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Modulation of crude glycerol fermentation by *Clostridium pasteurianum* DSM 525 towards the production of butanol

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ABSTRACT

High production yields and productivities are requisites for the development of an industrial butanol production process based on biodiesel-derived crude glycerol. However, impurities present in this substrate and/or the concentration of glycerol itself can affect the microbial metabolism. In this work, the effect of crude glycerol concentration on the production of butanol and 1,3-propanediol (1,3-PDO) by *Clostridium pasteurianum* DSM 525 is studied. Also, the effect of acetate and butyrate supplementation to the culture medium and the culture medium composition are evaluated. The results showed a marked effect of crude glycerol concentration on the product yield. The competitive nature of butanol and 1,3-PDO pathways has been evident, and a shift to the butanol pathway once using higher substrate concentrations (up to 35 g l^{-1}) was clearly observed. Butyrate supplementation to the culture medium resulted in a 45% higher butanol titre, a lower production of 1,3-PDO and it decreased the fermentation time. Acetate supplementation also increased the butanol titre but the fermentation was longer. Even though glycerol consumption could not be increased over 32 g l^{-1}, when the concentrations of NH$_4$Cl and FeCl$_2$ were simultaneously increased, the results obtained were similar to those observed when butyrate was supplemented to the culture medium; a 35% higher butanol yield at the expense of 1,3-PDO and a shorter fermentation. The results herein gathered suggest that there are other factors besides butanol inhibition and nutrient limitation that affect the glycerol consumption.

1. Introduction

Glycerol is an attractive substrate for anaerobic fermentation that has interesting advantages over simple sugars such as glucose. From the technical point of view, the highly reduced nature of this compound results in twice the amount of reducing equivalents when it is converted to glycolytic intermediates such as pyruvate, which leads to higher yields of reduced compounds such as butanol [1]. On the other hand, glycerol is a by-product in the production of biodiesel and ethanol, representing approximately 10% and 6% (w/w) of the

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production, respectively [2]. In this context, the rapid expansion that the biodiesel industry has experienced in the last decades has led to a surplus of crude glycerol in the market, thus a decrease in its price [3], making it an economically competitive substrate. As an example, the current spot price of crude glycerol is $0.20 per kilogram in the US (FOB Midwest) [4]. On the other hand, the price of corn (Central Illinois) is $0.19 per kilogram [4]. Therefore, if 1 kg of corn leads to 0.71 kg of glucose [5], the price of corn-derived glucose, considering only the raw material, can be estimated in $0.28 per kilogram.

The production of butanol and 1,3-PDO from either pure or crude biodiesel-derived glycerol by *Clostridium pasteurianum* has been reported by several authors [6—15]. However, only few researchers used high concentrations of crude glycerol and their results showed a great variability in terms of product yield, titre and productivity. A source of such variability could be the impurities present in the crude glycerol, which potentially include methanol, ash, free fatty acids and triglycerides. Therefore, extra care has been advised regarding the use of crude glycerol, especially in what concerns the remaining methanol, as well as the sodium or potassium salts since they are known to inhibit cell growth [16]. Taconi et al. [14] reported a decrease in the average yield of butanol and longer fermentation times for *C. pasteurianum* ATCC 6013 when using crude glycerol instead of pure glycerol. Venkataramanan et al. [15] found that linolenic acid, potentially present as non-reacted free fatty acid in biodiesel-derived crude glycerol, strongly inhibits glycerol utilization by *C. pasteurianum* ATCC 6013. Khanna et al. [11] used *C. pasteurianum* immobilized cells and found a pronounced decrease in the butanol production from crude glycerol concentrations higher than 25 g l^{-1}. On the other hand, glycerol itself at high concentrations rather than the impurities that crude glycerol could contain, seems to influence the *C. pasteurianum* metabolism [8].

In general, two steps, namely acidogenesis and solventogenesis, characterize solvent production by *Clostrdium* spp. Although the metabolic regulation systems are not fully elucidated, the metabolic pathways present in solventogenic clostridia are well established (Fig. 1).

Even though the conversion of butyrate to butanol does not occur in the absence of a reduced carbon source, it has been reported that the supplementation of the culture medium with this compound (and also with acetate) enhances and stabilizes solvent production in *Clostridium beijerinckii* [17—20]. However, the supplementation of butyrate as a strategy to increase butanol production from crude glycerol by *C. pasteurianum* has not been reported. Heyndrickx et al. [9] showed that externally added acetate has an effect on the product distribution in the fermentation of pure glycerol by *C. pasteurianum*. Contrarily, Harris et al. [21] reported that the supplementation of acetate or butyrate to the culture medium did not influence *C. pasteurianum* fermentation using glucose as the carbon source.

As Datta and Zeikus [22] pointed, a higher butanol titre obtained at the expense of butyrate must be accompanied by a change in the electron flow towards the production of solvents. This situation can be the result of a higher activity of the enzymes involved in the production of solvents from acetate or butyrate. Indeed, Chen and Blanchek [17] reported a higher CoA transferase activity and higher acetate kinase- and
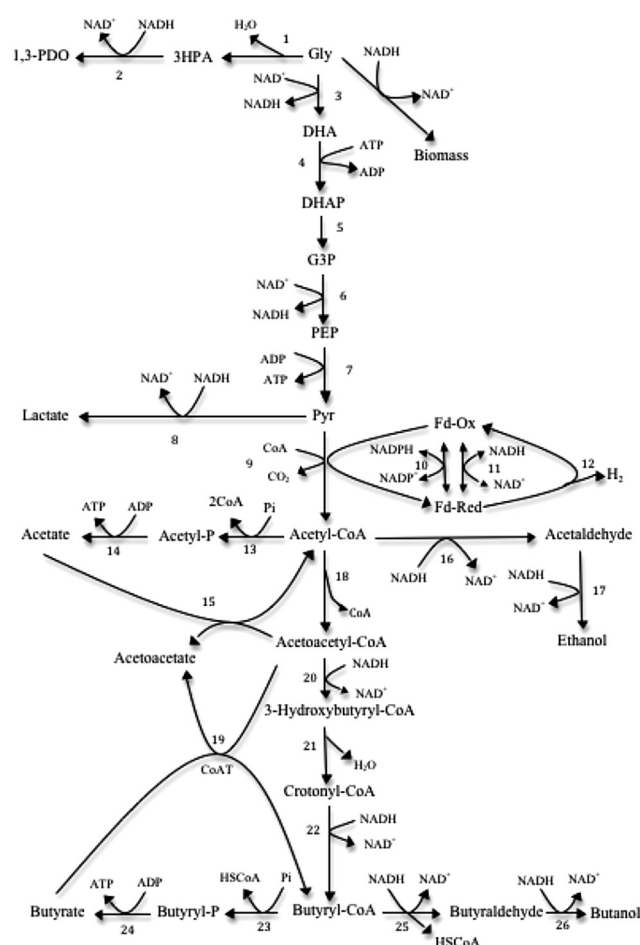
Fig. 1 — Metabolic pathways involved in glycerol degradation by *C. pasteurianum*. 1: glycerol dehydratase; 2: 1,3-propanediol oxidoreductase; 3: glycerol-3-phosphate dehydrogenase; 4: dihydroxyacetone kinase; 5: triose-phosphate isomerase; 6: glyceraldehyde-3-phosphate dehydrogenase; 7: pyruvate kinase; 8: lactate dehydrogenase; 9: pyruvate-ferredoxin oxidoreductase; 10: NADPH-ferredoxin oxidoreductase; 11: NADH-ferredoxin oxidoreductase; 12: ferredoxin hydrogenase; 13: phosphate acetyltransferase; 14: acetate kinase; 15:, acetoacetyl-CoA: acetate:CoA transferase; 16: acetaldehyde dehydrogenase; 17: ethanol dehydrogenase; 18: thiolase; 19: acetoacetyl-CoA: butyrate:CoA transferase; 20: β-hydroxybutyryl-CoA dehydrogenase; 21: crotonase; 22: butyryl-CoA dehydrogenase; 23: phosphotransbutyrylase; 24: butyrate kinase; 25: butaraldehyde dehydrogenase; 26: butanol dehydrogenase. Adapted from Malaviya et al. [13].

butyrate kinase specific activity in *C. beijerinckii* NCIMB 8052 grown in a culture medium supplemented with sodium acetate. Husemann and Papoutsakis [23] showed that the levels of NAD-dependent and NADP-dependent butyraldehyde dehydrogenase, which catalyses the conversion of butyryl-CoA to butyraldehyde (precursor of butanol), were more strongly induced in the presence of CO and butyric acid.

In this work, the effects of crude glycerol concentration and of the supplementation of the culture medium with acetate and butyrate on the production of butanol and 1,3-PDO by

C. pasteurianum DSM 525 are studied. Moreover, the composition of the culture medium is evaluated in order to identify possible nutrient limitations and thus to improve the production of butanol.

2.   Material and methods

2.1.   Strain maintenance and reactivation

C. pasteurianum DSM 525 was purchased from DSMZ (German collection of microorganisms and cell cultures). Freeze dried cells were reactivated in a semi-defined culture medium containing per litre of distilled water: 40 g glucose; 1 g yeast extract; 0.5 g KH_2PO_4; 0.5 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.2H_2O$; 3 g NH_4Cl; 4 g $NaHCO_3$; 0.5 g cysteine-HCl; 1 ml acid micronutrients solution (1.8 g l^{-1} HCl; 61.8 mg l^{-1} H_3BO_3; 61.3 mg l^{-1} $MnCl_2$; 943.5 mg l^{-1} $FeCl_2$; 64.5 mg l^{-1} $CoCl_2$; 12.9 mg l^{-1} $NiCl_2$; 67.7 mg l^{-1} $ZnCl_2$) and 1 ml alkaline micronutrients solution (0.4 g l^{-1} NaOH; 17.3 mg l^{-1} Na_2SeO_3; 29.4 mg l^{-1} Na_2WO_4; 20.5 mg l^{-1} Na_2MoO_4) under a N_2—CO_2 gas mixture (80%—20%) atmosphere. Stock cultures were kept at room temperature and were transferred to fresh medium periodically.

2.2.   Analytical methods

Acids, glycerol and 1,3-PDO were measured through high performance liquid chromatography (Aminex cation-exchange HPX-87H column) equipped with UV and RI detectors. The column was eluted isocratically with H_2SO_4 0.01 N using a flow rate of 0.7 ml min^{-1}. The column temperature was set at 60 °C. Ethanol and butanol were measured by gas chromatography (TR-WAX capillary column) equipped with a flame ionization detector. A temperature ramp (0.5 °C min^{-1}) was used for the column and the temperatures of the injector and detector were kept at 200 °C and 250 °C, respectively.

2.3.   Experimental procedure

The strain was anaerobically cultured in 500 ml serum bottles using a working volume of 200 ml. Ten per cent culture volume was repeatedly transferred to increasing biodiesel-derived crude glycerol concentrations using the semi-defined culture medium described in Section 2.1, in which the glucose was replaced by crude glycerol. Alternatively, the $NaHCO_3$—CO_2 buffer was changed by $CaCO_3$ and the serum bottles were pressurized with N_2. The crude glycerol used was kindly provided by a Portuguese biodiesel producer. The composition (w/w) of the crude glycerol was glycerol (85% min.), ash (6% max.), non-glycerol organic matter (2.5% max.), moisture (8—10%), and methanol (0.1% max.). For each crude glycerol concentration, four bottles were prepared. Samples were taken at regular time intervals from three bottles until the end of the fermentation and one bottle was used as inoculum to transfer cells in a vegetative stage (12—24 h). Alternatively, cells were transferred at the end of the fermentation, thus allowing them to sporulate. The crude glycerol concentrations used were 5, 10, 15, 20, 35 and 50 g l^{-1}.

The effect of butyrate on the fermentation of crude glycerol by *C. pasteurianum* DSM 525 was evaluated in culture media containing 5—50 g l^{-1} crude glycerol by using sodium butyrate in a concentration of 3.87 ± 0.42 g l^{-1}. The supplementation of sodium acetate at the same concentration was also evaluated in culture media containing 50 g l^{-1} crude glycerol. The experiments were conducted in parallel with those described above.

In order to obtain preliminary information about possible nutrient limitations and aiming to improve glycerol consumption and butanol production, the effect of different nutrients (NH_4Cl, $CaCO_3$, $FeCl_2$, microelements, salts, yeast extract, biotin and p-aminobenzoic acid) was evaluated by increasing their concentrations in independent experiments as showed in Table 1. The experiments were conducted in 160 ml serum bottles containing 60 ml of culture medium (50 g l^{-1} crude glycerol). The bottles were inoculated with 10% of an exponential culture of *C. pasteurianum* and the fermentation products were measured as described in Section 2.2. The results were statistically analysed using an F-test Two-Sample for Variances and subsequently a *t*-test assuming equal or unequal variances. In all experiments, the initial culture pH was adjusted to 6.8 ± 0.2 and cells were incubated at 37 °C without agitation.

Table 1 — Nutrient combinations used to identify possible nutrient limitations in cultures containing an initial glycerol concentration of 50 g l^{-1}.

Experiment	NH_4Cl (g l^{-1})	$CaCO_3$ (g l^{-1})	Yeast extract (g l^{-1})	Biotin (mg l^{-1})	p-aminobenzoic acid (mg l^{-1})	$FeCl_2$ (mg l^{-1})	Salts[a]	Microelements[b]
1	3	3	1	0	0	1	x 1	x 1
2	5	3	1	0	0	1	x 1	x 1
3	5	3	1	0	0	11	x 1	x 1
4	5	3	1	0	0	11	x 1	x 3
5	5	3	1	0.01	1	11	x1	x 3
6	5	5	2	0	0	11	x 1	x 3
7	5	5	1	0	0	11	x 1.5	x 3
8	5	10	1	0	0	11	x1	x 3
9	5	1.5	1	0	0	11	x1	x 3

[a,b] The notation x 1, x 1.5, x 3 indicates that the concentration of the corresponding nutrients in the culture medium was increased by a factor of 1, 1.5 and 3, respectively.

[a] Corresponds to KH_2PO_4, K_2HPO_4, and $MgSO_4$.$7H_2O$.

[b] Corresponds to trace elements solution 6 and solution 7.

Table 2 — Batch fermentation of crude glycerol by *C. pasteurianum* DSM 525 at 37 °C without pH control. Results represent the average of three independent assays ± S.D.

Initial glycerol (g l^{-1})	Glycerol consumption (g l^{-1})	Final butanol (g l^{-1})	Qbutanol (g l^{-1} h^{-1})	Final ethanol (g l^{-1})	Final 1,3-PDO (g l^{-1})	Q1,3-PDO (g l^{-1} h^{-1})	Final acetic acid (g l^{-1})	Final butyric acid (g l^{-1})	Final lactic acid (g l^{-1})
4.72[a]	4.71	0.17	0.003	0.06	1.80	0.032	0.74	0.87	0.33
8.87 ± 0.08	8.83 ± 0.08	0.77 ± 0.19	0.016 ± 0.004	0.18 ± 0.01	2.71 ± 0.21	0.051 ± 0.003	0.99 ± 0.05	0.94 ± 0.05	0.51 ± 0.07
16.69 ± 1.44	16.69 ± 1.44	2.21 ± 0.11	0.039 ± 0.001	0.34 ± 0.00	4.80 ± 0.16	0.051 ± 0.005	1.52 ± 0.12	1.63 ± 0.05	0.61 ± 0.05
19.33 ± 0.06	19.07 ± 0.17	2.42 ± 0.10	0.021 ± 0.002	0.26 ± 0.00	5.89 ± 0.23	0.053 ± 0.003	1.66 ± 0.10	1.83 ± 0.17	0.84 ± 0.10
32.80 ± 1.02	31.30 ± 1.18	6.71 ± 0.43	0.050 ± 0.008	0.59 ± 0.05	6.86 ± 0.51	0.060 ± 0.002	0.83 ± 0.08	0.43 ± 0.08	0.73 ± 0.18
44.13 ± 0.69	29.68 ± 1.43	6.73 ± 0.39	0.041 ± 0.002	0.68 ± 0.04	6.26 ± 0.27	0.077 ± 0.010	0.69 ± 0.03	0.21 ± 0.03	1.24 ± 0.04

Nomenclature: Qbutanol: butanol productivity; Q1,3-PDO: 1,3-PDO productivity.
[a] Only one out of three independent cultures produced butanol due to strain degeneration.

3. Results and discussion

Fast growth and almost complete substrate consumption was observed in experiments using up to 20 g l^{-1} initial crude glycerol concentration. Similar to previous reports about glycerol fermentation by *C. pasteurianum* [7,8,13], the main fermentation products found were acetic acid, butyric acid, lactic acid, butanol and 1,3- PDO. However, even though butanol and ethanol were produced in experiments using 5 g l^{-1} crude glycerol and in one out of three bottles containing 10 g l^{-1} crude glycerol, the solvent producing capacity of the cells was lost and only traces of alcohols were detected in the subsequent transfers. Higher production of acids, including isobutyric acid (not observed in normal cultures) and 1,3-PDO was detected when solvents were absent.

One of the main problems in the production of solvents using *Clostridium* spp., besides the toxicity of the end product butanol, is the so-called strain degeneration. This situation is basically the loss of the solvent capacity of the cells during repeated subculture in batch and continuous culture and it has been reported in *Clostridium acetobutylicum* and *C. beijerinckii* [24—26]. However, this issue has been rather neglected in *C. pasteurianum*.

The standard practice to avoid stain degeneration of *Clostridium* spp. has been to maintain cells as spores, which can be, heat activated and germinated when an inoculum is required [25]. On the other hand, it has been reported that *C. acetobutylicum* can be sub-cultured in a medium containing an excess of CaCO$_3$ for more than 200 days without losing its ability to produce solvents, and also that it is possible to restore the initial solvent yields by increasing the volume of the inoculum used [27]. Nevertheless, Taconi and collaborators [14] reported the loss of solvent capacity in *C. pasteurianum* ATCC 61013 although they used an excess of CaCO$_3$ and even when they centrifuged and transferred all the cells. Therefore, these strategies seem to be useless for *C. pasteurianum*. The addition of acetate [17,18] and butyrate [20,28] to the culture medium has also shown to prevent strain degeneration and to enhance solvent production.

In an attempt to restore the solvent production, and based on the information reported in literature, those cells cultured in 20 g l^{-1} crude glycerol that did not produce solvents were transferred to culture media with the following differences:

(a) 3 g l^{-1} of CaCO$_3$ and N$_2$ in the headspace instead of CO$_2$—NaHCO$_3$ (change in buffer system)
(b) Twice the volume of inoculum (20%)
(c) 4 g l^{-1} of sodium butyrate in the culture medium

Despite the new conditions used, the product profile was the same and the solvent production capacity of the cells could not be restored. This situation suggests that at least one of the enzymes involved in the conversion of butyrate to butanol (Fig. 1) was not present or was inactive.

New experiments were started from a stock culture using CaCO$_3$ as buffer agent instead of NaHCO$_3$—CO$_2$ and cells were transferred at the end of the fermentation, thus allowing them to sporulate. Even though in this new experimental set up strain degeneration was observed in experiments using 5 and 10 g l^{-1} of crude glycerol, it was still possible to select and transfer degeneration-resistant cells, thus guaranteeing that this situation was overcome through the rest of the work.

Cells were serially transferred to culture media containing 5, 10, 15, 20, 35, and 50 g l^{-1} of crude glycerol (Table 2). As in the first experiments, the main products found were butanol and 1,3-PDO besides minor quantities of ethanol and acids (acetic, n-butyric, lactic). Moreover, the competitive nature of butanol and 1,3-PDO pathways was evident, and a shift to the butanol pathway for higher crude glycerol concentrations (up to 35 g l^{-1}) was clearly observed (Fig. 2), which indeed is a positive fact for the industrial production of butanol. Further increases in crude glycerol concentration did not promote any improvement on the production of butanol.

The product profile herein obtained differs from the results reported by Biebl [7] and Dabrock et al. [8] who studied the influence of glycerol (pure) concentration using *C. pasteurianum* DSM 525. Biebl [7] found that the butanol/1,3 -PDO ratio varied considerably but without noticeable relation to the glycerol concentration up to 114.6 g l^{-1}. On the other hand, Dabrock et al. [8] found that up to 8% (w/v) glycerol, the ratio ethanol/butanol/1,3-PDO did not change and that higher glycerol concentrations favoured the production of 1,3-PDO at the expense of ethanol.

The strain was able to consume a maximum of 31.30 ± 1.18 g l^{-1} of glycerol, which resulted in 6.71 ± 0.43 g l^{-1} of butanol and 6.86 ± 0.51 g l^{-1} of 1,3-PDO. Figs. 3 and 4 illustrate the glycerol consumption and butanol, ethanol and 1,3-PDO production along the time for cultures containing 35
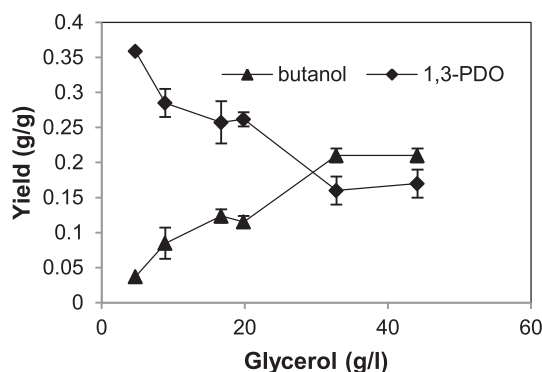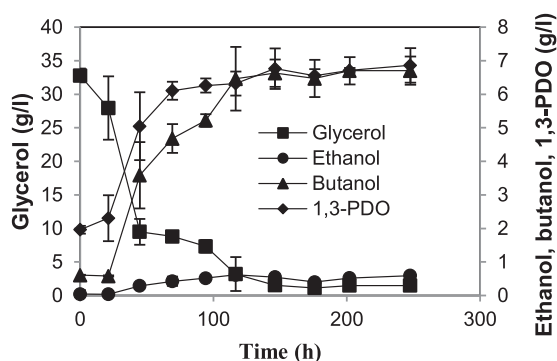
Fig. 2 – Butanol and 1,3-PDO yield *versus* glycerol concentration in batch fermentation of crude glycerol by *C. pasteurianum* DSM 525 at 37 °C without pH control. Results represent the average of three independent experiments ± S.D.

and 50 g l⁻¹ initial crude glycerol concentration, respectively. In both cultures, glycerol was rapidly consumed during the first 50 h, after which it was found a marked decrease in the consumption rate, probably due to butanol accumulation in the culture medium. As observed in all the experiments conducted, the amount of ethanol produced by the cells was residual in comparison with butanol, which is in agreement with the results presented by Biebl [7] and Malaviya et al. [13], although different from the ones presented by Dabrock et al. [8]. Interestingly, butanol was produced from the beginning of the fermentation and along with acids (data not shown), which differs from the classical biphasic ABE fermentation. Moreover, a clear association between butanol production and cell growth was reported by Biebl [7] using *C. pasteurianum* DSM 525 and glycerol at pH 6.0.

The fact that the strain was not able to consume more than 32 g l⁻¹ of glycerol is likely to be related with nutrient limitation, product inhibition, the pH of the culture medium and/or other physiological parameters. However, the accumulation of some toxic compound through the repeated sub-culturing cannot be discarded.

The glycerol consumption obtained in this work is slightly higher than the maximum glycerol consumption reported by

Fig. 3 – Batch fermentation of 35 g l⁻¹ crude glycerol by *C. pasteurianum* DSM 525 at 37 °C without pH control. Results represent the average of three independent experiments ± S.D.

— Gly — Butanol — Ethanol — 1,3-PDO
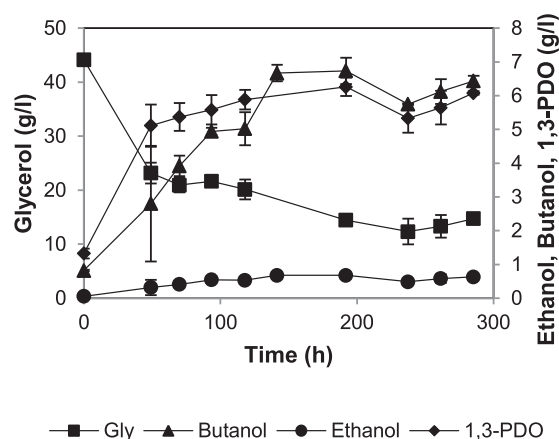
Fig. 4 – Batch fermentation of 50 g l⁻¹ crude glycerol by *C. pasteurianum* DSM 525 at 37 °C without pH control. Results represent the average of three independent experiments ± S.D.

Dabrock et al. [8] (27.6 g l⁻¹), but lower than the value reported by Biebl [7] (63.6 g l⁻¹). Regarding the production of butanol and 1,3-PDO, Dabrock et al. [8] showed an increase in butanol production from 2 to 8 % (w/v) glycerol in the culture medium, after which, its concentration remained more or less constant up to 17% (v/w). At this glycerol concentration the authors reported 3.33 g l⁻¹ of butanol and 10.04 g l⁻¹ of 1,3-PDO. The fermentations herein conducted resulted in a higher titre and yield of butanol, which is probably related with the composition of the culture medium used in the fermentation. In fact, Dabrock et al. [8] used a semi-defined culture medium containing an unusually high concentration of biotin (0.24 mg l⁻¹), which has been reported as a required growth factor for the production of 1,3-propanediol by *Clostridium butyricum* [29]. Also, the authors used a low concentration of nitrogen source (0.66 g l⁻¹), which has been reported to favour the production of 1,3-PDO by *C. pasteurianum* DSM 525 [30].

On the other hand, Biebl [7] reported a maximum glycerol consumption of 63.6 g l⁻¹ using an initial concentration of 114.6 g l⁻¹. The authors also reported the production of 14 g l⁻¹ of butanol, which is surprisingly high for the wild-type strain in comparison with the results herein obtained and other reports from literature. The main difference between the experiments conducted by Biebl [7] and the ones of the present work is the use of a pH-controlled reactor.

The pH and operational parameters have been recognized as important factors in the fermentation of glycerol by *C. pasteurianum*. Dabrock et al. [8] showed that in continuous culture at pH values below 5.5 the growth of *C. pasteurianum* and the substrate turnover started to decrease, and that at pH values below 4.8 the steady-state conditions could not be obtained anymore. Similarly, Biebl [7] studied the effect of pH on glycerol (pure) fermentation by *C. pasteurianum* DSM 525 in a pH-controlled reactor over a pH range of 4.5–7.5 and found that at pH 4.5 only part of the substrate was used after a prolonged fermentation time. The author reported that the optimal pH for butanol production was 6.0. Even though a low pH could limit the consumption of glycerol by stopping the fermentation, it should be mentioned that the lowest pH value

Table 3 — Batch fermentation of crude glycerol by C. pasteurianum DSM 525 in culture media supplemented with sodium butyrate at 37 °C without pH control. Results represent the average of three independent assays ± S.D.

Initial glycerol (g l⁻¹)	Glycerol consumption (g l⁻¹)	Final butanol (g l⁻¹)	Qbutanol (g l⁻¹ h⁻¹)	Final ethanol (g l⁻¹)	Final 1,3-PDO (g l⁻¹)	Q1,3-PDO (g l⁻¹ h⁻¹)	Final acetic acid (g l⁻¹)	Initial butyric acid (g l⁻¹)	Butyric acid consumption (g l⁻¹)	Final lactic acid (g l⁻¹)
4.83 ± 0.13	4.80 ± 0.14	0.36 ± 0.04	0.007 ± 0.001	0.06 ± 0.00	1.36 ± 0.07	0.024 ± 0.002	0.60 ± 0.08	3.68 ± 0.04	0.40 ± 0.12 (production)[b]	0.31 ± 0.02
8.26[a]	8.26	1.91	0.033	0.16	1.68	0.032	1.20	4.21	1.03	0.33
15.71 ± 2.39	15.71 ± 2.39	3.32 ± 0.18	0.060 ± 0.003	0.28 ± 0.01	3.30 ± 0.39	0.035 ± 0.003	1.80 ± 0.13	4.03 ± 0.49	0.36 ± 0.63	1.40 ± 0.16
19.47 ± 0.47	18.50 ± 0.41	4.21 ± 0.06	0.036 ± 0.005	0.36 ± 0.01	3.49 ± 0.08	0.026 ± 0.001	1.66 ± 0.10	4.36 ± 0.14	0.18 ± 0.9	2.26 ± 0.14
31.57 ± 0.58	28.18 ± 0.59	8.95 ± 0.44	0.084 ± 0.003	0.48 ± 0.06	3.48 ± 0.06	0.010 ± 0.001	0.94 ± 0.18	3.37 ± 0.17	2.58 ± 0.20	1.86 ± 0.28
42.86 ± 0.80	27.54 ± 2.54	9.75 ± 0.45	0.094 ± 0.003	1.07 ± 0.65	3.04 ± 0.05	0.008 ± 0.003	0.67 ± 0.03	3.57 ± 0.03	2.69 ± 0.07	2.04 ± 0.32

Nomenclature: Qbutanol: butanol productivity; Q1,3-PDO: 1,3-PDO productivity.
[a] Only one out of three independent cultures produced butanol due to strain degeneration.
[b] The final concentration of butyric acid was 0.40 ± 0.12 g l⁻¹ higher than the concentration measured at the beginning of the fermentation.

measured in the experiments conducted in the present work was 4.8, however usual values were over 5.0.

Malaviya et al. [13] developed a hyper-producing mutant of *C. pasteurianum* by random chemical mutagenesis. In preliminary experiments, the authors compared the mutant strain with the parent strain in flask cultures using 80 g l⁻¹ of glycerol and reported an average butanol production of 10.8 g l⁻¹ and 7.7 g l⁻¹, respectively, which is similar to the results herein obtained. No information on the glycerol consumption was provided in that report. Similarly to Biebl [7] and Dabrock et al. [8], the authors reported that when the same experiment was conducted in a pH-controlled reactor (pH above 4.5 or 4.8), the butanol concentrations increased to 10 g l⁻¹ and 13.7 g l⁻¹ for the parent and mutant strain, respectively. The authors further studied several parameters using the mutant strain, such as initial cell density, inoculum age and initial pH. In these experiments, conducted in serum bottles, they reported a maximum butanol production of 9.4 ± 0.02 g l⁻¹. Again, when the mutant strain was grown in a pH-controlled reactor, the butanol production increased to 14.2 ± 0.12 and 15.5 ± 0.09, respectively. It is worth to note that this is almost the same value found by Biebl [7] for the wild-type *C. pasteurianum* DSM 525.

Regarding a possible negative effect of the impurities present in crude glycerol on cell metabolism, it is important to stress that it is unlikely that methanol concentrations in the culture medium are high enough to exert a severe inhibition of the cell metabolism. As an example, it was reported that 40 g l⁻¹ methanol caused 61% inhibition in the cell growth of *C. acetobutylicum* [31]. The maximum concentration of methanol in the crude glycerol used in this work was 0.1% (w/w), which could result in a maximum concentration of 0.5 g l⁻¹ of this compound in a culture medium containing 50 g l⁻¹ of crude glycerol. Moreover, Venkataramanan et al. [15] studied the impact of impurities in biodiesel-derived crude glycerol on the fermentation by *C. pasteurianum* ATCC 6013 and found that salts and methanol had no negative effects on the growth and metabolism of the microorganism. However, the possible presence of linolenic acid in the crude glycerol could affect glycerol consumption, especially at high concentrations.

Overall, the supplementation of the culture medium with sodium butyrate resulted in higher butanol titres (Table 3). Using 50 g l⁻¹ of crude glycerol, the final butanol concentration was 9.75 ± 0.45 g l⁻¹ *versus* 6.73 ± 0.39 g l⁻¹ obtained in the control. The concentration of 1,3-PDO reached 3.04 ± 0.05 g l⁻¹ *versus* 6.26 ± 0.67 g l⁻¹ obtained in the control. The glycerol consumption was not notoriously affected by the butyrate supplementation, being in this case 27.54 ± 2.54 g l⁻¹. The fact that the supplementation of the culture medium with sodium butyrate resulted in higher butanol titres demonstrates that the glycerol consumption in the control cultures was not limited by butanol inhibition.

It is important to stress that a higher butanol titre obtained as a result of butyrate addition does not necessarily means a higher butanol on glycerol yield since butyrate can be directly converted into butanol via butyryl-CoA − butyraldehyde − butanol and/or the reverse phosphotransbutyrylase - butyrate kinase metabolic pathway as it has been demonstrated in *C. acetobutylicum* [32,33] and suggested in *C. beijerinckii* [34]. However, butyrate was significantly consumed only in the
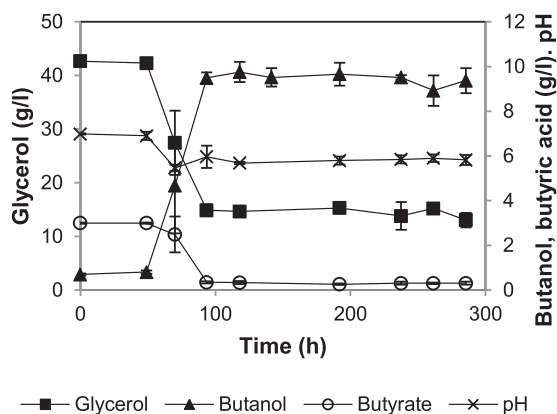
cultures with 35 and 50 g l^{-1} of initial crude glycerol concentrations.

The culture with 50 g l^{-1} initial crude glycerol concentration in which sodium butyrate was supplemented resulted in the consumption of 2.69 ± 0.07 g l^{-1} of butyric acid. On the contrary, the control culture produced 0.21 ± 0.03 g l^{-1} of this compound. If we consider an equimolar conversion of butyrate to butanol, and that all external butyrate was converted to butanol [35], the amount generated by this way would be 2.3 g l^{-1}, which is almost the difference observed between the butanol production in the culture where external butyrate was added and the control (3.02 ± 0.60 g l^{-1}).

Another effect of the butyrate supplementation to the culture medium was a decrease in the fermentation time, which combined with the higher titre obtained, resulted in more than twice the volumetric butanol productivity (0.094 ± 0.003 g l^{-1} h^{-1} vs. 0.041 ± 0.002 g l^{-1} h^{-1}) in the fermentation with 50 g l^{-1} initial crude glycerol concentration. Consumption of butyric acid started after a marked pH drop from 6.9 ± 0.1, to 5.5 ± 0.1 and accompanied the production of butanol, (Fig. 5). After butyric acid was consumed, butanol reached its maximum concentration and the pH increased to a maximum of 5.9 ± 0.2.

Cultures supplemented with sodium butyrate showed the same yield behaviour as those without external butyrate addition, i.e. an increase in the butanol yield at the expense of 1,3-PDO as the crude glycerol concentration was increased. Fig. 6 illustrates the 1,3-PDO and butanol yield assuming that butanol was formed only by the glycerol consumed.

Even though the results obtained clearly show that it is possible to increase the butanol volumetric productivity in the fermentation of crude glycerol by *C. pasteurianum* by supplementing the culture medium with butyrate, it is worth mentioning that economic factors could hamper the use of this compound as co-substrate. However, innovative solutions have been proposed in the literature. Especially interesting are those involving two-stage fermentations in which the first stage is dedicated to the production of butyric acid
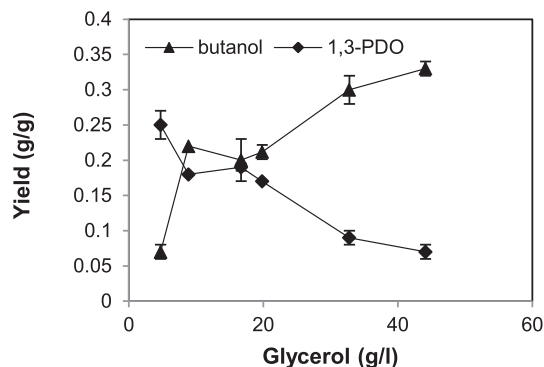
Fig. 6 — **Butanol and 1,3-PDO yields *versus* glycerol concentration in a batch fermentation using crude glycerol and sodium butyrate. Experiments were conducted at 37 °C without pH control Results represent the average of three independent assays ± S.D.**

either using the same [36] or a different strain [37]. Moreover, the use of waste-derived feedstock for this purpose could improve the process economy, although each substrate should be individually evaluated [38]. On the other hand, butyric acid is usually present in various waste streams from agricultural and industrial processes [34].

The supplementation of sodium acetate to culture media with 50 g l^{-1} crude glycerol resulted also in a higher butanol production by *C. pasteurianum*. However, the butanol titre obtained was lower than in those cultures in which sodium butyrate was used (8.95 ± 0.27 g l^{-1} *versus* 9.75 ± 0.45 g l^{-1}). Furthermore, the fermentation was longer than the control cultures and the ones in which sodium butyrate was supplemented. Similar to those cultures in which sodium butyrate was used, glycerol consumption was not significantly affected (33.5 ± 1.95 g l^{-1}).

Even though more butanol could be formed from butyrate and therefore the yield of butanol reported is "apparent", an interesting fact is the lower yield of 1,3-PDO that was obtained. This situation shows that the supplementation of the culture medium with sodium butyrate affects the electron flow in the fermentation of crude glycerol by *C. pasteurianum*. The reducing equivalents formed during glycolysis are redirected to the production of butanol instead of 1,3-PDO (and probably hydrogen). This is in agreement with the findings reported in the literature and supports the hypothesis that butyrate supplementation is responsible for a higher activity of the enzymes involved in the production of butanol from this compound.

The results herein gathered, demonstrated that it is possible to obtain 9.75 ± 0.45 g l^{-1} of butanol, thus suggesting that cells did not consume more glycerol as a result of nutrient limitation and not due to butanol inhibition. Therefore, the evaluation of the culture medium composition was considered necessary at this point. The results of the experiments described in Table 1 (Section 2.3) are shown in Table 4.

Although it was not possible to increase the glycerol consumption by adjusting the culture medium composition, the experiments conducted resulted in significant differences in the product distribution. In particular, an increase in the concentration of NH$_4$Cl from 3 to 5 g l^{-1} (Experiment 2) led to

Fig. 5 — **Butyric acid consumption and pH behaviour during the butanol production from glycerol by *C. pasteurianum* DSM 525 in batch culture at 37 °C without pH control. In this experiment 50 g l^{-1} of crude glycerol and 36 mM of sodium butyrate were used. Results represent the average of three independent assays ± S.D.**

Table 4 – Evaluation of effect of the culture medium composition on the final yields of butanol and 1,3-PDO. The experiments were conducted in batch at 37 °C without pH control and using 50 g l^{-1} crude glycerol.

Experiment[a]	Glycerol initial (g l^{-1})	Glycerol consumption (g l^{-1})	Butanol final (g l^{-1})	1,3-PDO final (g l^{-1})	Y Butanol (g g^{-1})	Qbutanol (g l^{-1} h^{-1})	Y 1,3-PDO (g g^{-1})	Q1,3-PDO (g l^{-1} h^{-1})
1 (control)	44.13 ± 0.69	29.68 ± 1.43	6.73 ± 0.39	6.26 ± 0.27	0.20 ± 0.01	0.041 ± 0.002	0.17 ± 0.01	0.077 ± 0.010
2	49.03 ± 0.86	31.97 ± 0.69	8.28 ± 0.39	3.18 ± 0.26	0.23 ± 0.01	0.069 ± 0.004	0.08 ± 0.01	0.026 ± 0.007
3	46.94 ± 0.28	29.61 ± 0.30	8.95 ± 0.08	1.78 ± 0.24	0.27 ± 0.01	0.119 ± 0.003	0.06 ± 0.01	0.026 ± 0.002
4	44.94 ± 3.25	28.38 ± 4.37	8.80 ± 0.39	1.90 ± 0.16	0.28 ± 0.04	0.117 ± 0.004	0.07 ± 0.02	0.019 ± 0.007
5	46.16 ± 1.05	26.54 ± 0.55	7.92 ± 0.22	1.69 ± 0.15	0.26 ± 0.01	0.099 ± 0.006	0.06 ± 0.01	0.021 ± 0.001
6	46.08 ± 2.81	24.08 ± 2.25	6.57 ± 0.24	5.00 ± 0.26	0.24 ± 0.02	0.073 ± 0.002	0.21 ± 0.01	0.054 ± 0.005
7	47.54 ± 1.69	25.18 ± 0.87	6.72 ± 0.07	5.34 ± 0.21	0.23 ± 0.01	0.064 ± 0.03	0.21 ± 0.01	0.058 ± 0.006
8	47.04 ± 0.51	25.57 ± 0.86	6.26 ± 0.11	4.99 ± 0.04	0.21 ± 0.01	0.032 ± 0.004	0.19 ± 0.01	0.031 ± 0.009
9	45.98 ± 1.05	17.53 ± 0.94	4.18 ± 0.07	2.98 ± 0.19	0.19 ± 0.01	0.035 ± 0.002	0.17 ± 0.01	0.021 ± 0.001

Nomenclature: Y butanol: yield of butanol from glicerol; Y 1,3-PDO: yield of 1,3-PDO from glycerol; Qbutanol: butanol productivity; Q1,3-PDO: 1,3-PDO productivity.

[a] Experiments defined in Table 1.

butanol and 1,3-PDO yields of 0.23 ± 0.01 g g^{-1} and 0.08 g g^{-1}, respectively, which were statistically different ($p = 0.03$ and $p = 0.07$) from the ones obtained in the control (0.20 ± 0.01 g g^{-1} and 0.17 ± 0.02 g g^{-1}, respectively). Using 5 g l^{-1} of NH$_4$Cl, an increase in the FeSO$_4$ concentration to 11 mg l^{-1} (Experiment 3), further increased the butanol yield to 0.27 ± 0.01 g g^{-1}, while the 1,3-PDO yield decreased to 0.06 ± 0.01 g g^{-1}, being both values significantly different ($p = 0.02$ and $p = 0.04$) from the ones obtained in Experiment 2. This condition led to the higher butanol productivity, corresponding to 0.119 ± 0.003 g l^{-1} h^{-1}. The concentration of butanol and 1,3-PDO reached 8.95 ± 0.08 g l^{-1} and 1.78 ± 0.24 g l^{-1}, respectively. The glycerol consumption was not significantly affected (29.61 ± 0.30 g l^{-1}).

The increase of the salts concentration (Experiment 4), as well as the supplementation of biotin and p-aminobenzoic acid (Experiment 5) did not promote any improvement in the consumption of glycerol or production of butanol. Also, from experiments 6 to 9, it is clear that changes in the CaCO$_3$ concentration (buffer agent) negatively affected the *C. pasteurianum* metabolism by decreasing the consumption of glycerol, which is especially notorious when a concentration of 1.5 g l^{-1} was used. These results confirm the importance of pH control in the fermentation of glycerol by *C. pasteurianum*.

The influence of the culture medium composition on the product distribution in the fermentation of glycerol by *C. pasteurianum* has been previously reported [8,30]. In particular, Moon et al. [30] reported that the concentration of the nitrogen source is an important variable and that a high concentration of (NH$_4$)$_2$SO$_4$ negatively affects 1,3-PDO, favouring the production of butanol, which is in agreement with the results herein obtained, although the nitrogen source used in this work was NH$_4$Cl.

Iron is part of at least four important proteins directly related with the electron flow in *Clostridium* spp. namely, pyruvate-ferredoxin oxidoreductase (PFOR) [39], ferredoxin [40], hydrogenases I and II [41] and iron-containing alcohol dehydrogenases [42]. The results herein obtained are in agreement with the ones reported by Dabrock et al. [8] who found that iron limitation in the fermentation of glycerol by *C. pasteurianum* DSM 525 somehow inhibits butanol production, which they related to the presence of iron-containing alcohol

dehydrogenases. In *C. pasteurianum* it is possible to identify three major electron sinks; 1,3-PDO, hydrogen and butanol/ethanol. Thus, the results reported by those authors and the ones herein gathered suggest that an iron limitation leads to a low butanol dehydrogenase activity, forcing the cell to favour the production of 1,3-PDO as a way to regenerate NAD$^+$, probably accompanied with a high hydrogen production. Conversely, the presence of iron in sufficient amounts leads to the production of butanol and a lower production of hydrogen. It is worth to note that the production of butanol consumes twice the amount of reducing equivalents than the production of 1,3-PDO (Fig. 1). A more detailed study on the effect of nitrogen and iron on the production of butanol by *C. pasteurianum*, including hydrogen evolution measurements, should be conducted in order to validate these assumptions.

4. Conclusions

In this work, we found that high concentrations of biodiesel-derived crude glycerol favour the production of butanol over 1,3-PDO. Also, it was demonstrated that it is possible to modulate the outcome of the fermentation towards the production of butanol by supplementing the culture medium with sodium butyrate or by adjusting the concentration of key nutrients.

Significant differences in glycerol consumption and butanol production are reported in the literature and also in comparison with the results herein obtained, thus reflecting the complexity of the regulatory mechanisms present in this strain. These differences can be partially explained by the culture media composition, but undoubtedly other variables are also involved in the process. The results gathered in this work suggest that there are other factors besides butanol inhibition and nutrient limitation that affect glycerol consumption.

Acknowledgements

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