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Kinetic modelling of 2,3-butanediol production by *Raoultella terrigena* CECT 4519 resting cells: Effect of fluid dynamics conditions and initial glycerol concentration

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ABSTRACT

Biodiesel-derived glycerol was biologically converted to 2,3-butanediol, an organic compound with multiple industrial applications. Recent studies showed that *Raoultella terrigena* CECT 4519 is an effective biocatalyst for the bioprocess under conventional growing conditions. In the present work, a novel biocatalyst composed by *R. terrigena* resting cells was evaluated for the first time. Fluid dynamic conditions has been optimized to maximize 2,3-butanediol production in terms of titre, yield, and selectivity. Regarding the effect of initial glycerol concentration in batch runs, no substrate inhibition was detected in the studied conditions (concentrations between 45 and 250 g/L were used). Employing pure glycerol as carbon source, 82.0 g/L 2,3-butanediol titre was achieved, whereas 76.5 g/L were reached using raw glycerol. These numbers involve an achieved yield respect to maximal theoretic yield of 79% and 77%, respectively. A successful kinetic modelling of the bioprocess was developed and it is able to describe both the evolution of concentrations of relevant components with time and the rates calculated at experimental time values. Estimated specific 2,3-butanediol production rate was 0.034 $g_{2,3\text{-BDO}}/g_X \cdot h$, while the estimated empirical pseudo-stoichiometric coefficient glycerol/2,3-butanediol was 2.52 $g_{Gly}/g_{2,3\text{-BDO}}$. The obtained results showed that *R. terrigena* resting cells is a promising biocatalyst, which provides new opportunities for developing and scaling-up glycerol biorefineries.

1. Introduction

The current energy and climate crisis derived from the use of non-renewable resources has encouraged the search for new sustainable fuels, such as biodiesel. Biodiesel has several environmental advantages respect to petroleum-based diesel: higher biodegradability, lower pollutant emissions, and also a reduced carbon footprint. However, its price is currently not competitive with fossil fuels [1].

Linked to biodiesel production, around 10% (w/w of oil) of raw glycerol is obtained as by-product in the process. Typically, the purity of raw glycerol is around 50–55% and it also contents impurities, such as water, methanol, salts, and fatty acids. In the last decades, the increasing production of biodiesel has caused a large surplus of raw glycerol with a concomitant drastic price reduction [2]. The integration of glycerol biorefineries coupled to biodiesel facilities for the simultaneously co-production of energy, biofuels and chemicals, such as organic acids and diols, could play an important role on the biodiesel plant's profitability [3].

Glycerol constitutes a simple 3-carbon molecule that can be easily metabolized as carbon source by a great number of bacteria, such as *Klebsiella, Aeromonas, Clostridium, Enterobacter, Gluconobacter* [4,5] and yeast, such as *Saccharomyces, Candida* and *Yarrowia* [6,7]. Therefore, the scientific community is paying much attention to develop and optimize new biotechnological processes to transform glycerol into value-added products.

Specifically, 2,3-butanediol (2,3-BDO) is a highly versatile bulk chemical with numerous direct and indirect applications in agriculture (antibacterial agent), pharmaceutical industry (precursor for synthesis

Abbreviations: 2,3-BDO, 2,3-butanediol; Ac, Acetoin; X, Biomass; g_X/L CDM, Cell Dry Mass concentration; EtOH, Ethanol; $g_{Gly}/g_{2,3\text{-BDO}}$ $y_{Gly/2,3\text{-BDO}}$. Glycerol Gly, Empirical pseudo-stoichiometry coefficient glycerol from 2,3-BDO; LA, Lactic acid; $g_{2,3\text{-BDO}}/g_X \cdot h$ $q_{2,3B}$, Specific 2,3-BDO production rate; % η, Yield respect to the maximal theoretic yield.

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of high-value drugs, biomarker, antiseptic, cryoprotectant), food industry (flavouring agent), and polymer industry (antifreezing, chain initiator in polyurethane intermediates reactions, precursor for synthesis of thermoplastic polymers) [8].

The main challenges for the process industrial implementation are due to safety, raw material costs and downstream operation considerations [8]. Industrial bioprocess safety requires employing biocatalysts belonging to risk group 1. To date, the highest 2,3-BDO titre from glycerol have been produced by microorganisms belonging to risk 2, such as *K. oxytoca* [9]. On the other hand, recovery 2,3-BDO from fermentation broth is still a barrier to the large-scale implementation. Several unit operations based on distillation, membrane separation, solvent extraction, and ion exchange are being investigated [10–12]. Independently of the downstream operations, achieving a high 2,3-BDO titre in the broth is a key factor that determines the effectiveness of the purification.

In this sense, several authors have studied fed-batch fermentations employing growing cells as a process strategy to efficiently avoid substrate inhibition and enhance 2,3-BDO productivity [9,13–15]. On the contrary, only few studies have been focused on seeking alternative biocatalyst states for the bioprocess. Liu et al. [16] studied the biotransformation of glucose into 2,3-BDO employing resting cells of *K. pneumoniae* and *Bacillus subtilis*. Recently, Kay and Jewett [17] have developed a free-cell catalytic system based on a recombinant *Escherichia coli* strain lysate employing glucose and biomass hydrolysate as substrates. However, the 2,3-BDO concentration in these studies were relatively modest (12.5 g/L and 7.2 g/L, respectively).

Resting cells are characterized by nutrient limitation in broth to avoid cell growth. In growing cells bioprocesses, cofactors, such as NADH and NAD(P)H, can be regenerated by means of relevant metabolic pathways. Moreover, NADH and ADP are cofactors directly consumed in 2,3-BDO metabolic pathway [18,19]. Therefore, 2,3-BDO production can be also deeply influenced by the availability of theses energetic-molecules that could be reduced along the fermentation. Cofactors regeneration has been previously studied as a key factor for other bioprocess [20–22].

Recently, *Raoultella terrigena* CECT 4519 growing cells, a risk group 1 microorganism [23], have been proposed as an effective biocatalyst to produce high 2,3-BDO concentration employing raw glycerol as sole carbon source. The published results showed that 2,3-BDO is a non-growth associated metabolite under the studied conditions [15,24]. There is no information in literature regarding the use of resting cells as biocatalysts to produce 2,3-BDO. Employing resting cells as biocatalyst for non-growth associated metabolites production could enhance the main product yield and reduce by-products formation. Therefore, the purpose of this operation strategy is to separate the growth and production processes to evaluate if the established submerged culture procedure can be improved. Based on these points, in the present work *R. terrigena* under non growing conditions (resting cells) is proposed as a novel biocatalyst for this bioprocess in order to improve it, as a proof of concept.

In aerobic bioprocesses, such as 2,3-BDO production, oxygen plays a key factor in cell growth, substrate consumption, cell maintenance, and metabolites production. When optimising an aerobic process, operational conditions (aeration and agitation) must be carefully set to maintain environmental and nutritional conditions (temperature, pressure, pH, nutrients, mixing, hydrodynamics stress, etc.) [25]. Specifically, 2,3-BDO production takes place by means of a metabolic pathway that is extremely sensitive to oxygen supply [13,26–29]. The influence of carbon source availability on 2,3-BDO productivity and product distribution has also been demonstrated [15,24,30,31].

The present work aims to assess the feasibility of 2,3-BDO production through glycerol bioconversion employing *R. terrigena* CECT 4519 resting cells as biocatalyst, as a proof-of-concept. To reach this goal, the influence of the most influenced variables in the bioprocess (stirring speed as well as the initial glycerol concentration) on 2,3-BDO production in batch experiments has been studied. Last, but not least, a kinetic model is proposed and applied to predict the evolution of the experimental data. Based on the kinetic analysis, model parameters (empirical pseudo-stoichiometric coefficient and specific production rate) have been estimated.

2. Materials and methods

2.1. Microorganism and biocatalyst production

In the present work, *R. terrigena* CECT 4519 was selected as biocatalyst. Previous publications have shown the efficacy of this bacterial strain to produce 2,3-butanediol from pure and waste glycerol employing growing cells [15,24,28].

Bacterial stock was stored at − 80 °C in a solution composed by glycerol-saline serum mixture (50:50% w/w). Experimental approach for biocatalyst production consisted in three serial growth steps that ensured a reproducible procedure as well as biomass amplification for production experiments. Modified synthetic M9 minimal salts 2x was employed as growth medium (composition: 30 g/L pure glycerol; 1.5 g/L yeast extract; NH_4Cl 2 g/L; 6 g/L KH_2PO_4; 12 g/L Na_2HPO_4; 1 g/L NaCl; 0.246 g/L $MgSO_4.7H_2O$; 0.011 g/L $CaCl_2$) [24].

At first, two successive cultures were carried out in 250-mL non-baffled shaken flask with 50 mL of working volume, operating at 30 °C at 210 rpm in an orbital shaker. The duration of each step was 14 h and 4 h, respectively, being initial dry biomass concentration 0.1 g_X/L. Afterwards, the third step was carried out in a 3-L Biostat® B-Plus (Sartorius AG Germany) with a working volume of 2 L in order to ensure enough biomass concentration. The employed bioreactor consisted of a stirred non-baffled cylindrical tank. Biomass obtained in second step was used as inoculum of the bioreactor; being initial dry biomass concentration 0.25 g_X/L. Operating conditions were set considering the optimum values determined in previous works (temperature: 30 °C; airflow: 1.5 vvm; stirring speed: 400 rpm; pH was left to freely evolve from 6.9 to 5.5 and subsequently maintained at this acid pH value using 2 M NaOH and 2 M HCl solutions) [24,28]. The duration of this third step was 20 h.

Afterwards, cells were harvested from broth by centrifugation (9000 rpm; 5 min at room temperature). The pellet was washed twice with Phosphate Buffered Saline (PBS) to eliminate residual growth medium. Finally, cells were suspended into PBS and used immediately in the subsequent resting cells experiments.

2.2. 2,3–butanediol production from glycerol employing resting cells biocatalyst

Batch experiments employing resting cells were carried out in a 1-L Biostat® B-Plus (Sartorius AG Germany) with a working volume of 0.5 L. Biocatalyst concentration was fixed at 20 g_X/L and temperature was set at 30 °C, the aeration was kept at 1.5 vvm of the air flow rate, pH was controlled at 5.5 using 2 M NaOH and 2 M HCl solutions. The production medium used in this setup was PBS.

Two different sets of experiments were performed: stirring speed was studied from 200 to 500 rpm and initial glycerol concentration was set at different values: 45, 70, 125, 210, and 260 g_{Gly}/L, employing pure glycerol (Panreac, ref. 151339), as well as raw glycerol provided by a Spanish biodiesel manufacturing plant (210 g_{Gly}//L). Raw glycerol samples analysis yielded the following composition: glycerol (from 55% to 85%w/w), chloride salts (from 17.3 to 56.6 g/L), phosphate salts (from 2.54 to 6.14 g/L), and a low amount of methanol (~0.06%w/w). This study was carried out at the best stirred speed value obtained from the first set of experiments (400 rpm).

All of the experiments described in this work have been performed in triplicate, and only the mean value has been presented. The global experimental error was lower than 5%.

2.3. Analytical methods

Cell dry mass concentration (CDM) was measured by means of spectrophotometric technique (Shimadzu UV–visible spectrophotometer UV-1603). Dry cell weight was linearly related to the optical density (OD) of the broth at 600 nm, according to:

$$\text{CDM } (g_X/L) = 1.616 \cdot OD \quad (1)$$

Dilution of the sample is required to keep the absorbance value within the range of linear variation ($0.1 < OD < 0.7$).

Broth composition was determined by means of HPLC technique. Samples were previously centrifuged (14,000 *g*; 10 min) to remove solids. Glycerol, 2,3-BDO and other metabolic by-products (acetate, lactate, acetoin and ethanol) were quantified using a Rezex RHM-Monosaccharide H+ (8%) column (300 × 7.8 mm, Phenomenex) and both refractive index and diode array detectors (Agilent Technologies, 1100 Series). A sulphuric acid Milli-Q water solution (0.01 M) flowing at 0.6 mL/min was employed as mobile phase. Column temperature was set at 65 °C, while refractive index detector operated at 55 °C. 2,3-BDO concentration corresponds to the total product concentration (*meso*-2,3-butanediol + (2*R,3R*)-2,3-butanediol + (2*S,3S*)-2,3-butanediol).

2.4. Calculations

Aspen Custom Modeler© software was employed to fit the kinetic models to experimental data in order to estimate kinetic parameters. The set of differential equations forming the kinetic model was integrated using the numerical implicit Euler method, so a multiple-response method to fit the model to the data was employed. The kinetic parameters were estimated by minimizing the difference between experimental observations and model simulation according to "least squares method" by an adaptive non-linear least-squares algorithm (NL2SOL).

For the purpose of the validation of the proposed model, physicochemical and statistical criteria were applied. The main statistical criteria were goodness-of-fit parameters: F-Fischer's value (F), the sum of squared residuals (SQR), the residual mean square error (RMSE), and percentage of explained variance (VE). They have been defined in a previous paper [5] but, in short, the first three parameters contemplate the mean difference between experimental data and data estimated using the kinetic model, while the latter indicates how the model predicts the temporal change of the concentrations of relevant components in the reacting system.

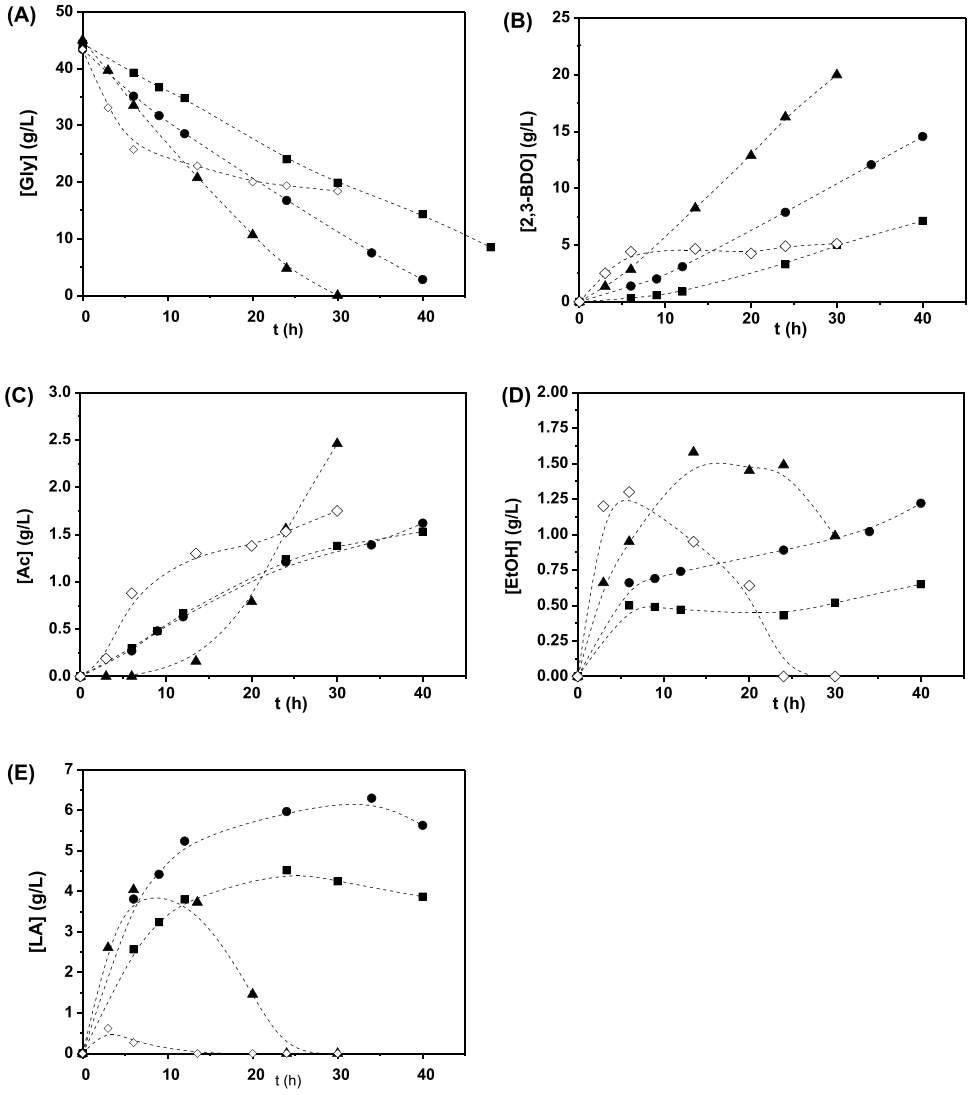

Fig. 1. Evolution of (A) glycerol consumption, (B) 2,3-BDO production, (C) acetoin production, (D) ethanol production, and (E) lactic acid production in batch experiments employing *R. terrigena* resting cells as biocatalyst (20 g/L). *Key:* 200 rpm (Filled square); 300 rpm (Filled circle); 400 rpm (Filled triangle); 500 rpm (Blank diamond). *Operational conditions:* biocatalyst concentration: 20 g_X/L; temperature: 30 °C; air flow rate: 1.5 vvm; pH: 5.5 (controlled); Initial glycerol concentration: 45 g_{Gly}/L.

3. Results and discussion

3.1. Influence of stirring speed on 2,3-BDO production

In this section we show several results on the influence of stirring speed on 2,3-BDO production employing *R. terrigena* resting cells as biocatalyst. Batch experiments employing a constant initial glycerol concentration (45 g_{Gly}/L) and a constant biocatalyst concentration (20 g_X/L) were performed at four stirring speed conditions: 200, 300, 400 and 500 rpm. The stirring speed range was selected based on previous results employing *R. terrigena* growing cells as biocatalyst [28]. The evolution of glycerol consumption, 2,3-BDO production, and by-products generation are shown in Fig. 1.

As indicated in this figure, between 200 and 400 rpm there is an increase in glycerol consumption and 2,3-BDO production rates, reaching a peak at the higher stirring speed mentioned. However, when we increased the agitation speed to 500 rpm, a sharp deceleration in both parameters was observed. In consequence, it seems that there is a narrow range of oxygen supply that maximizes this diol production. For lower oxygen supply levels, bioprocess takes places slowly; but, on the contrary, 2,3-BDO production is not effective. Since intermediate metabolite in 2,3-BDO pathway is acetoin, its production is completely linked to 2,3-BDO and its maximal final concentration is observed at 400 rpm. Regarding to ethanol and lactic acid production, both molecules are more reduced metabolites than 2,3-BDO, so their production is boosted at lower values of oxygen supply (200–300 rpm). Similar influence of stirring speed on metabolite distribution employing *R. terrigena* growing cells has been previously reported [28]. Stirring speed range between 250 and 400 rpm enhanced 2,3-BDO production, reaching 5.8 $g_{2,3\text{-BDO}}$/L when 30 g_{Gly}/L of carbon source was used. In addition, anaerobic metabolites, such as succinic acid, lactic acid, and ethanol, are maximized at lower oxygen supply conditions (stirring speed lower than 400 rpm).

Since there is no availability of nutrients for growing in a resting cells system, a higher value of stirring speed (500 rpm) leads to arrest biocatalyst activity after 5 h of fermentation. However, growing cells system still works under higher agitations (until 1600 rpm). Between 700 and 1600 rpm metabolism is complete focused on growth and respiration pathways (maximal biomass concentration reached 22.5 g_X/L, and produced carbon dioxide was 14.7 g_{CO2}/L). At these conditions, phenomena dealing with oxidative and hydrodynamic stress could take place in resting cells system, as it was observed in conventional submerged fermentation using growing cells [28].

In order to study metabolite distribution at each condition, yield and selectivity for each product have been studied. Yield (η) is expressed as the percentage of the ratio between the yield (product concentration/consumed glycerol concentration) and the maximal theoretic yield, which is calculated applying the electron balance [32]. The values for the theoretical maximum yields of the different metabolites from glycerol are: for 2,3-BDO 0.62 $g_{2,3\text{-BDO}}/g_{Gly}$; for acetoin from 0.67 g_{Ac}/g_{Gly}; for ethanol 0.58 g_{EtOH}/g_{Gly}, and for lactic acid 1.14 g_{LA}/g_{Gly}. The influence of stirring speed on product yield respect to maximal theoretic yield is shown in Fig. 2A. According to yield data, the metabolism, when the biocatalyst is a suspension of resting cells, focuses on producing 2,3-BDO, being this diol the main product in broth after 40 h of fermentation. Lactic acid yield is negligible for all the studied conditions. Between 200 and 400 rpm, a residual ethanol yield (around 5%) is obtained. It should be highlighted that 2,3-BDO yield respect to maximal theoretic yield reached employing 400 rpm is around 90%, so the use of this resting cells system leads to a very effective glycerol conversion to 2,3-BDO.

Selectivity is defined as the amount of a product per total amount of generated products, and it is expressed as a percentage. This is an interesting parameter to discuss about product distribution. Influence of stirring speed on product's selectivity is shown in Fig. 2B. Here, it is evidenced that oxygen supply plays an important role on this parameter: under lower oxygen supply conditions, anaerobic fermentative metabolites (lactic acid, ethanol) are produced. However, 2,3-BDO is the main product for all the studied conditions. 2,3-BDO selectivity is maximized (85%) when fixing the stirring speed at 400 rpm. Therefore, the best operational stirring speed is 400 rpm and it was set at that value for subsequent runs in this work (studying glycerol initial concentration and type).

3.2. Influence of initial glycerol concentration on 2,3-BDO production

To improve final 2,3-BDO concentration, different batch experiments were carried out employing increasing initial concentrations of pure glycerol (45, 70, 125, 210 and 260 g_{Gly}/L). Also, biodiesel-derived raw glycerol was used as carbon substrate at an initial concentration of 210 g/L. Evolution of glycerol concentration (Figs. 3A) and 2,3-BDO production (Fig. 3B) are shown in Fig. 3.

As it can be seen in Fig. 3A, the use of glycerol concentration below 210 g_{Gly}/L, a total consumption of substrate is reached, even if we employ raw glycerol as the carbon source. Since resting cells are characterized by nutrient limitation in broth, exhaustion of required cofactors in metabolic pathway for 2,3-BDO production could be taking place. In longer experiment (after 150 h fermentation), biocatalyst is also getting old. As a consequence, substrate consumption slows down and the complete substrate exhaustion is not achieved. The evolution of glycerol concentration in all the experiments shows parallel trends, so the rate of glycerol consumption is constant regardless of the initial substrate concentration. As a conclusion, the studied glycerol concentration range does not inhibit bioprocess when using resting cells unlike what is observed with growing cells [20].

Regarding 2,3-BDO production, the lower is the initial glycerol

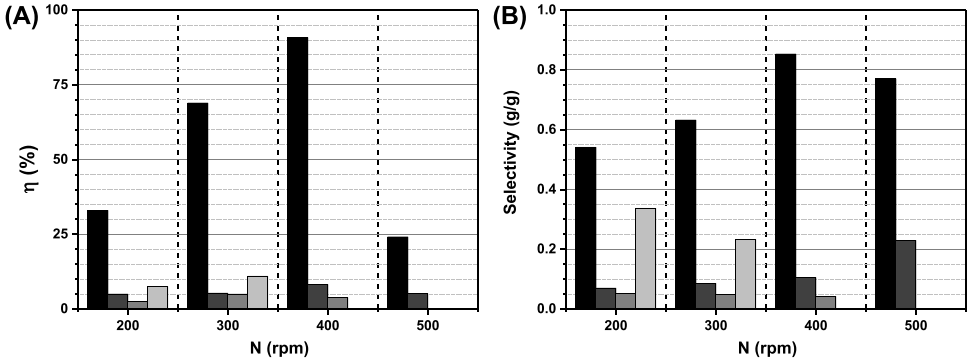

Fig. 2. Influence on stirring speed on (A) product yield respect to maximal theoretic yield, (B) product selectivity *Key*: 2,3-BDO (Black column); Acetoin (Dark grey column); Ethanol (Grey column); Lactic acid (Light grey column). *Operational conditions*: biocatalyst concentration: 20 g_X/L; temperature: 30 °C; air flow rate: 1.5 vvm; pH: 5.5 (controlled); Initial glycerol concentration: 45 g_{Gly}/L.

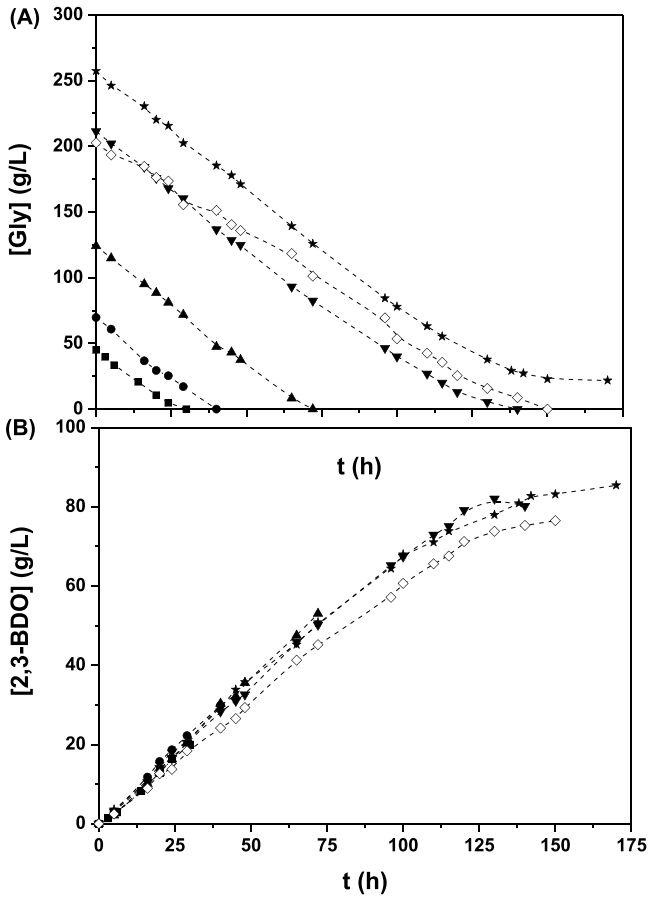

Fig. 3. Influence of initial glycerol concentration employing *R. terrigena* resting cells as biocatalyst on (A) glycerol consumption, and (B) 2,3-butanediol production. *Key:* Pure glycerol concentration: (Filled square) 45 g_{Gly}/L; (Filled circle) 70 g_{Gly}/L; (Filled triangle) 125 g_{Gly}/L; (Filled inverted triangle) 210 g_{Gly}/L; (Filled star) 260 g_{Gly}/L; Raw glycerol concentration: (Blank diamond) 210 g_{Gly}/L. *Operational conditions:* biocatalyst concentration: 20 g_X/L; temperature: 30 °C; air flow rate: 1.5 vvm; pH: 5.5 (controlled); Stirring speed: 400 rpm.

concentration, the faster substrate exhaustion and the lower 2,3-BDO titre are achieved. Maximal 2,3-BDO concentration reached is 85.4 $g_{2,3BD}$/L, using 260 g_{Gly}/L initial glycerol concentration. It must be highlighted that, in contrast to production employing growing cells, 2,3-BDO production starts at the very beginning of the fermentation, perhaps due to the high biocatalyst concentration (when using growing cells, a lag phase for 2,3-BDO production was observed [20]). 2,3-BDO production rate present the same constant value regardless the initial substrate concentration used. Although 2,3-BDO production rate is slightly slower employing raw glycerol, the final titre is similar to the one obtained when using pure glycerol: 76.5 $g_{2,3BD}$ /L and 80.5 $g_{2,3BD}$ /L, respectively.

It should be pointed out that the evolution of substrate consumption as well as product generation along the time is quite similar for all the experiment, which means that the bioprocess is not inhibited by the substrate or the product concentrations.

Fig. 4-A shows the influence of initial glycerol concentration using *R. terrigena* resting cells as biocatalyst on 2,3-BDO yield respect to maximal theoretic yield. It can be observed that, between 45 and 125 g_{Gly}/L, 2,3-BDO yield remains constant, being the final yield around 85–90%. These results show the effectiveness of resting cells to transform glycerol into 2,3-BDO, being really close to the maximum achievable amount. However, higher initial glycerol concentrations lead to a slightly reduction of yield, achieving 65% when 260 g_{Gly}/L is used.

Similar tendency is observed for 2,3-butanediol productivity, showed in Fig. 4-B. Maximal productivity reaching 0.75 $g_{2,3BD}$ /(L·h) employing an initial glycerol concentration between 70 and 125 g/L. Regarding biodiesel-derived glycerol experiment, decrease in yield (77%) and productivity (0.51 $g_{2,3BD}$ /(L·h)) has been observed. Slowing down of the bioprocess can be ascribed to the impurities contained in the raw glycerol.

Acetoin and ethanol yields respect to the maximal theoretic yield reached at the end of the fermentation for each condition are shown in Fig. 4-C and D, respectively. Lactic acid production was not detected in any experiment. Acetoin and ethanol yields achieved values lower than 4%. Indeed, employing an initial glycerol concentration of 210 g_{Gly}/L, acetoin yield is lower than 1.5% and there is not ethanol in the broth. It have to be pointed out that 2,3-BDO is always the main product regardless of the substrate concentration used and by-products concentrations are negligible (below 2 g/L).

Table 1 summarized the published studies on 2,3-BDO production from pure and raw glycerol as sole carbon source using different risk 1-strains in batch and fed-batch operational mode as well as the results showed in this paper. 2,3-BDO yield respect to maximal theoretic yield has been calculated to compare results showed in the present work to published results. This comparison establishes that, currently, *R. terrigena* is the most effective biocatalyst to achieve a high concentration of the product. Comparing these results to the previous published research employing growing cells as biocatalyst [20], it is highlighted that productivity has been enhanced employing resting cells. Although the results showed in the present work could be improved using, i.e. fed-batch operation, cell immobilization and/or regeneration of co-factors, it has been confirmed that *R. terrigena* resting cells are a novel viable biocatalyst for the biotransformation of raw glycerol into 2,3-BDO.

3.3. Kinetic modelling 2,3-BDO production employing resting cells as biocatalyst

Based on the experimental results, a kinetic model (Eqs. 2–5) is proposed to describe the observed tendencies. Due to the observation of experimental results (Fig. 5) only one reaction can be considered that it is shown as Equation (2); moreover, a linear trend is observed, so, a constant reaction rate can be assumed. The only variable to be considered is the biomass concentration. The kinetic equation is shown in Eq. (3). The production rates for the two compounds considered in this model are summarized in Eqs. (4) and (5), for 2,3-BDO and glycerol, respectively. An empirical pseudo-stoichiometric coefficient, $y_{2,3\text{-BDO/Gly}}$ ($g_{2,3\text{-BDO}}/g_{Gly}$), is also required to relate the amount of glycerol consumed to produce 1 g of product (Eq. 5).

Reaction stoichiometry: $$y_{Gly/2,3-BDO}\cdot Gly \xrightarrow{r} 2,3-BDO \quad (2)$$

Reaction rate: $$r\left(g_{2,3-BDO}/L\cdot h\right) = q_{2,3-BDO}\cdot[X] \quad (3)$$

2,3 – BDO production rate: $$R_{2,3-BDO} = \frac{d[2,3-BDO]}{dt}\left(g_{2,3-BDO}/L\cdot h\right) = r \quad (4)$$

Glycerol consumption rate: $$R_{Gly} = -\frac{d[Gly]}{dt}\left(g_{Gly}/L\cdot h\right) = -y_{Gly/2,3-BDO}\cdot r \quad (5)$$

The fitting of the proposed kinetic equation to experimental data (glycerol, 2,3-BDO and biomass concentration) was carried out to estimate the value of the only kinetic parameter. The fitting of the model to all data but the one obtained at 260 g/L (at different glycerol initial concentration) has been performed. Experiment with 260 g/L as initial glycerol concentration has been not considered for the kinetic analyses due to the deceleration occurred in the last hours of the fermentation. Experimental data relating to different initial glycerol concentrations

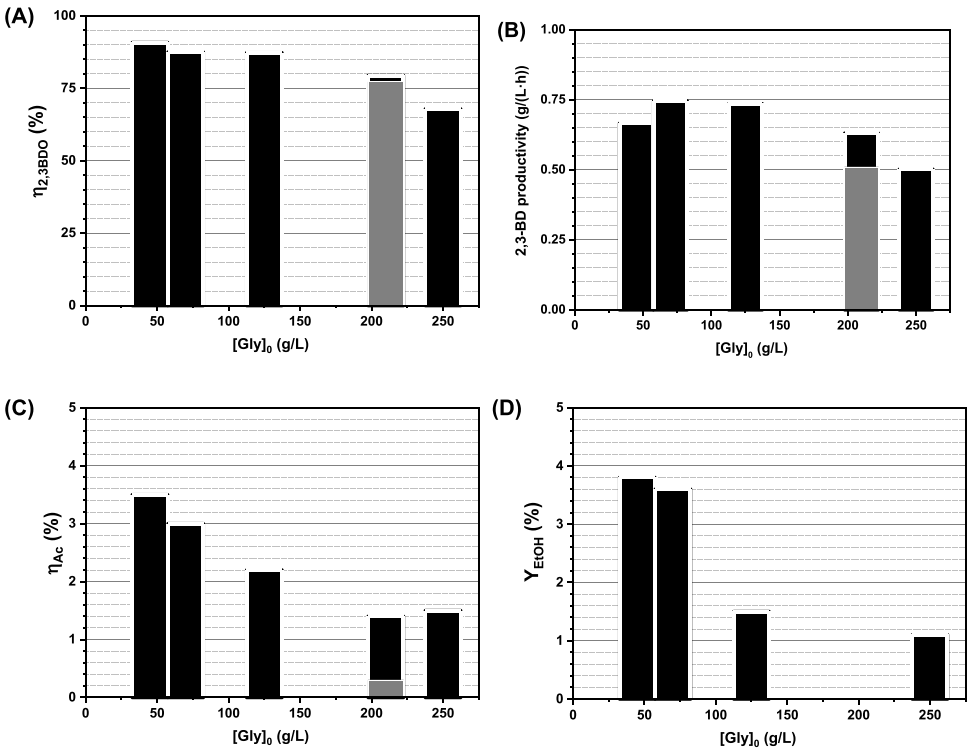

Fig. 4. Influence of initial glycerol concentration employing *R. terrigena* resting cells as biocatalyst on (A) 2,3-BDO yield respect to maximal theoretic yield, (B) 2,3-butanediol productivity, (C) Acetoin yield respect to maximal theoretic yield, and (D) Ethanol yield respect to maximal theoretic yield. *Key:* Pure glycerol (Black columns), Raw glycerol (Grey column). *Operational conditions:* biocatalyst concentration: 20 g_X/L; temperature: 30 °C; air flow rate: 1.5 vvm; pH: 5.5 (controlled); Stirring speed: 400 rpm.

Table 1
Summary of published studies on 2,3-BDO production from pure and raw glycerol as sole carbon source using different risk 1-strains in batch and fed-batch operational mode.

Employed Biocatalyst (strain/state)	Operational mode	Type of glycerol	Titre ($g_{2,3\text{-BDO}}$ /L)	η (%)	Productivity ($g_{2,3\text{-BDO}}$/ (L·h))	Reference
Bacillus amyloliquefaciens/ Growing cells	Batch	Pure	42.6	85	1.07	[33]
	Fed-batch	Raw	43.1	78	0.45	[34]
R. ornithinolytica/ Growing cells	Batch	Pure	62.4	36	0.74	[14]
	Fed-batch	Raw	78.1	72	0.87	
Serratia proteamaculans/ Growing cells	Batch	Pure	18.4	40	0.15	[35]
R. planticola / Growing cells	Batch	Pure	33.6	54	1.40	[36]
R. terrigena / Growing cells	Batch	Pure	49.4	68	0.39	[15]
	Fed-batch	Pure	90.5	88	0.52	
	Fed-batch	Raw	80.5	90	0.40	
***R. terrigena*/ Resting cells**	Batch	**Pure**	**82.0**	**79**	**0.63**	**This work**
	Batch	**Raw**	**76.5**	**77**	**0.51**	

(between 45 and 210 g/L), including experiment with raw glycerol source (210 g/L), have been used together to fit the proposed model. The results of this fit are shown in Table 2.

Fig. 5 shows both the experimental data evolution and the kinetic model prediction for substrate and product concentrations for the experiments at different initial glycerol concentration, including pure and raw substrate. It can be concluded that experimental results of all runs are perfectly described by the model previously commented, independently of the initial glycerol concentration and the type of carbon source employed (pure and raw). This is also reflected by the value of the statistical parameters dealing with the fitting, which are shown in Table 2. The F-Fisher calculated value (F_{cal}) is much higher than the tabulated one (F_{tab}) for a 95% of confidence interval, so the null-hypothesis test is fulfilled and the proposed kinetic model accurately reflects reality. SQR and RMSE values were acceptable in all fittings. Finally, the value of VE is quite close to 100%, which indicates the goodness of the fittings and the temporal trends estimated with the model and further reinforced the validity of the proposed kinetic model. It can be concluded that, under the studied conditions, the specific 2,3-BDO production rate is 0.034 ± 0.009 $g_{2,3\text{-BDO}}/(g_X \cdot h)$ and the pseudo-stoichiometric coefficient is 2.52 ± 0.16 $g_{Gly}/g_{2,3\text{-BDO}}$.

4. Conclusions

This is the first time that 2,3-BDO production employing *R. terrigena* resting cells has been described. The obtained results showed that *R. terrigena* resting cells not only focused their metabolism on 2,3-BDO production pathway but also were not inhibited by initial substrate concentration. Both characteristics are key advantages in comparison to bioprocess carried out by growing cells. Successful 2,3-BDO production results were reached employing pure glycerol as carbon source (82.0 g/L), as well as biodiesel-derived glycerol (76.5 g/L).

In short, *R. terrigena* resting cells are a novel viable biocatalyst in order to develop an industrial bioprocess based on separate growth and production units. The proposed bioprocess means a breakthrough respect to the conventional submerged fermentation using growing cells, enhancing the overall bioprocess effectiveness. Although these results provide promising insights, further researches on continuous operational mode, cofactors regeneration and cell immobilization are advisable.

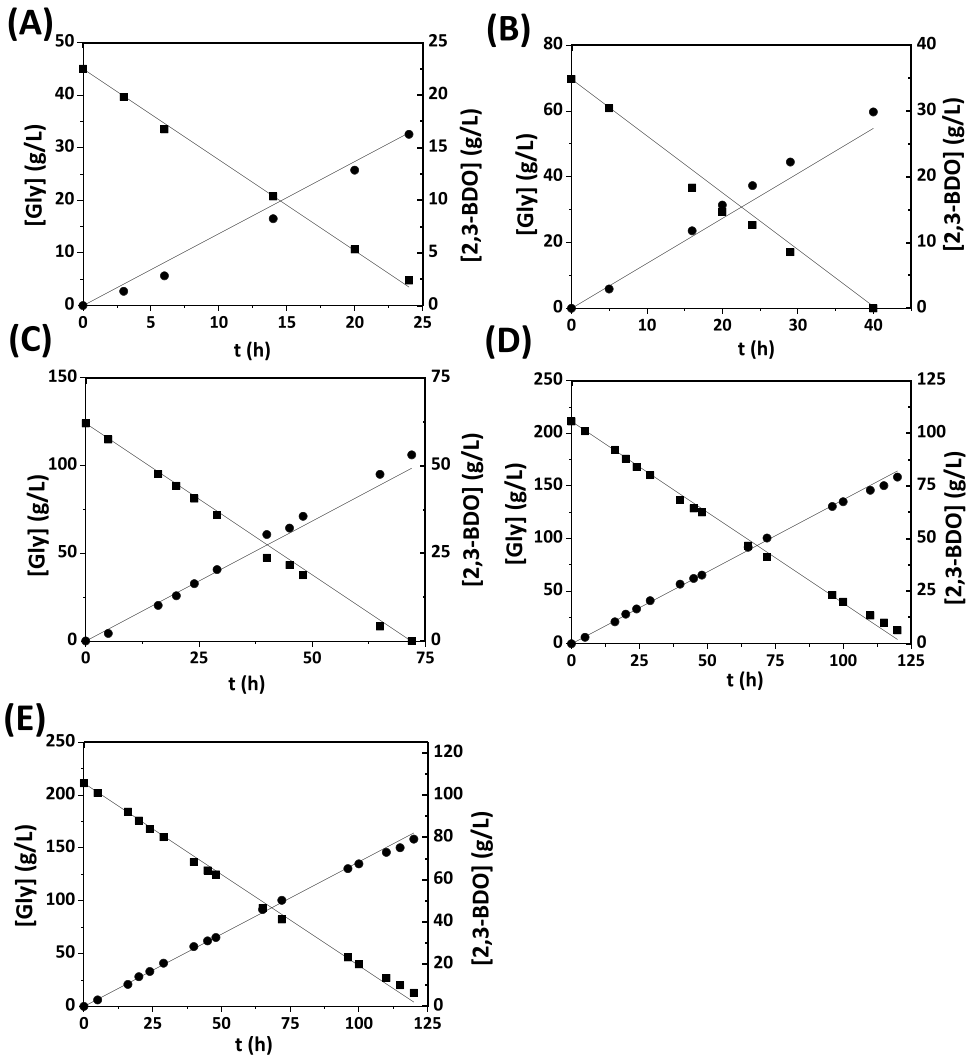

Fig. 5. Experimental evolution (points) and kinetic model prediction (lines) of substrate and product concentrations for each experiment with the following initial glycerol concentration: (A) 45 g/L pure glycerol, (B) 70 g/L pure glycerol, (C) 125 g/L pure glycerol, (D) 210 g/L pure glycerol, and (E) 210 g/L raw glycerol. *Key:* glycerol concentration (Filled square); 2,3-BDO concentration (Filled circle). *Operational conditions:* biocatalyst concentration: 20 g_X/L; temperature: 30 °C; air flow rate: 1.5 vvm; pH: 5.5 (controlled); Stirring speed: 400 rpm.

Table 2
Estimated model and statistical parameters obtained from the fit the proposed model to the experimental date from the initial (pure and raw) glycerol concentration experiments.

Model Parameters	Value
$q_{2,3-BDO-}(g_{2,3-BDO}/L.H)$	0.034 ± 0.009
$y_{Gly/2,3-BDO}\left(g_{Gly}/g_{2,3-BDO}\right)$	2.52 ± 0.16
Statistical parameters	Value
$F_{cal}/F_{tab\ 95\%}$	$9.42{\cdot}10^3$ / 19.5
SQR	64.2
RMSE	4.11
VE (%)	99.4

CRediT authorship contribution statement

Vanessa Ripoll: Investigation, Data curation, Methodology, Writing – original draft. **Miguel Ladero:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Victoria E. Santos:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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