjan [34] reported enhanced 1,3-PD production at pH 8.0 and attributed this observation to the expression level of enzymes, especially glycerol dehydratase and 1,3-PD oxidoreductase. Hongwen et al. [35] determined that at pH 7.0 the activities of glycerol dehydrogenase, 1,3-PD oxidoreductase, and glycerol dehydratase were at their maximal levels in *K. pneumoniae*. Kongjan et al. [34] reported that the optimum pH for 1,3-PD production with *Enterobacter* sp. is 8.0, while the reports for *K. pneumoniae* indicate that the optimal value is between 6.0 and 7.5 [36–39].

Not all fermentation processes are performed with a constant pH setpoint. Ji et al. [37] developed a fermentation strategy which included a forced fluctuation of pH between 6.3 and 7.3. This resulted in reduced amounts of by-products produced and an increased 1,3-PD production [37]. A similar approach was also employed by Petrov and Stoyanov [5] who reported a 10% increase of the maximal concentration of 1,3-PD obtained. Fermentation with a pH shift was thus planned by us. The initial pH setpoint was 7.5, as the production rate of 1,3-PD at this pH was higher than at 7.0 (Figure 5). After 24 h of the process, the pH was set to 7.0. As no significant changes in final 1,3-PD concentration were observed (Figure 7), a constant pH value of 7.0 was selected for use in further research.

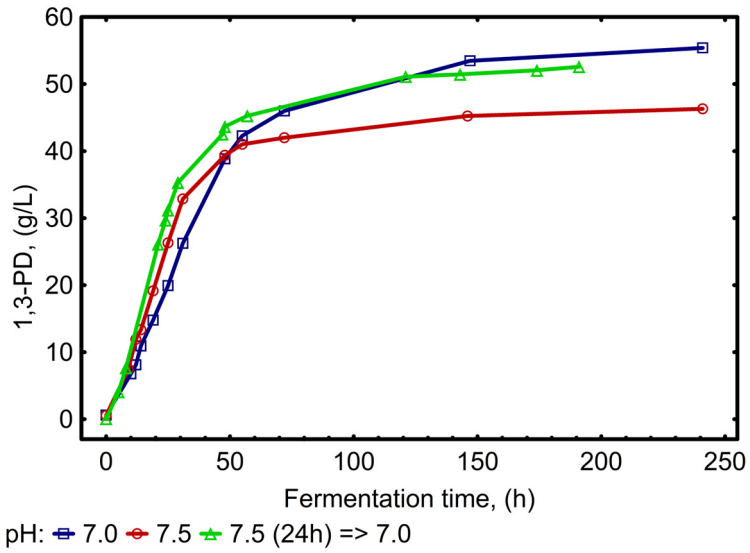


Figure 7. Comparative kinetics of 1,3-propanediol (1,3-PD) production with *C. freundii* AD119 in fed-batch cultures at a maintained pH = 7.0 or pH = 7.5 and in a process with an initial pH value of 7.5 then shifted to 7.0 after 24 h.

3.3. Impact of Stirring Rate on Products Formation

The stirring rate was reported to influence the production of 1,3-PD in *K. pneumoniae* and *Shimwella blattae* [19,33,35,38]. To optimize this parameter for *C. freundii* AD119, four different stirring values were tested, from 40–160 rpm. The kinetics of the fed-batch fermentations performed using these conditions are presented in Figure 8.

There were no significant differences in the final concentration of 1,3-PD, lactic acid, succinic acid, and ethanol obtained at stirring rate values ranging from 40 to 160 rpm (Table 3).

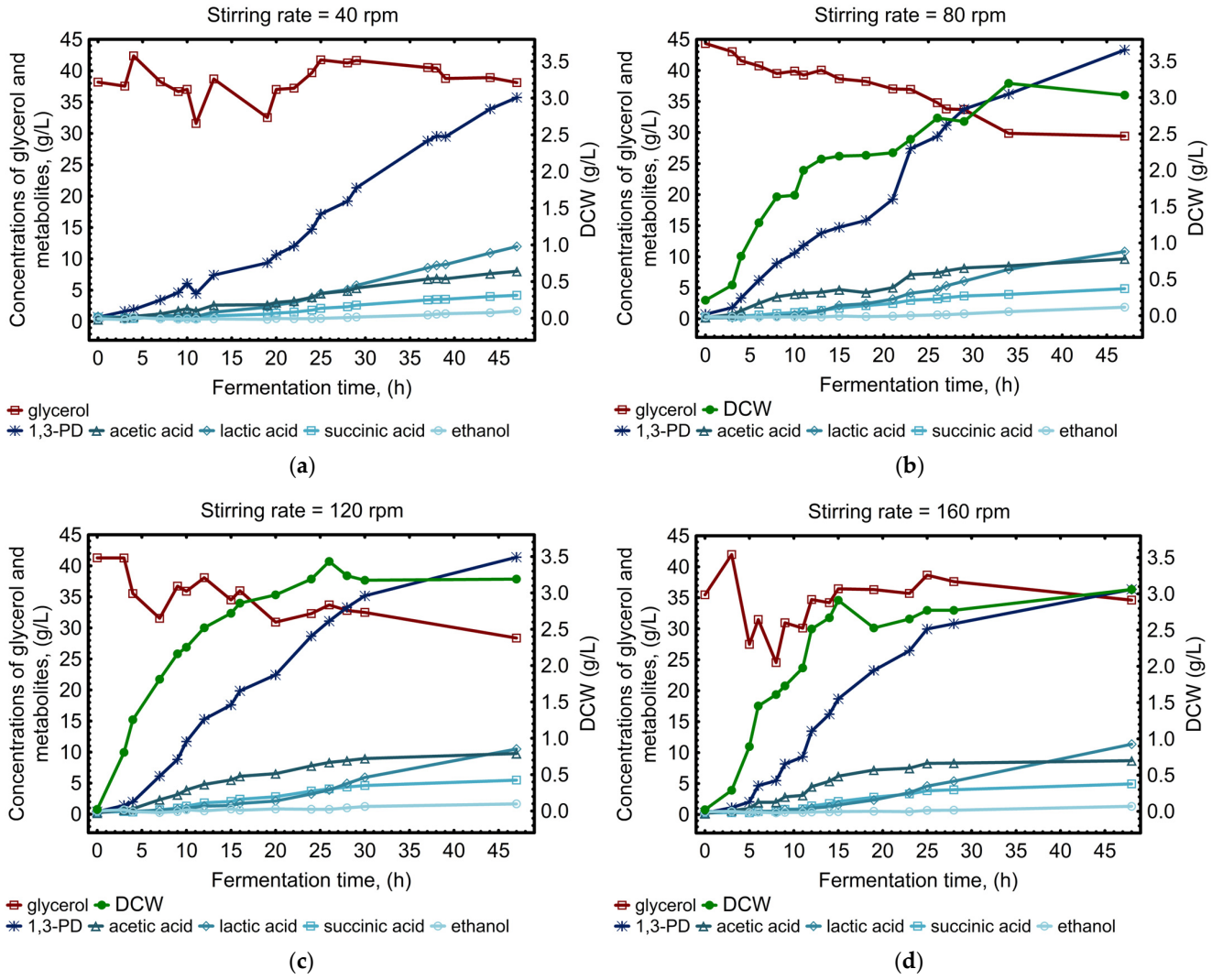


Figure 8. Time course of dry cell weight (DCW) and metabolite production (1,3-propanediol (1,3-PD), acetic acid, lactic acid, succinic acid, ethanol) with *C. freundii* AD119 in a fed-batch culture with a fixed temperature of 30 °C and pH value of 7.0 at a stirring rate of (**a**) 40 rpm, (**b**) 80 rpm, (**c**) 120 rpm, and (**d**) 160 rpm.

Although no premature cessation of the fermentations was observed, the concentration of 3-HPA was analyzed. It did not exceed 4.5 mmol/g of bacterial cell dry mass in any of the batches. However, the higher the stirring rate was, the lower the observed concentration of 3-HPA (Figure 9). The reported optimal values of the stirring rate range between 110 and 350 rpm [19,33,38,40]. These depend on the strain and the type of bioreactor used. Excessively vigorous stirring has been found to decrease 1,3-PD production, probably due to over-aeration of the medium [40], or to have a negative effect on bacterial growth [33]. Excessively low stirring rate, on the other hand, led to the accumulation of 3-HPA [16,38]. The value of 80 rpm was selected as the optimal stirring rate, as it prevents foaming and additional, unjustified power demand.

Table 3. Final concentrations of metabolites (1,3-propanediol (1,3-PD), lactic acid, acetic acid, succinic acid, ethanol) and dry cell weight (DCW) in fed-batch cultures of *C. freundii* AD119 at various stirring rate values.

Metabolites	Stirring Rate (rpm)			
	40	80	120	160
1,3-PD (g/L)	35.72 ± 1.49 [a]	43.30 ± 2.07 [a]	41.39 ± 0.15 [a]	36.35 ± 1.83 [a]
Lactic acid (g/L)	11.96 ± 1.49 [a]	10.82 ± 0.05 [a]	10.49 ± 0.16 [a]	11.35 ± 0.11 [a]
Acetic acid (g/L)	8.01 ± 0.18 [a]	9.65 ± 0.04 [b]	9.76 ± 0.16 [b]	8.69 ± 0.39 [ab]
Succinic acid (g/L)	4.18 ± 0.42 [a]	4.85 ± 0.04 [a]	5.47 ± 0.15 [a]	4.95 ± 0.30 [a]
Ethanol (g/L)	1.70 ± 0.58 [a]	1.86 ± 0.09 [a]	1.66 ± 0.30 [a]	1.32 ± 0.03 [a]
DCW (g/L)	ND	3.03 ± 0.14 [a]	3.19 ± 0.03 [a]	3.06 ± 0.07 [a]
3-HPA (mmol/g DCW) *	ND	4.12 ± 0.34 [a]	3.37 ± 0.49 [a]	2.94 ± 0.59 [a]

* maximum concentration of 3-HPA achieved; Means ± SD with different superscript letters in the same row differ significantly ($p < 0.05$).

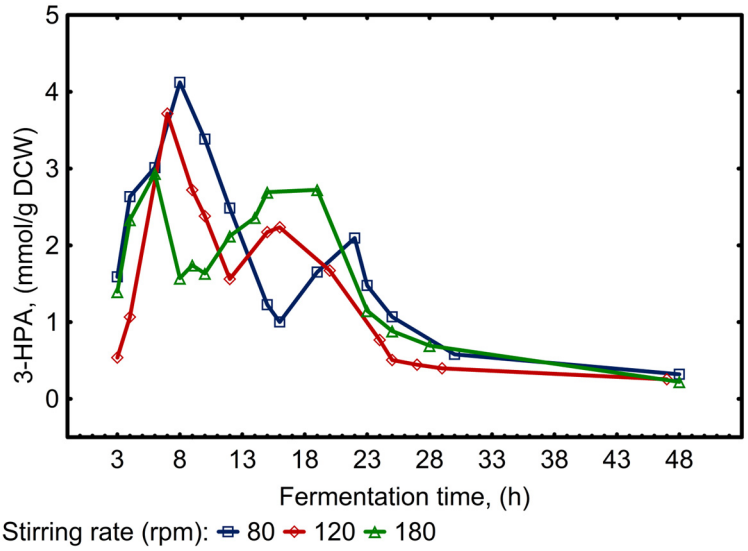


Figure 9. Production of 3-hydroxypropionaldehyde (3-HPA) with *C. freundii* AD119 in fed-batch cultures with a fixed temperature of 30 °C and pH value of 7.0 at various stirring rate values.

3.4. Impact of Feeding Strategy

Considering the importance of the glycerol concentration on biomass growth and 1,3-PD synthesis, two different feeding strategies were tested. As in all the previous experiments, the pH was maintained using an automatic addition of glycerol–potassium hydroxide mixture. An initial glycerol concentration of 40 g/L was used in both strategies. The difference between them lied in the glycerol concentration in the fermenting medium. The first strategy assumed maintaining a relatively high glycerol concentration (approximately 25 g/L) throughout the process. In the second strategy, the concentration of glycerol was allowed to decrease to a significantly lower value of less than 15 g/L. The kinetics of both processes are presented in Figure 10, and a summary of the most important process parameters is given in Table 4.

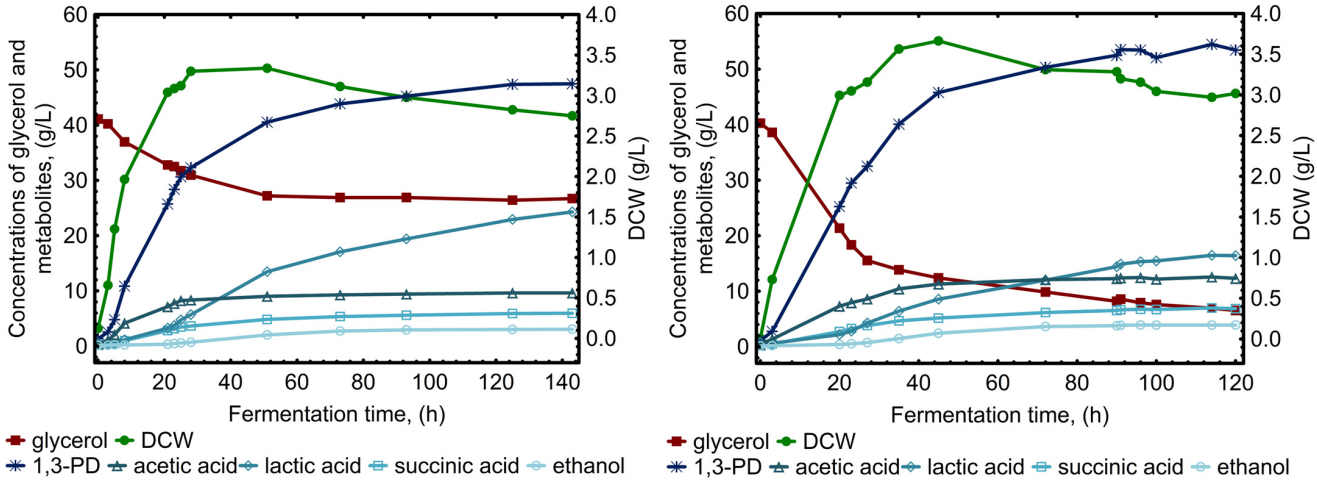


Figure 10. Time course of dry cell weight (DCW) and metabolite production (1,3-propanediol (1,3-PD), acetic acid, lactic acid, succinic acid, ethanol) with *C. freundii* AD119 in a fed-batch culture using different feeding strategies: (**a**) maintaining a relatively high glycerol concentration and (**b**) maintaining a relatively low glycerol concentration after 27 h of the process.

Table 4. Comparison of glycerol fermentation with *C. freundii* AD119 using different feeding strategies.

Strategy *	**I**	**II**
Time (h)	144	120
1,3-PD (g/L)	47.48 ± 0.76	53.44 ± 1.19
Glycerol utilization (%)	82.05 ± 0.78	95.15 ± 1.15
Yield (%)	38.91 ± 1.30	40.96 ± 1.82
Volumetric productivity (g/(L·h)	0.33 ± 0.01	0.44 ± 0.01
Lactic acid (g/L)	24.29 ± 2.54	16.41 ± 2.37
Acetic acid (g/L)	9.62 ± 0.40	12.32 ± 0.45
Succinic acid (g/L)	5.95 ± 0.16	6.87 ± 0.13
Ethanol (g/L)	3.06 ± 0.49	3.92 ± 0.06
Biomass (g/L)	2.82 ± 0.21	3.02 ± 0.30

* Strategy I: maintaining a relatively high glycerol concentration. Strategy II: maintaining a relatively low glycerol concentration after 27 h of the process.

With the use of the first feeding strategy, a maximum concentration of 1,3-PD of 47.48 g/L was obtained with a volumetric productivity of 0.33 g/(L·h) and yield of 38.91%. Glycerol was utilized in 82.05%. The by-products obtained were as follows: lactic acid (24.29 g/L), acetic acid (9.62 g/L), succinic acid (5.95 g/L), and ethanol (3.06 g/L). The dry cell weight at the end of the process was 2.82 g/L. Using the second feeding strategy, a higher concentration of 1,3-PD was obtained (53.44 g/L) with an improved volumetric productivity (0.44 g/(L·h). Higher concentrations of by-products, with the exception of lactic acid, were also obtained. The second strategy resulted in obtaining lactic acid in a decreased concentration. The production of lactic acid is generally unwanted during the microbial 1,3-PD fermentation as it decreases the conversion yield of glycerol to the diol by reducing the amount of available $NADH_2$ [5,37,41]. In both cases, the rate of 1,3-PD production decreased with time which may likely be a result of the accumulation of fermentation products or the impurities contained in crude glycerol used as the carbon source [12,27].

So far, the highest 1,3-PD concentration was obtained using the *C. freundii* FMCC-B 294 (VK-19) strain in a fed-batch culture, and this was equal to 68.1 g/L [33]. The microbial production of 1,3-PD still attracts the attention of researchers. New studies are published

regularly containing reports on the bioconversion of glycerol to this diol with different microorganisms, including *C. freundii* [42,43], *K. pneumoniae* [44,45], *C. butyricum* [46,47].

4. Conclusions

The ability of *C. freundii* strains to convert glycerol into 1,3-PD is well known. However, the efficiency of this production depends on the features of the strain itself, medium composition, fermentation conditions, and bioprocess operation modes (batch, fed-batch, continuous cultures). In this work, 53.44 g/L of 1,3-PD was obtained in a fed-batch culture with *C. freundii* AD119 performed at a temperature of 30 °C, pH of 7, and a stirring rate of 80 RPM. The obtained product concentration proves the potential of this strain. Moreover, certain conditions, such as a temperature of 33°C or higher, or a pH of 6.5, triggered premature termination of the fermentation that coincided with increased levels of 3-HPA in the fermentation liquid. To the best of our knowledge, this report is the first to present data on the impact of culture conditions on the accumulation of 3-HPA in glycerol fermentation with *C. freundii*.

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