Biomass and Bioenergy 91 (2016) 37–47

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: http://www.elsevier.com/locate/biombioe

Research paper

Pilot scale fed-batch fermentation in a closed loop mixed reactor for the biotransformation of crude glycerol into ethanol and hydrogen by *Escherichia coli* MG1655

O. Cofré [a, b], M. Ramírez [a], J.M. Gómez [a, *], D. Cantero [a]

[a] Universidad de Cádiz, Campus de Excelencia Internacional Universitario ceiA3, Departamento de Ingeniería Química y Tecnología de Alimentos, Facultad de Ciencias, Av. República Saharaui s/n, 11510, Puerto Real, Cádiz, Spain
[b] Universidad de Valparaíso, Escuela de Ingeniería en Medioambiente, Facultad de Ingeniería, Patricio Lynch 1, Playa Ancha, Valparaíso, Chile

ARTICLE INFO

Article history:
Received 4 November 2014
Received in revised form
25 February 2016
Accepted 25 April 2016

Keywords:
Biotransformation
Escherichia coli
Ethanol
Fed-batch culture
Glycerol
Pilot scale

ABSTRACT

Glycerol is a low-cost carbon source that can be used to produce chemicals like ethanol or hydrogen (H_2). In the work described here, the biotransformation of crude glycerol, obtained from a biodiesel production process, into ethanol and H_2 by *Escherichia coli* MG1655 was studied for batch and fed-batch operating modes. No difference was found between the use of crude glycerol rather than food-grade glycerol as the main carbon source. Three concentrations of crude glycerol were studied for fed-batch experiments under constant and exponential feeding regimes. No nutrients were added during the feeding step and a crude glycerol-water solution was fed into the reactor. The exponential feeding regime with 37.7 g L^{-1} of crude glycerol in the feed gave the best overall results, with 100% of fed crude glycerol consumed, a final ethanol concentration of 7.58 ± 1.52 g L^{-1} and an H_2 yield of 0.56 mol mol^{-1} of fed crude glycerol. The process was studied on a pilot scale (working volume: 200 L) in a closed loop mixed reactor, giving an ethanol concentration of 8.5 ± 1.70 g L^{-1}, thus indicating that scale-up of the process is possible. Fed-batch mode under an exponential feeding regime is a promising strategy to increase ethanol and H_2 production and crude glycerol utilization given that previous studies concerning the biotransformation of glycerol to ethanol and H_2 by *Escherichia coli* have mainly been performed in batch mode. Hydrodynamic characterization of the reactors was performed to establish conditions that would allow an approach to a complete mixing regime in all experiments.

1. Introduction

Glycerol is a versatile carbon and energy source with many potential applications in industrial fermentation. Most research in this area has focused on the use of glycerol in the production of solvents such as 1,3-propanediol [1–3]. A total of 1 kg of glycerol is generated for every 10 kg of biodiesel produced [4], therefore a huge amount of glycerol is produced as a byproduct in the biodiesel industry. The recent increase in biodiesel production has resulted in an excess of glycerol that traditional industries (e.g. cosmetics and food) cannot absorb [5]. In this respect, some commodity chemicals such ethanol and hydrogen (H_2) can be produced biotechnologically using glycerol as the carbon source in conjunction with microorganisms. This approach can also reduce our current fossil fuel dependency [6]. This bioconversion would directly benefit the environment by providing biodegradable compounds, promoting the use of biodiesel and reducing the emission of greenhouse gases [7]. The development of processes for converting inexpensive glycerol into higher value products [8,9] is expected to make biodiesel production more economical, and this will encourage the building of more biodiesel processing plants.

As a result of the situation outlined above, it is necessary to find alternative uses for this glycerol overproduction, preferably by identifying a high-value use for this by-product.

It is well known that glycerol can be used to obtain ethanol and H_2 by anaerobic fermentation [10–14]. Nevertheless, a few studies have been published concerning the bioconversion of crude glycerol by *Escherichia coli* [6]. Most of the studies reported in the literature have involved the use of pure glycerol in order to prevent microbial growth inhibition due to the presence of impurities

* Corresponding author.
E-mail address: josemanuel.montesdeoca@uca.es (J.M. Gómez).

http://dx.doi.org/10.1016/j.biombioe.2016.04.015
0961-9534/

[13,15,16]. The use of pure glycerol, however, does not solve the problem of the excess glycerol produced industrially. Hence, it is necessary to improve the bacterial transformation process to include the use of crude glycerol.

In our previous work [17], we optimized the culture medium for the biotransformation of glycerol into ethanol by *E. coli* under anaerobic conditions by considering biomass productivity as a response variable for small-scale bioreactors (volume of 0.5 L, batch fermentations). In the study reported here, fermentations were carried out in bench-scale bioreactors (volume of 5.5 L) and attention was focused on the use of crude glycerol supplied directly from a biodiesel plant without any pre-treatment, with the associated economic advantages [18] for a future scale-up of the process. Indeed, previous studies concerning the transformation of glycerol into ethanol and H_2 found in the literature were mostly carried out in batch mode in Erlenmeyer flasks or 0.5 L working volume mini-reactors [19–21], thus meaning that it is necessary to proceed to the next level and perform the biotransformation of crude glycerol in a higher working volume in order to verify the influence of fermentation mode on the process. Fed-batch operation is utilized to achieve high cell densities, productivity, and yields of the desired products. In this mode of operation, the addition of nutrients during the process allows higher product concentrations to be achieved [22,23]. The aim of this work was to study the bioconversion of crude glycerol into ethanol and H_2 under anaerobic conditions by *E. coli* in two fermentation modes (batch and fed-batch mode) in order to assess the effect of operating mode on fermentation with a wild-type strain prior to scaling this process up, and is the first pilot-scale fed-batch fermentation of crude glycerol into ethanol using *E. coli* in a closed loop mixed reactor.

2. Materials and methods

2.1. Microorganism, glycerol source and culture media

E. coli wild-type MG1655, CGSC#6300, was obtained from the *E. coli* Genetic Resource Center (Coli Genetic Store Center, Department of Molecular, Cellular & Developmental Biology at Yale University). The strain was kept in 15% glycerol stocks at −80 °C.

Two types of glycerol raw material were used: crude glycerol and food-grade glycerol. Food-grade glycerol was 99% pure (GL0027, Ph Eur, USP, BP, FCC, E422) (Scharlab S.L., Spain) and was used as control for the crude glycerol biotransformation process. Crude glycerol was obtained from a biodiesel production plant (Abengoa Bioenergy S.A.) located in San Roque (Cádiz, Spain). Crude glycerol was obtained after transesterification and fatty acid methyl ester (FAME) separation steps and was used without any further treatment. The crude glycerol composition and selected chemical and physical properties are shown in Table 1.

The screening for initial glycerol concentration (0–60 g L^{-1}) (data not shown) performed in a previous stage resulted in three possible values or levels for a subsequent experimental design. As described in Cofré et al. [17], the culture medium was optimized in order to achieve maximum biomass productivity and three glycerol levels were tested (10, 20, 30 g L^{-1}). The final composition of the optimized medium was (g L^{-1}): crude glycerol (10), Na_2SO_4 (0.0806), NaCl (0.0152), $MgSO_4 \cdot 7H_2O$ (0.0310) and peptone (4.25).

Table 1
Crude glycerol composition and selected chemical and physical properties.

Parameter	Value	Unit
Glycerol	83.3	%(w w^{-1})
Water	8.4	%(w w^{-1})
NaCl	4.7	%(w w^{-1})
Methanol	0.06	%(w w^{-1})
Ash (including NaCl)	4.9	%(w w^{-1})
NGOM[a]	3.34	%(w w^{-1})
pH	5.3	
Density (ρ)	1.26	kg L^{-1}

[a] Non-glycerol organic matter (olefins, diglycerides, triglycerides, etc.).

2.2. Bioreactors

Two autoclavable glass bioreactors (Applikon Biotechnology B.V., Netherlands) with a total volume of 7 L and a working volume of 5.5 L (R5) were used. The bioreactors were equipped with temperature (internal coil), pH and agitation speed control. The temperature and agitation speed were maintained at 37 °C and 150 rpm, respectively. The pH was controlled at 6.30–6.35 by adding NaOH solution (2 M) using a pH controller (AX466 dual controller, ABB S.A, Spain) and a pH electrode (TB551, ABB S.A., Spain). Argon was used as the inert gas (Ar quality 5.0, Abelló Linde S.A., Spain). The argon stream was sterilized using a filter with a 0.2 μm pore size (SLG05010, Millipore, USA).

The substrate feed was controlled using a peristaltic pump (L/S fixed-speed drive 30 rpm, Masterflex®, Cole-Parmer, USA) connected to a programmable logic module (LOGO! 12/24RC, Siemens AG, Spain). The off-gas stream passed through a condenser with ethylene glycol/water circulation (4 °C) in order to prevent evaporation effects or stripping phenomena. All bioreactors and culture media were sterilized at 121 °C and 1 atm.

A pilot-scale closed loop mixed reactor with a working volume of 200 L and a gas diffusion membrane, shown in Fig. 1, was utilized. The temperature was kept constant at 37 °C by circulating water through the coil of the reactor via an immersion thermostat (Termotronic, Selecta, Spain), pH was maintained at 6.30–6.35 by addition of NaOH 6.25 M. The inert gas (Ar) was bubbled at 0.65 L min^{-1} to produce H_2 displacement. Stirring of the medium was performed by internal recirculation, using two centrifugal pumps connected in parallel (MP20R, Selecta, Spain). Fresh inert gas was injected during fermentation (Ar quality 5.0, Abelló Linde SA, Spain) after filtration through a 0.2 μm filter (SLG05010, Millipore, USA).

The off-gas was passed through a condenser containing a circulating ethylene glycol/water mixture at 4 °C using a flow circulator (F12, Julabo Labortechnik GmbH, Germany).

To increase agitation of the system a membrane compressor (N035.3AN18, KNF Neuberger Group, Germany), which allowed an internal loop to be created, was mounted, maintaining a constant gas recirculation of 24 L min^{-1}.

Fresh medium was fed into the reactor using a peristaltic pump (L/S Standard drive Masterflex Cole-Parmer) connected to a programmable LOGO! 12/24RC logic device (Siemens, Spain).

2.3. Inoculum preparation and fermentation conditions

Four stages were carried out in order to grow the inoculum until an optical density of 0.6 (at 600 nm) was achieved. The inoculum was prepared from a single colony in a tube (10 mL), which this was transferred from the tube to a serum bottle (50 mL) and then from the serum bottle to a bioreactor (500 mL) according to the procedure described by Cofré et al. [17]. The inoculum size used in this study was 10% (v/v) for each fermentation. According to authors such as Dharmadi et al. [12] and Murarka et al. [11], the addition of argon is necessary to remove the H_2 produced during the fermentation of glycerol from the system and to prevent the detrimental effects that this gas has on the process. In fact, these authors established that the absence of a stream of argon harmed

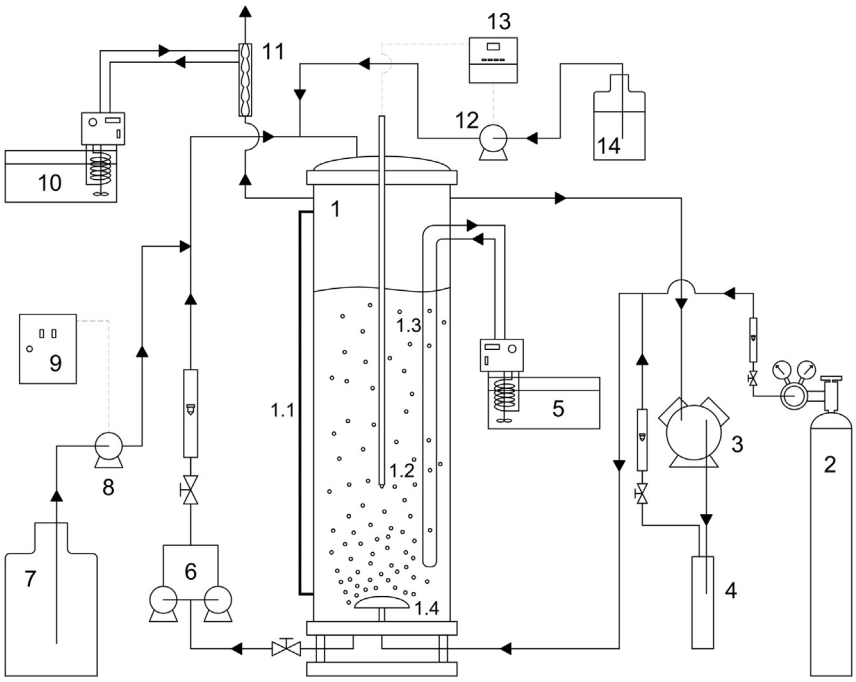

Fig. 1. Pilot-scale closed-loop mixed reactor system. 1) reactor working volume: 200 L (1.1 liquid level meter, 1.2 pH probe, 1.3 heating coil, 1.4 gas diffusion membrane), 2) Ar bottle, 3) compressor, 4) rotameter, 5) immersion bath (water at 37 °C), 6) water recirculation pump, 7) feeding tank, 8) peristaltic pump for feeding, 9) programmable logic module, 10) cooling system (ethylene glycol/water at 4 °C), 11) condenser, 12) peristaltic pump (NaOH), 13) pH controller/transmitter, 14) NaOH 6.25 M.

the process and the resulting glycerol consumption and biomass growth were only 20% and 0.15 g L^{-1}, respectively.

The effect of inert gas (Ar) supply (fermentation volume of 5.5 L) on batch mode fermentation was studied. Three experiments were carried out: (i) food-grade glycerol as substrate and Ar supplied (2.5×10^{-3} vvm) (B1), (ii) crude glycerol as substrate and Ar supplied (2.5×10^{-3} vvm) (B2), and (iii) crude glycerol as substrate and no inert gas stream (B3). The initial concentration of glycerol was 9.6 ± 1.4 g L^{-1}.

The fed-batch fermentation mode was studied under two feeding regimes: (i) constant feed at two crude glycerol concentrations (fermentation F1 at 12.6 g L^{-1} and fermentation F2 and 37.7 g L^{-1}), and (ii) exponential feed at three crude glycerol concentrations (12.6, 37.7 and 62.8 g L^{-1}, F3, F4 and F5, respectively). All fed-batch fermentations involved a previous 2 L batch step inoculated with 500 mL of exponential culture followed by a feeding step to give a final volume of 5.5 L. Details of the exponential feeding equation are given in Section 3.2.

The pilot reactor was inoculated with 5 L of culture at an optical density of approximately 0.6 (600 nm), previously cultured in a reactor R5. The culture medium used during the batch step corresponded to the optimized culture medium, completing a volume of 50 L. Once the discontinuous phase had been completed, the feeding step under an exponential function was started up to a final fermentation volume of 197.5 L. The fermentation received an identification code (RP).

2.4. Analytical methods

Biomass was determined by measuring the optical density at 600 nm. Glycerol and ethanol concentrations were determined by HPLC (LaChrom Elite®, VWR, Spain) using a system equipped with an HPX-87H organic acid column (Bio-Rad, USA). The operating conditions were 5 mM H_2SO_4 (mobile phase) at 0.6 mL min^{-1} and a column temperature of 50 °C. Samples were pre-filtered (pore size 0.22 μm), placed into cryotubes, immediately frozen and stored until analysis. H_2 concentration was determined by gas chromatography (450-GC, Bruker, Spain) using a system equipped with a Poraplot Q FLOT FS 25 m × 0.53 mm × 20 μm (CP7554, Agilent Technologies S.A, Spain) column and TCD detector. The operating conditions were as follows: oven temperature 33 °C, detector temperature 150 °C, filament temperature 250 °C, injector temperature 150 °C. Gas samples were collected in Tedlar® bags connected to the off-gas bioreactor.

The coefficients of variation (CVs) for concentration measurements of biomass, glycerol, ethanol and H_2 were 4.39, 9.90, 20.03 and 4.78%, respectively (obtained from replicates of 100 experiments working with 2 mL of culture medium at a fermentation time of 48 h (data not published)).

2.5. Kinetic parameters

The specific growth rate (μ; h^{-1}) was estimated by plotting total cell concentration against fermentation time in a log-linear plot, for batch fermentation. The slope of the curve thus obtained during exponential growth was used as the specific growth rate. Yields ($Y_{X/S}$, $Y_{P/S}$), expressed as g g^{-1} of cell or ethanol per glycerol, respectively, were calculated as the increase in cell mass or ethanol per glycerol consumed once the cultures reached the stationary phase.

2.6. Hydrodynamic characterization of reactors

Hydraulic tests were performed for R5 and pilot reactors by constructing RTD (residence time distribution) curves. A three channel multimeter 44 (Crison Instruments S.A., Spain) that can be used to measure and regulate the electric conductivity (E.C.), a temperature board (two relays and two 4–20 mA outputs) and a conductivity cell (scale 0.5–80000 μS/cm, 0–100 °C) was used for this purpose. A solution of NaCl (4 M) was used as tracer and variations of E.C. with time were recorded at the output of the reactor.

The effect of agitation speed and gas bubbling were also evaluated. The conditions studied for R5 (at a constant volume of 5.5 L) were agitation speed (75, 150, 300 rpm) each one with 20% and 100% of Ar flow rate, with 20% representing the design gas flow rate used in each of the actual fermentations performed. The conditions studied for RP (at constant gas recirculation 24 L min^{-1}) were a working volume of 50, 125, 200 L each, with 50% and 100% liquid recirculation, with 100% representing the fraction of the design liquid recirculation flow rate used in the actual fermentations (200 L h^{-1}).

3. Results and discussion

3.1. Batch fermentations

The results obtained show that the use of crude glycerol did not have a negative effect on the glycerol fermentation process as the glycerol consumed, ethanol produced and biomass growth were of same order of magnitude for fermentations B1 (Fig. 2a) and B2 (Fig. 2b). The only exception was for fermentation B3 (Fig. 2c), where an H_2 inhibition effect was observed. The final biomass concentration reached for fermentations B1 and B2 was 0.235 ± 0.047 g L^{-1}, this value being similar to a previously reported biomass concentration of 0.27 g L^{-1} [11]. However, the biomass growth in B3 was lower, with a value of 0.126 ± 0.005 g L^{-1} due to H_2 inhibition.

The specific growth rate (μ), glycerol to biomass yield ($Y_{X/S}$) and glycerol to ethanol yield ($Y_{P/S}$) values obtained for fermentation B1 were, respectively, 0.03 h^{-1}, 0.031 ± 0.003 g g^{-1} and 0.48 ± 0.10 g g^{-1} (96% of the maximum theoretical value according to Cintolesi et al. [20]). The $Y_{X/S}$ and $Y_{P/S}$ values for fermentation B2 were 0.038 ± 0.003 g g^{-1} and 0.57 ± 0.11 g g^{-1} (114% of the maximum theoretical), respectively. The value of $Y_{P/S}$ cannot be higher than 0.5 g g^{-1} if glycerol is the only carbon source. It can be seen from the results in Table 1 that crude glycerol contains other carbon compounds. As such, other carbon sources could be used by *E. coli* or the high CV for ethanol concentration measurements (20.03%) could be responsible. Thus, the results are quite similar and the use of crude glycerol did not affect the yields.

The final ethanol concentrations achieved were 3.82 ± 0.76 and 3.64 ± 0.73 g L^{-1} for fermentations B1 and B2, respectively. These values are consistent with previously reported data for similar experimental conditions using pure glycerol [11].

It has been reported in the literature [7,24–27] that the impurities present in crude glycerol could act as inhibitors of the fermentation process. In our case, the glycerol supplied had a methanol content of 0.06% (w w^{-1}), and this could have an inhibitory effect on the bacterial population. We therefore evaluated the possibility of working with crude glycerol directly, without any prior purification step. It can be seen from Fig. 2 that there was no significant difference between the use of crude glycerol and food-grade glycerol for fermentations B1 and B2. Crude glycerol can therefore be used without any pre-treatment. The absence of inhibition is a great advantage because it avoids the need for a pre-treatment step and this will lead to cost savings in a scaled-up process.

H_2 production was followed for B2 and B1, with the latter considered as the control. For crude glycerol (B2) a specific H_2 productivity of 2.5 ± 0.11 mmol g^{-1} h^{-1} (4.4 ± 0.44 mmol $g_{crude\ glycerol consumed}^{-1}$) was obtained after a fermentation time of 50 h (Table 2). The H_2 productivity was lower when using food-grade glycerol (B1) as substrate, with a value of 1.48 ± 0.07 mmol g^{-1} h^{-1} (4.5 ± 0.45 mmol $g^{-1}_{crude\ glycerol\ consumed}$). The performance of the fermentation using crude glycerol (B2) was therefore better than that obtained using food-grade glycerol (B1). Ito et al. [28] used a continuously packed bed reactor with a working volume of 60 mL and *Enterobacter aerogenes H-101* and obtained a maximum H_2 production rate of 30 mmol L^{-1} h^{-1} with glycerol from a biodiesel plant. This value is 20 times higher than the rate estimated for B2 (1.3 ± 0.06 mmol L^{-1} h^{-1}), although the glycerol to H_2 yield reported by these authors was 7.7 mmol $g_{crude\ glycerol}^{-1}$ for an initial crude glycerol concentration of 10 g L^{-1}, while the H_2 yield for B2 was of the same order as indicated above. Moreover, Vlassis et al. [29] investigated the production of H_2 from glycerol under anaerobic batch conditions and used a mesophilic anaerobic sludge previously treated to eliminate methanogenic populations. These authors studied the effect of initial glycerol concentration and found that the best results were obtained for an initial concentration of 8.3 g_{COD} L^{-1} (6.85 $g_{glycerol}$ L^{-1}) and an initial glycerol feed of 31.8 mL g^{-1} with a fermentation time of 50 h. For B1, the H_2 produced with respect to the initial glycerol fed into the system was about two times higher at 74.3 ± 7.6 mL g^{-1}. Furthermore, the results for glycerol to ethanol yield are similar to those obtained in other studies concerning H_2 production from formate [30] or organic waste [31].

In the case of fermentation B3, crude glycerol consumption decreased from 65 ± 6.4% to 21 ± 2.1%, the final ethanol concentration decreased by 59% from 3.64 ± 0.73 to 1.49 ± 0.30 g L^{-1} and the final biomass concentration was 0.12 ± 0.005 g L^{-1}. H_2 production was also adversely affected, with only 21.6 ± 1.0 mmol of total H_2 (1.8 ± 0.18 mmol $g^{-1}_{crude\ glycerol\ consumed}$) produced. Therefore, bubbling a stream of Ar through the system to remove H_2 as it is produced has a positive effect on the process in terms of glycerol consumption, ethanol production, biomass growth and H_2 production.

An estimation of substrate demand was carried out from the results obtained in these experiments. The definition of the substrate to biomass yield ($Y_{X/S}$) enables a relationship to be found between the rates of substrate uptake (d(SV)/dt) and biomass growth (d(XV)/dt) according to Equation (1):

$$\frac{d(SV)}{dt} = -\frac{1}{Y_{X/S}}\frac{d(XV)}{dt} \quad (1)$$

The biomass growth rate was evaluated by approximation to ΔX/Δt from the fermentation profile obtained for fermentation B2 as the difference between the beginning of the growth phase and the beginning of the stationary phase. The biomass growth rate was 2.59 ± 0.11·10^{-3} g L^{-1} h^{-1}, the glycerol uptake (−Δ(SV)/Δt) was 0.4078 ± 0.08 g h^{-1} and $Y_{X/S}$ was 0.035 ± 0.007 g g^{-1}.

In our previous work aimed at optimizing the culture medium [17], the consumption of glycerol and ethanol production continued even though biomass reached a steady state. This trend was repeated on the larger scale (working volume of 5.5 L) and we therefore considered it of interest to study the biotransformation under fed-batch mode. Thus, biomass growth could be achieved in the first stage and then, in a second phase, glycerol could be fed into the system to produce ethanol and H_2.

3.2. Fed-batch fermentations

These experiments were carried out by adding nutrients exclusively at the batch step and only a solution of crude glycerol in water was fed into the reactor in the feeding step. The batch step was carried out until the biomass reached an O.D. ≈ 0.6 (600 nm) and then the feeding step was started.

Two fermentations were carried out with a constant feeding regime (F1 and F2). The feed flow rate was calculated by considering the glycerol uptake (0.4078 g h^{-1}) obtained in the batch fermentation (B2) and a crude glycerol concentration in the inlet medium of 12.6 g L^{-1}. The constant feed flow rate was

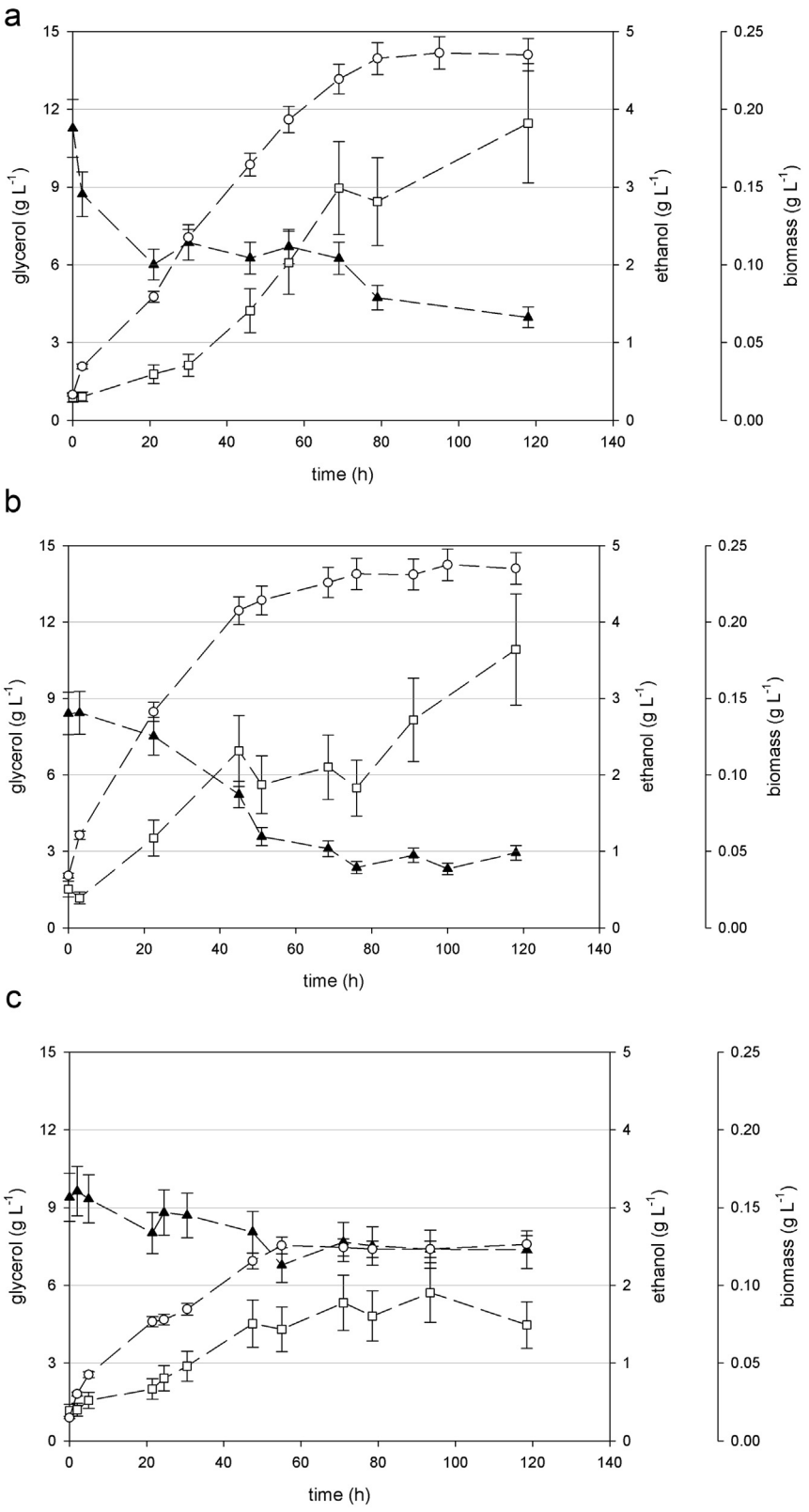

Fig. 2. Profiles for batch fermentations. (a) Food-grade glycerol as substrate with Ar supplied (B1). (b) Crude glycerol as substrate with Ar supplied (B2). (c) Crude glycerol as substrate with no inert gas stream (B3). (□) ethanol concentration, (▲) glycerol concentration, (○) biomass concentration.

0.68 mL min^{-1}, therefore the theoretical consumption time was 108 h. For fermentation F2 the feed flow rate was kept constant and the crude glycerol concentration in the inlet medium was increased to 37.7 g L^{-1}.

The crude glycerol mass consumptions for F1 and F2 were 27.5 ± 5.5 and 28.6 ± 5.7 g, respectively. The results obtained in

Table 2
Summary of main results for batch and fed-batch fermentations studied.

Fermentation	Modality/feed condition	Working volume (L)	Duration (h)	Glycerol consumed (%)	Final ethanol (g L^{-1})	H_2 produced (mmol)	H_2 produced (mmol/$g_{glycerol\ consumed}^{-1}$)
B1	Batch/–	5.5	118	65 ± 6.4	3.82 ± 0.77	181 ± 8.7	4.5 ± 0.45
B2	Batch/–	5.5	118	65 ± 6.4	3.64 ± 0.73	132 ± 6.3	4.4 ± 0.44
B3	Batch/–	5.5	118	21 ± 2.1	1.49 ± 0.30	22 ± 1.1	1.8 ± 0.18
F1	Fed-batch/constant feed 12.6 g L^{-1}	5.5	100	51 ± 5.0	3.16 ± 0.63	167 ± 8.0	6.2 ± 0.61
F2	Fed-batch/constant feed 37.7 g L^{-1}	5.5	100	24 ± 2.4	3.53 ± 0.71	281 ± 13.4	9.8 ± 0.97
F3	Fed-batch/exponential feed 12.6 g L^{-1}	5.5	88	99 ± 1.0	4.12 ± 0.83	361 ± 17.3	10.1 ± 1.00
F4	Fed-batch/exponential feed 37.7 g L^{-1}	5.5	88	77 ± 7.6	6.28 ± 1.26	523 ± 25.0	6.4 ± 0.63
F4*	Fed-batch/exponential feed 37.7 g L^{-1}	5.5	160	100 ± 9.9	7.58 ± 1.52	–	–
F5	Fed-batch/exponential feed 62.8 g L^{-1}	5.5	88	61 ± 6.0	8.59 ± 1.72	680 ± 32.5	6.3 ± 0.62
F5*	Fed-batch/exponential feed 62.8 g L^{-1}	5.5	160	84 ± 8.3	8.68 ± 1.74	782 ± 37.4	5.2 ± 0.51
RP	Fed-batch/exponential feed 37.7 g L^{-1}	200	160	52.7 ± 5.2	5.4 ± 1.08	449.18 ± 21.47	0.2 ± 0.01
RP*	Fed-batch/exponential feed 37.7 g L^{-1}	200	250	100 ± 9.9	8.5 ± 1.70	–	–

* Considering feeding step and post-batch step.

batch experiments were quite similar, therefore higher crude glycerol concentrations were not studied. The crude glycerol biotransformation percentages were 51 ± 5.0% and 24 ± 2.4% for F1 and F2, respectively (Table 2). The final ethanol concentrations were 3.16 ± 0.63 g L^{-1} (F1) and 3.53 ± 0.71 g L^{-1} (F2) and the mean value for biomass growth was 0.212 ± 0.009 g L^{-1} at 80 h of fermentation, including the prior batch step. F1 gave the lowest H_2 production of 167 ± 8.0 mmol (6.2 ± 0.61 mmol $g_{crude\ glycerol\ consumed}^{-1}$), with F2 producing 281 ± 13.4 mmol (9.8 ± 0.97 mmol $g_{crude\ glycerol\ consumed}^{-1}$). However, both of these values are higher than that obtained in the batch fermentation (B2) (Table 2). The profiles for ethanol, glycerol and biomass are shown in Fig. 3 and H_2 production is represented in Fig. 4.

Three fermentations (F3, F4 and F5) were carried out in the exponential feeding regime. The feed flow rate was calculated by considering the specific growth rate (μ_0) and $Y_{X/S}$ obtained in fermentation B2; i.e., 0.029 h^{-1} and 0.035 ± 0.007 g g^{-1}, respectively. The initial flow rate (F_0, Equations (2) and (3)) was estimated by considering an initial biomass concentration of 0.31 g L^{-1}, a batch volume of 2 L and a substrate concentration in the feed stream (S_F) of 62.8 g L^{-1} (concentration used in fermentation F5).

$$\frac{dV}{dt} = F_0 e^{\mu_0 t} \tag{2}$$

where for a constant specific growth rate [32].

$$F_0 = \frac{\mu_0 \cdot X_0 \cdot V_0}{Y_{X/S} \cdot S_F} \tag{3}$$

The same exponential feeding equation was used for F4 and F3 but the substrate concentrations in the feed stream were decreased to 37.7 and 12.6 g L^{-1}, respectively. The total masses of crude glycerol fed into the system were 36.3, 106 and 177 g for F3, F4 and F5, respectively. Profiles for biomass, ethanol, glycerol and H_2 were obtained for all fermentations. The feeding step took 88 h and a post-batch step of 72 h (F4* and F5*) was considered to observe the evolution of crude glycerol consumption and ethanol production. The profiles for the three sets of conditions are shown in Fig. 5 and the results for crude glycerol consumption, ethanol production and yields for biomass and ethanol are summarized in Table 2.

The results obtained under an exponential feeding regime are better than those obtained in constant feed and batch fermentations (Table 2). The best results for fed-batch fermentation were obtained for F4*, with 100% of crude glycerol consumed and a final ethanol concentration of 7.58 ± 1.52 g L^{-1}. A higher ethanol concentration was reached for F5 but the glycerol consumption was lower, with values of 61 ± 6.0% and 84 ± 8.3% for F5 and F5*, respectively. The ethanol and H_2 productions from crude glycerol reported by Yazdani and Gonzalez [6] are higher than the results obtained in this study. However, these authors worked with a genetically modified strain SY03. Similarly, Yang et al. [16], who worked with *E.* coli YL15, obtained a final ethanol concentration of 1 g L^{-1} and a glycerol to ethanol yield about 20% of that obtained in this work. A comparison with previously reported results for H_2 and ethanol production using glycerol as the carbon source is possible from the results shown in Table 3. In this study the operation mode was optimized when working with a wild-strain of *E. coli*. It is therefore highly likely that the use of genetically modified strains in fed-batch reactors will allow higher ethanol concentrations and/or H_2 production to be achieved, as has been reported in the literature [33].

In order to explore the use of glycerol without dilution, two fermentations were carried out under constant-volume fed-batch mode [34] (data not shown). Pure crude glycerol (without water) was added as fresh feed at the same mass flow rate as for F4. During total fed-batch step time (88 h), a crude glycerol final concentration of 24 g L^{-1} in the reactor was achieved and crude glycerol consumption reached just 30%. This result suggests that dilution of crude glycerol with water is necessary, as indicated in the literature [11] and discussed in our previous work.

3.3. Pilot scale fed-batch fermentations

The same operational conditions as for F4 were replicated on a pilot scale (RP) and an initial specific feeding rate (ISFR) was calculated for both experiments according to:

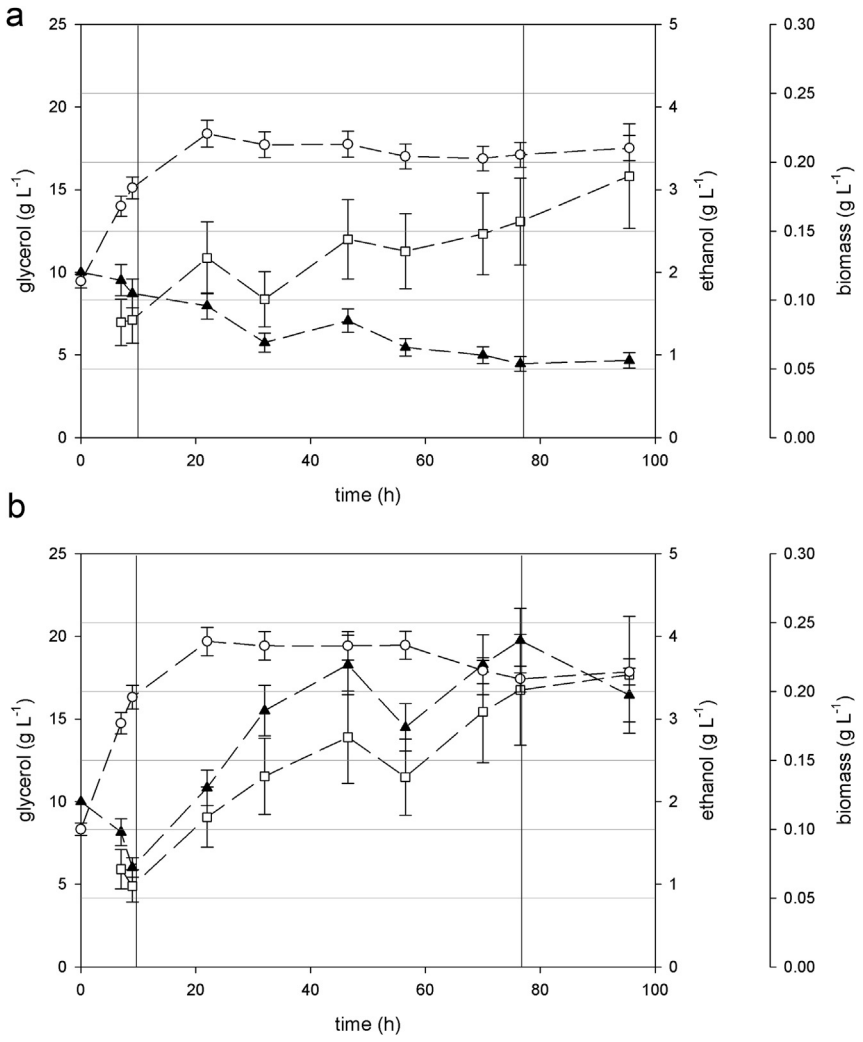

Fig. 3. Profiles for fed-batch fermentations with a constant feeding regime. Crude glycerol concentrations in inlet medium: (a) 12.5 g L^{-1} (F1); (b) 37.7 g L^{-1} (F2). (□) ethanol concentration, (▲) glycerol concentration, (○) biomass concentration.

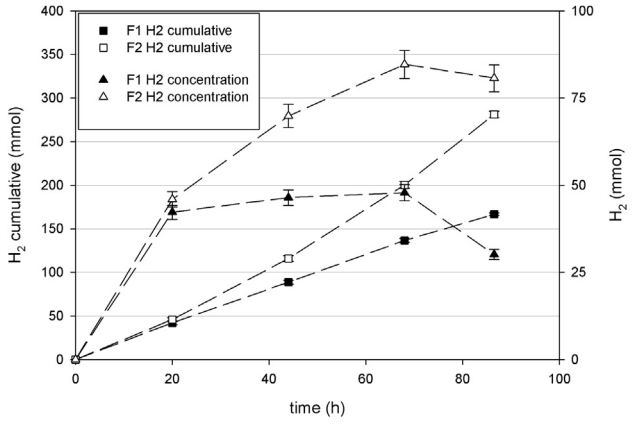

Fig. 4. Hydrogen production in fed-batch fermentations with a constant feeding regime; F1 and F2.

$$ISFR = \frac{\left(F \cdot S_f\right)_0}{(X \cdot V)_0} \quad (4)$$

An ISFR value of 0.6456 g g^{-1} h^{-1} was calculated for F4 and used in RP.

In light of the results obtained in R5, the pilot scale experiment was carried out by adding nutrients exclusively in the batch step, with only a solution of crude glycerol in water being fed into the reactor during the feeding step. The process was divided into three stages: batch of 63.5 h (S1), fed-batch of 96 h (S2) and post-batch of 92 h (S3). The final biomass concentration at the end of S1 was 1.19 g L^{-1}, which is about four times the biomass concentration obtained for R5 at the end of the batch stage. This result can be explained by the presence of O_2 in the system. Indeed, Durnin et al. [21] reported a biomass concentration of about 1.7 g L^{-1} when working with *E. coli* BW25113 in batch mode under microaerobic conditions with an initial glycerol concentration of 18 g L^{-1}. The ethanol concentration reported by these authors is 5.5 g L^{-1} and is therefore higher than the concentration obtained at the end of S1 (3.5 ± 0.70 g L^{-1}). However, the initial crude glycerol concentration in S1 was 10 g L^{-1}, thus meaning that if ethanol yield is compared, a value of 0.28 and 0.35 g g^{-1} of consumed glycerol was obtained by Durnin et al. [21] and in this work, respectively. Microaerobic conditions have been justified in terms of the incorporation of an electron acceptor (O_2) and to avoid the need to add tryptone or yeast extract [21]. However, although *E. coli* can utilize glycerol as a

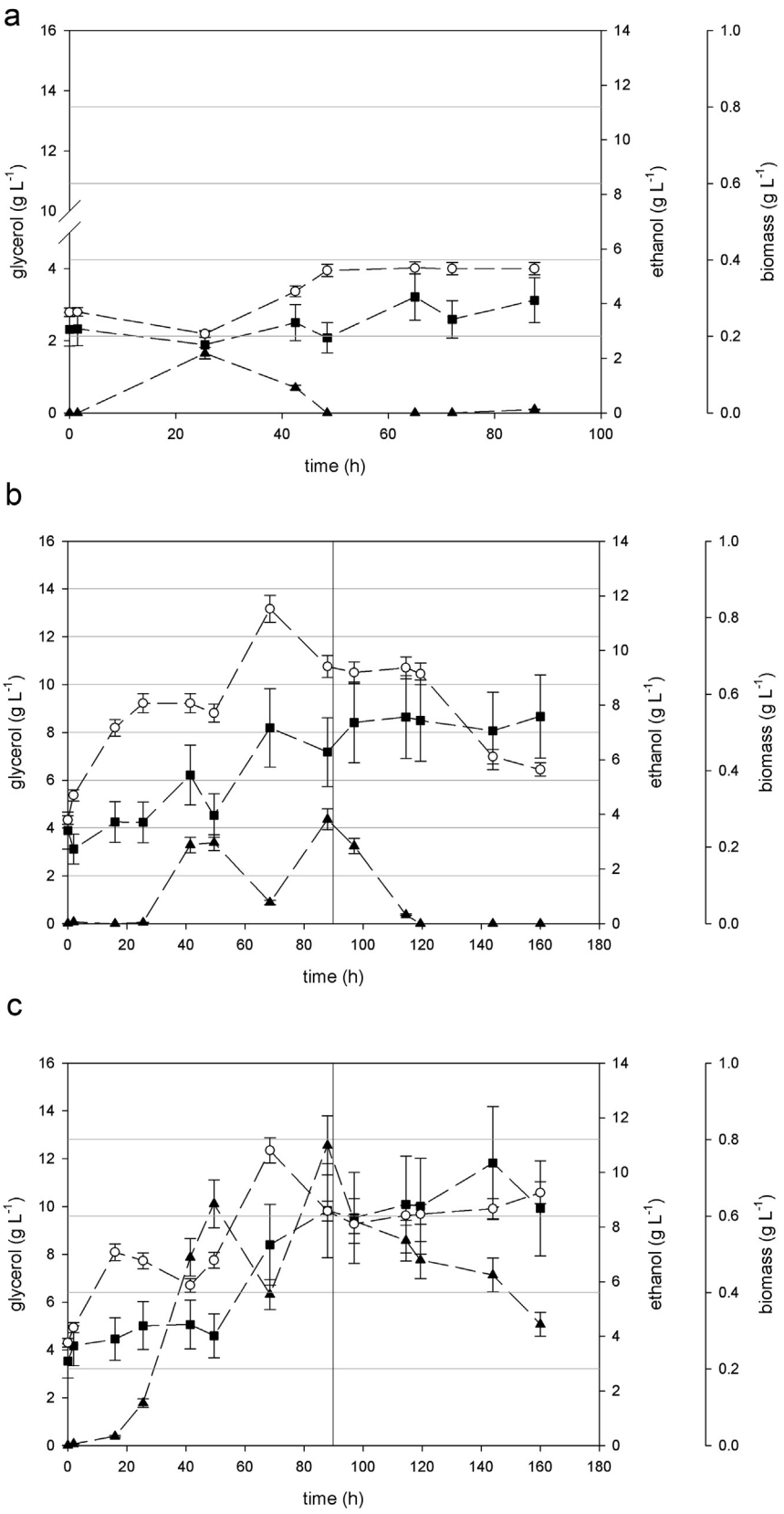

Fig. 5. Profiles for fed-batch fermentations with an exponential feeding regime. Crude glycerol concentrations in inlet medium: (a) 12.6 g L^{-1} (F3); (b) 37.7 g L^{-1} (F4); (c) 62.8 g L^{-1} (F5). (□) ethanol concentration, (▲) glycerol concentration, (○) biomass concentration.

substrate in the presence of oxygen, this results in enhanced growth rather than the production of reduced compounds. Jitrwung and Yargeau [35] studied the effect of the presence of oxygen during the production of hydrogen from glycerol with

Table 3
Summary table about crude glycerol fermentation by strains of *E. coli* to produce ethanol and hydrogen.

Strain	Carbon source	Nitrogen source	Ethanol production (mol mol^{-1})	H_2 production (mol mol^{-1})	Reference
E. coli SS1	PG	Peptone and yeast extract	1.00	0.24	[13]
E. coli KC3	PG	Peptone and yeast extract	0.102	–	[16]
E. coli YL15	PG	$(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_2H$-citrate	0.206	–	[16]
E. coli SY03	CG	Tryptone	1.01	1.02	[6]
E. coli BL21	CG	$(NH_4)_2SO_4$	0.04	–	[9]
E. coli (pARD33)	CG	$(NH_4)_2SO_4$	0.26	–	[9]
E. coli MG1655	CG	Tryptone and yeast extract	0.92	–	[20]
E. coli MG1655	CG	none	0.66	0.56	This work (F4)

Enterobacter aerogenes in serum bottles of 125 mL, and concluded that a reduction in the amount of oxygen present during the initial steps of the process to near-zero levels increased the production of hydrogen. However, these authors note that a complete absence of oxygen is not beneficial and suggest that an argon-oxygen mixture containing 2.5–7.5% oxygen should be used to pressurize the serum bottles during the inoculation stage. The use of microaerobic conditions early in the process is a condition that can be studied in the future; however, the addition of oxygen must be very well controlled and in low proportions. During this study, preference was given to studying the biotransformation of glycerol into ethanol and hydrogen under anaerobic conditions, in line with most of the published literature in this field [6,10,20,28,36,37]. However, anaerobic conditions are difficult to achieve when working with an RP, therefore another chemical compound, such as sodium sulfite, is added to avoid any type of inhibitory or toxic effects on *E. coli*. The presence of oxygen during the early stages of the fermentation carried out in RP may be supported by the fact that analysis of the gas stream leaving the reactor showed an oxygen concentration of 3.4% in this gas stream at the end of S1.

Profiles of crude glycerol consumed and biomass and ethanol produced are shown in Fig. 6, where the vertical lines indicate the borders between the three stages mentioned. During S2, the yield of biomass relative to crude glycerol consumed ($Y_{X/S}$) was equal to 0.03 g g^{-1} and in the case of ethanol $Y_{P/S}$ reached a value of 0.43 g g^{-1}, with an ethanol concentration of the order of 5.4 ± 1.08 g L^{-1}. This value for the final ethanol concentration is of a similar magnitude to the results obtained in R5 and is 14% lower than the result obtained for F4. At the conclusion of S2 the system had achieved a crude glycerol consumption of 52.7%, reaching a concentration in the medium of 10.6 g L^{-1}. Moreover, biomass reached a steady state after 155 h of fermentation and remained at a constant value of 0.65 g L^{-1} up to 250 h. After S3, all the crude glycerol initially present had been consumed and the maximum ethanol concentration was reached. Indeed, during S3 crude glycerol was completely consumed within 68 h (≈75% of total time), which also coincided with the maximum ethanol concentration reached (8.15 ± 1.63 g L^{-1}). The overall behavior of RP was similar to that of F4. As regards crude glycerol consumption and ethanol production during the post batch (S3), the maximum ethanol concentration achieved in F4* was equal to 7.58 ± 1.51 g L^{-1} whereas in RP* (S1+S2+S3) the maximum concentration was higher and equal to 8.51 g L^{-1}. Hydrogen production in the system was monitored at the same time, with total output being 449.18 mmol (0.19 mmol H_2 $g^{-1}_{glycerol}$, 160 h), which is equivalent to a hydrogen volume of approximately 0.05 L L^{-1} of reactor (at 1 atm and 25 °C). The mass of crude glycerol consumed is equivalent to $4.2 \cdot 10^{-3}$ L $g^{-1}_{glycerol}$, a value well below that obtained in R5 (0.14 L $g^{-1}_{glycerol}$). The oxygen content in the off-gas stream from the reactor, expressed as a percentage, was also determined. After fermentation for 66 h, this value was maintained below 1% until the end of fermentation. The results for crude glycerol consumption, ethanol production and yields for biomass and ethanol are presented in Table 2.

3.4. Hydrodynamic characterization of the reactor

A real reactor can present a flow pattern with some deviations from ideal plug flow or mixed flow [38]. The residence time distribution (RTD) or exit age distribution function (E_t) represent the

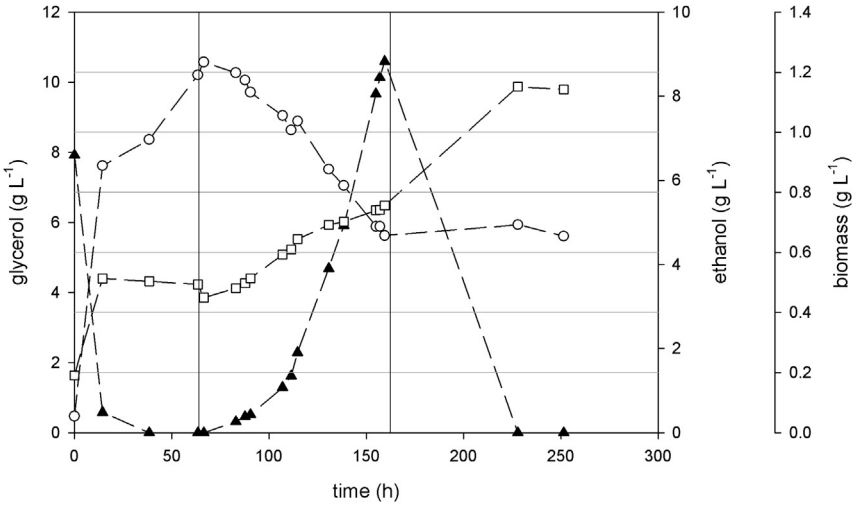

Fig. 6. Profiles for pilot-scale fed-batch fermentation (RP) with an exponential feeding regime. Crude glycerol concentrations in inlet medium 37.7 g L^{-1}, (□) ethanol concentration, (▲) glycerol concentration, (○) biomass concentration.

time spent in a vessel by the flowing material. E_t can be represented graphically with respect to time (t) and the area under the curve must be equal to 1, according to:

$$\int_0^\infty E_t dt = 1 \tag{5}$$

For an ideal system E_t can be described as an exponential decay. In fact, considering M to be the mass of tracer and C a measure of tracer presence (i.e. concentration) at the output of the system, and v to be the volumetric flow through the vessel, the E_t function for an ideal mixed-flow pattern can represented as shown in Equation (6):

$$E_t = \frac{v}{V} e^{-\frac{v}{V}t} \tag{6}$$

When a deviation from the ideal flow pattern occurs, compartment models can be utilized. In this work two different compartment models and an ideal mixed-flow model were considered. The compartment models utilized here were the dead zones model (model 1) and the short-circuiting model (model 2) [39]. The E_t function for model 1 can be represented as shown in Equation (7).

$$E_t = \frac{v}{V_m} e^{-\frac{v}{V_m}t}, \quad con \quad V = V_m + V_d \tag{7}$$

Where V_m is the mixed volume fraction and V_d the non-mixed volume fraction.

The E_t function for model 2 is shown in Equation (8):

$$E_t = \frac{v_a^2}{Vv} e^{-\frac{v_a}{V}t}, \quad con \quad v = v_a + v_b \tag{8}$$

where v_a is the active flow passing through the mixing region and v_b is the bypass flow.

The E_t curves obtained for R5 (data not shown) showed that working at 150 rpm and 20% Ar (2.5×10^{-3} vvm) allowed operation under mixed conditions very close to an ideal model of complete mixing, with a 97.6% match with respect to working volume ($r^2 = 0.9869$ for model 1). These conditions ensure the adequate distribution of nutrients, biomass and products within the reactor for all fermentations performed.

The mixing system in the RP reactor corresponds to a liquid recycle stream drawn from the bottom and fed to the top of the reactor. The effectiveness of this mixing system can be affected by the total volume of liquid in the reactor. For this reason, a study of six different conditions was performed, with the liquid recirculation flow and the liquid volume inside the reactor being selected as study variables. The degree of fit of the data obtained for the E_t function for models 1 and 2 was evaluated. A good correlation ($r^2 > 0.9950$) was obtained for each condition, and a summary of the results is shown in Table 4 (where σ_θ^2 is dimensionless variance).

A liquid recirculation of 100% corresponds to the design flow used during fermentation and is equivalent to 200 L h^{-1}. The worst condition in terms of degree of mixing corresponded to condition 1, where the fraction of actual flow that crossed the mixing zone was less than 50%. For this condition, there was a break in the curve of E_t function at about 3500 s. As such, a replica of the test was performed and the same effect was observed at about 3800 s. Consequently, this mixing condition was not used at any time during the fermentation of crude glycerol. The degree of fit of each of the models considered was determined from the E_t curves resulting from the study of conditions 2 to 6. Model 2 provided the best fit for all conditions studied, with the exception of conditions 5 and 6. Indeed, 95% of the flow effectively crossed the mixing zone for condition 4, which is a minor deviation from the ideal model, a situation that can clearly be seen in Fig. 7. This small variation, and the fact that the actual flow rate in the mixing zone for condition 2 was equal to 87.47%, justifies the use of a liquid recirculation flow rate of 100%. In the case of conditions 5 and 6, model 1 provided a dead volume of 5% and 15%, respectively. After fermenting crude glycerol for 63.5 h, the feeding step commenced, with an initial volume of 50 L. To complete the period of 138.5 h (effective feeding of 75 h), an additional volume of 123 L was added to give a maximum volume of 197.5 L and 159.5 h of fermentation (21 h more than above). For this reason, and although a higher degree of mixing was achieved with 50% liquid recirculation at the limit point of 200 L, it was decided to maintain a constant liquid recirculation of 100% throughout the fermentation. It is considered that by the end of fermentation, the dead volume fraction is between 5% and 15%.

4. Conclusions

Biotransformation of crude glycerol into ethanol and H_2 with *E. coli* has been studied and it has been demonstrated that the use

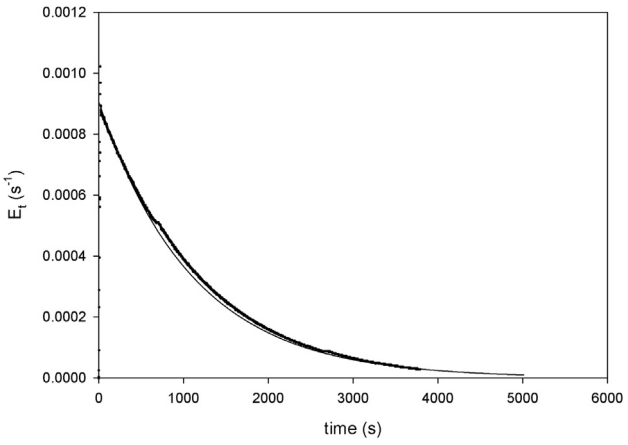

Fig. 7. Comparison for exit age distribution function E_t (real and ideal pattern) for condition 4 in RP. (■) Real E_t, (-) Ideal E_t.

Table 4
Summary of analytical results for dead zones (model 1) and short-circuiting model (model 2) in RP (pilot scale fed-batch reactor fermentation).

Condition	Volume	Recirculation rate	t_m (s)	σ_θ^2	r^2	V_m (L)	v_a/v (%)
1	50 L	50%	1381.5	0.4956	0.9952	n.m.	49.16
2	50 L	100%	1031.3	0.6455	0.9996	n.m.	87.47
3	125 L	50%	1036.9	0.6767	0.9972	n.m.	81.82
4	125 L	100%	1005.8	0.7153	0.9991	n.m.	95.09
5	200 L	50%	1388.9	0.7165	0.9994	189.86	n.m.
6	200 L	100%	1249.2	0.6795	0.9991	177.94	n.m.

n.m.: did not fit the model.

of glycerol without any prior purification step is possible. The beneficial effect of displacing the H_2 produced with a stream of Ar has also been verified. The experimental results show that a fed-batch mode under an exponential feeding regime is a promising strategy to increase ethanol and H_2 production and the use of crude glycerol. It is important to mention that this work has allowed the effect of operating mode on the fermentation process with a wild-type strain of *E. coli* to be assessed, with the product yields obtained being in the same range as in other reported studies with genetically modified strains. The hydrodynamic characterization of R5 and RP reactors has allowed us to approach the ideal model of complete mixing for fermentation experiments. As such, the biotransformation of crude glycerol in RP is viable and reproducible compared to that performed in R5, with final values for crude glycerol consumption and ethanol concentration being comparable to those obtained on a scale 36-times smaller.

Acknowledgments

This work was supported by the Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía, through the Project for Excellence P09-TEP-4830 co-funded by FEDER Programme 2007–2013 and by the Fundación Carolina (Spain)-University of Cádiz (Spain)-University of Vaparaíso (Chili) Doctorate Scholarship.

References

[1] B.R. Oh, J.W. Seo, S.Y. Heo, W.K. Hong, L.H. Luo, J.H. Son, et al., Fermentation strategies for 1,3-propanediol production from glycerol using a genetically engineered *Klebsiella pneumoniae* strain to eliminate by-product formation, Bioprocess Biosyst. Eng. 35 (1–2) (2012) 159–165.
[2] K.-K. Cheng, J.-A. Zhang, D.-H. Liu, Y. Sun, H.-J. Liu, M.-D. Yang, et al., Pilot-scale production of 1,3-propanediol using *Klebsiella pneumoniae*, Process Biochem. 42 (4) (2007) 740–744.
[3] C.E. Nakamura, G.M. Whited, Metabolic engineering for the microbial production of 1,3-propanediol, Curr. Opin. Biotechnol. 14 (5) (2003) 454–459.
[4] J.Á. Siles López, MdlÁ. Martín Santos, A.F. Chica Pérez, A. Martín Martín, Anaerobic digestion of glycerol derived from biodiesel manufacturing, Bioresour. Technol. 100 (23) (2009) 5609–5615.
[5] T. Willke, K.D. Vorlop, Industrial bioconversion of renewable resources as an alternative to conventional chemistry, Appl. Microbiol. Biotechnol. 66 (2) (2004) 131–142.
[6] S. Yazdani, R. Gonzalez, Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products, Metab. Eng. 10 (6) (2008) 340–351.
[7] S.J. Sarma, S.K. Brar, E.B. Sydney, Y. Le Bihan, G. Buelna, C.R. Soccol, Microbial hydrogen production by bioconversion of crude glycerol: a review, Int. J. Hydrogen Energy 37 (8) (2012) 6473–6490.
[8] X. Shen, Y. Lin, R. Jain, Q. Yuan, Y. Yan, Inhibition of acetate accumulation leads to enhanced production of (R,R)-2,3-butanediol from glycerol in *Escherichia coli*, J. Ind. Microbiol. Biotechnol. 39 (11) (2012) 1725–1729.
[9] P. Shah, F.S. Chiu, J.C. Lan, Aerobic utilization of crude glycerol by recombinant *Escherichia coli* for simultaneous production of poly 3-hydroxybutyrate and bioethanol, J. Biosci. Bioeng. 117 (3) (2014) 343–350.
[10] S. Yazdani, R. Gonzalez, Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry, Curr. Opin. Biotechnol. 18 (3) (2007) 213–219.
[11] A. Murarka, Y. Dharmadi, S.S. Yazdani, R. Gonzalez, Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals, Appl. Environ. Microbiol. 74 (4) (2008) 1124–1135.
[12] Y. Dharmadi, A. Murarka, R. Gonzalez, Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering, Biotechnol. Bioeng. 94 (5) (2006) 821–829.
[13] S.N. Suhaimi, L.-Y. Phang, T. Maeda, S. Abd-Aziz, M. Wakisaka, Y. Shirai, et al., Bioconversion of glycerol for bioethanol production using isolated *Escherichia coli* SS1, Braz J. Microbiol. 43 (2) (2012) 506–516.
[14] V. Sanchez-Torres, M.Z. Mohd Yusoff, C. Nakano, T. Maeda, H.I. Ogawa, T.K. Wood, Influence of *Escherichia coli* hydrogenases on hydrogen fermentation from glycerol, Int. J. Hydrogen Energy 38 (10) (2013) 3905–3912.
[15] A. Chatzifragkou, S. Papanikolaou, Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes, Appl. Microbiol. Biotechnol. 95 (1) (2012) 13–27.
[16] L. Yang, Z. Zhu, W. Wang, X. Lu, Microbial recycling of glycerol to biodiesel, Bioresour. Technol. 150 (2013) 1–8.
[17] O. Cofré, M. Ramírez, J.M. Gómez, D. Cantero, Optimization of culture media for ethanol production from glycerol by *Escherichia coli*, Biomass Bioenerg. 37 (2012) 275–281.
[18] R. Dobson, V. Gray, K. Rumbold, Microbial utilization of crude glycerol for the production of value-added products, J. Ind. Microbiol. Biotechnol. 39 (2) (2012) 217–226.
[19] K. Tran, T. Maeda, T. Wood, Metabolic engineering of *Escherichia coli* to enhance hydrogen production from glycerol, Appl. Microbiol. Biotechnol. 98 (10) (2014) 4757–4770.
[20] A. Cintolesi, J.M. Clomburg, V. Rigou, K. Zygourakis, R. Gonzalez, Quantitative analysis of the fermentative metabolism of glycerol in *Escherichia coli*, Biotechnol. Bioeng. 109 (1) (2012) 187–198.
[21] G. Durnin, J. Clomburg, Z. Yeates, P.J.J. Alvarez, K. Zygourakis, P. Campbell, et al., Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*, Biotechnol. Bioeng. 103 (1) (2009) 148–161.
[22] M.S.I. Mozumder, H. De Wever, E.I.P. Volcke, L. Garcia-Gonzalez, A robust fed-batch feeding strategy independent of the carbon source for optimal polyhydroxybutyrate production, Process Biochem. 49 (3) (2014) 365–373.
[23] M. Rocha, R. Mendes, O. Rocha, I. Rocha, E.C. Ferreira, Optimization of fed-batch fermentation processes with bio-inspired algorithms, Expert Syst. Appl. 41 (5) (2014) 2186–2195.
[24] J.A. Posada, C.A. Cardona, Design and analysis of fuel ethanol production from raw glycerol, Energy 35 (12) (2010) 5286–5293.
[25] J.A. Posada, J.M. Naranjo, J.A. López, J.C. Higuita, C.A. Cardona, Design and analysis of poly-3-hydroxybutyrate production processes from crude glycerol, Process Biochem. 46 (1) (2011) 310–317.
[26] J.A. Posada, L.E. Rincón, C.A. Cardona, Design and analysis of biorefineries based on raw glycerol: addressing the glycerol problem, Bioresour. Technol. 111 (2012) 282–293.
[27] Z. Chi, D. Pyle, Z. Wen, C. Frear, S. Chen, A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation, Process Biochem. 42 (11) (2007) 1537–1545.
[28] T. Ito, Y. Nakashimada, K. Senba, T. Matsui, N. Nishio, Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process, J. Biosci. Bioeng. 100 (3) (2005) 260–265.
[29] T. Vlassis, G. Antonopoulou, K. Stamatelatou, G. Lyberatos, Anaerobic treatment of glycerol for methane and hydrogen production, Glob. Nest J. 14 (2) (2012) 149–156.
[30] P. Bakonyi, N. Nemestóthy, J. Ramirez, G. Ruiz-Filippi, K. Bélafi-Bakó, *Escherichia coli* (XL1-BLUE) for continuous fermentation of bioH2 and its separation by polyimide membrane, Int. J. Hydrogen Energy 37 (7) (2012) 5623–5630.
[31] D. Liu, Bio-hydrogen Production by Dark Fermentation from Organic Wastes and Residues, Department of Environmental Engineering: Technical University of Denmark, 2008.
[32] L.A. Passarinha, M.J. Bonifacio, J.A. Queiroz, Application of a fed-batch bioprocess for the heterologous production of hSCOMT in *Escherichia coli*, J. Microbiol. Biotechnol. 19 (9) (2009) 972–981.
[33] J. Lee, S.Y. Lee, S. Park, A.P.J. Middelberg, Control of fed-batch fermentations, Biotechnol. Adv. 17 (1) (1999) 29–48.
[34] C. Beum Park, S. Bok Lee, Constant-volume fed-batch operation for high density cultivation of hyperthermophilic aerobes, Biotechnol. Tech. 11 (5) (1997) 277–281.
[35] R. Jitrwung, V. Yargeau, Optimization of media composition for the production of biohydrogen from waste glycerol, Int. J. Hydrogen Energy 36 (16) (2011) 9602–9611.
[36] R. Gonzalez, A. Murarka, Y. Dharmadi, S.S. Yazdani, A new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in *Escherichia coli*, Metab. Eng. 10 (5) (2008) 234–245.
[37] A. Murarka, Metabolic Flux Analysis of Fermentative Carbon Metabolism in *Escherichia coli*, Rice University, 2008.
[38] O. Levenspiel, The Chemical Reactor Omnibook: Oregon St Univ Bookstores, 1979.
[39] I. Capela, M.J. Bilé, F. Silva, H. Nadais, A. Prates, L. Arroja, Hydrodynamic behaviour of a full-scale anaerobic contact reactor using residence time distribution technique, J. Chem. Technol. Biotechnol. 84 (5) (2009) 716–724.