HIGH 2,3-BUTANEDIOL PRODUCTION FROM GLYCEROL BY *Raoultella terrigena* CECT 4519

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ABSTRACT

Bioconversion of derived-biodiesel glycerol into 2,3-butanediol has received recently much attention due to its increasing surplus. It is widely used as bulk chemical for several industrial applications. The influence of initial glycerol concentration on 2,3-butanediol production in batch runs has been studied. A concentration higher than 140 g/L produces an inhibitory effect on the final 2,3-butanediol concentration as well as on its production rate. In batch operation mode, the best yield was obtained employing 140 g/L as initial glycerol concentration, being 71% respect to the theoretical maximum yield (Based on these results, fed-batch operation mode was carried out to improve 2,3-butanediol production. The titre of 2,3-butanediol was 90.5 g/L employing pure glycerol as carbon source and 80.5 g/L employing raw glycerol. The 2,3-butanediol yield was also improved through the fed-batch operation (90 % respect to the theoretical maximum yield). To date, this concentration is the highest produced amount employing as biocatalyst a non-pathogenic bacterium (level 1).

KEYWORDS

Biorefinery; Raoultella terrigena; 2,3-butanediol; Raw glycerol.

- **HIGHLIGHTS**High 2,3-butanediol concentration was achieved employing a class 1 microorganism.
- Initial substrate inhibition has been described in batch experiments.
- Yield respect to the theoretical maximum yield reaches 90 %.
- Fed-batch run employing pure glycerol leads to 90.5 g/L of 2,3-butanediol.

1 INTRODUCTION

Nowadays, the progressive exhaustion of the non-renewable petroleum sources has triggered an energy worldwide crisis. Besides the search for new sustainable fuels, the development of alternative synthesis routes of bulk chemical is required to complement or substitute the conventional petrochemical ones. In this sense, the advances in the biotechnological industry involve a great contribution in this field (Wilke et al. 2004).

2,3-butanediol (2,3-BD) is an interesting commodity chemicals with a large number of industrial applications, as a feedstock for rubber, plastic, and solvents production processes. 2,3-BD dehydration reaction is used to produce methyl ethyl ketone, which is employed as an effective fuel additive and as a solvent for resins and lacquers. In addition, 2,3-BD is used as antifreeze agent and as octane booster for fuels (Celinska and Grajek 2009; Villet et al. 1981).

During the last decade, many authors have been focusing on the search for renewable carbon sources based on industrial wastes to produce 2,3-BD by biotechnological routes. The latest published studies have been summarized in Table 1. Most of the studied feedstock proceed from agricultural and food industry wastes, whose composition is variable and sometimes includes inhibitory compounds (Palmqvist et al. 2000). To date, the highest 2,3-BD concentration has been reached employing sugar molasses as feedstock. Glucose is the main sugar in this waste, which is, usually, the preferred carbon source fastly uptaken and metabolized by several bacteria (Martinez-Gomez et al. 2012). Although glycerol is an energy-poor carbon source, a number of microorganisms are able also to grow on glycerol as sole carbon source, and metabolize it by means of both oxidative and reductive pathways (Da Silva et al. 2009).

In the framework of circular economy, raw glycerol, the waste derived from the biodiesel production, stands out as a promising alternative carbon source for fermentation bioprocesses. The increase of biodiesel demand according to European policies, such as the Directives 2003/30/EC and 2009/27/EC, has caused a large surplus of raw glycerol. As a result, the drastic

fall of its price and the presence of impurities make the conventional glycerol applications related to pharmaceutical, food and cosmetic industries not enough to cope with glycerol production (Gerpen 2005; Pagliaro et al. 2007; Santibañez et al. 2011).

In order to establish an industrial-scale bioprocess, safety is the key aspect. Non-pathogenic microorganisms belonging to risk group 1 are the most desirable biocatalysts. To date, the best 2,3-BD producer biocatalysts belong to risk group 2 (*Klebsiella pneumonia* and *K. oxytoca)*, (Cho et al. 2015; Petrov and Petrova, 2010). Recently, *Raoultella terrigena* CECT 4519 has been reported as a promising biocatalyst of the bioconversion process of raw glycerol into 2,3-BD, which accomplishes the safety requirements (Ripoll et al. 2016). According to the available information, this bacterial strain belongs to biosafety level 1 (BacDive, CECT). Therefore, it is considered as non-pathogenic biocatalyst.

The aim of the present work is to study 2,3-BD production by means of glycerol bioconversion employing *R. terrigena* CECT 4519 as biocatalyst. To reach this goal, the influence of initial glycerol concentration on 2,3-BD production in batch experiments has been studied. Based on the obtained results, different profiles for glycerol addition in fed-batch experiments were carried out to enhance the 2,3-BD titre. In addition, a study regarding the use of industrial raw glycerol as sole carbon source has been also performed.

2 MATERIALS AND METHODS

2.1 *Microorganism and inoculum procedure*

In the present work, *R. terrigena* CECT 4519 has been employed as biocatalyst. The ability of this strain to produce 2,3-butanediol had been previously reported (Ripoll et al. 2010). Strain maintenance and inoculum growth were carried out based on the established protocols previously published (Ripoll et al. 2010; Rodriguez et al. 2017). Cells were stored at -80 ºC in a glycerol-saline serum solution (50:50 % w/w). Two growth steps were required to ensure a reproducible inoculum from the stored cellular stock. Both growth phases were carried out in 250-mL unbaffled shaken flask with 50 mL of working volume, operating at 30 ºC and 210 rpm

in an orbital shaker. The duration of each step was 14 h and 4 h, respectively. The initial dry biomass concentration was 0.1 g/L in both cases.

2.2 *2,3-butanediol production experiments*

Batch and fed-batch fermentations were carried out in a 3-L Biostat® B-Plus (Sartorius AG Germany) with a working volume of 2-L. The employed bioreactor configuration consisted of a stirred unbaffled cylindrical tank. Medium composition was formulated employing the following amount (per litre of deionized water): 2 g of NH_4Cl, 6 g of KH_2PO_4, 12 g of Na_2HPO_4, 1 g of NaCl, 0.246 g of $MgSO_4 \cdot 7H_2O_2$, 0.011 g of $CaCl_2$ and 1.5 g of yeast extract. Different initial glycerol concentration between 35 and 210 g/L were used, employing pure glycerol (Panreac, ref. 151339) as well as industrial raw glycerol. Raw glycerol has been provided by a Spanish biodiesel manufacture plant. Raw glycerol samples contented 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).

At the beginning of each experiment, 10 % v/v of inoculum were added to reach an initial biomass concentration of 0.25 g/L. Operational conditions were set according to earlier results (Ripoll et al. 2010; Rodriguez et al. 2017). Operational temperature and stirring speed were set at 30 ºC and 400 r.p.m., respectively. Airflow rate was fixed at 1.5 v.v.m. Initial pH was 7.0 and it was not controlled until it reached a value of 5.5 during the fermentation. From that moment on, pH was controlled at 5.5 employing acid and basic solutions (HCl 2M and NaOH 2M). Broth samples were collected during the experiment in order to determine the evolution of the fermentation (biomass and metabolite concentrations).

Batch runs were carried out employing initial glycerol concentration between 35 and 210 g/L. Fed-batch experiments were carried out at the same operational conditions as batch runs (temperature, stirring speed, airflow rate, pH profile and initial biomass concentration). Different feeding profiles were performed by means of a pulse of 200 mL of a concentrated

112 solution of glycerol, dissolved in the culture medium, supplied into the bioreactor. Before the
113 introduction of the pulse, 200 mL of the broth were removed from the vessel.

114 **2.3** ***Analytical methods***

115 The evolution of the biomass concentration was determined by means of the measure of the
116 optical density at 600 nm (Shimadzu UV-visible spectrophotometer UV-1603). The cell dry
117 mass concentration (CDM) was related proportionally to the optical density (OD) of the broth at
118 600 nm, according to the following equation:

119 $CDM\ (g/L) = 1.616 \cdot OD_{600}$ **(1)**

120 The consumption of glycerol and the production of the metabolites (acetate, lactate, 2,3-BD,
121 acetoin and ethanol) were quantified by a HPLC technique, using a Rezex RHM-
122 Monosaccharide H^+ (8 %) column (300×7.8 mm, Phenomenex), and both refractive index and
123 diode array detectors (Agilent Technologies, 1100 Series). Broth samples were previously
124 centrifuged (14000*g*; 10 min) in order to remove the cells. The employed mobile phase was
125 H_2SO_4 (0.01M) flowing at 0.6 mL/min. Column temperature was set at 65 °C, while refractive
126 index detector operated at 55 °C.

127 2,3-BD is an organic alcohol with three different isoforms (*meso*-2,3-butanediol, (*R, R*)-2,3-
128 butanediol and, (*S, S)*-2,3-butanediol). The analytical technique employed does not afford for
129 quantify independently each stereoisomer. Therefore, 2,3-BD concentration corresponds to the
130 total product concentration (*meso*-2,3-butanediol + (*R, R*)-2,3-butanediol + (*S, S)*-2,3-
131 butanediol).

132 **3 RESULTS AND DISCUSSION**

133 **3.1** ***Influence of initial glycerol concentration on 2,3-butanediol production***

134 In order to determine the influence of initial carbon source on the bioprocess, 6 batch
135 experiments were carried out employing different initial pure glycerol concentration (35, 80,
136 110, 140, 190, and 210 g/L).

For each run, the final broth composition has been summarized in Table 2. According to the results, 2,3-BD is the main fermentation product independently of the initial substrate amount. This fact shows that the selected operational conditions are favourable to address metabolism pathways to 2,3-BD production (Ripoll et al. 2010; Rodriguez et al. 2017).

The final broth composition affects not only to the achieved concentration of the target product, but also to the distribution of products and by-products. Broth composition plays an important role in the economic viability of the process due to the high cost of downstream operations (Ji et al. 2011). In this study, the biotransformation takes place under a great selectivity to the 2,3-BD. On the other hand, organic acids and ethanol concentrations are negligible in comparison to the ones of the diol and its precursor compound in the pathway, acetoin. This is considered as an important advantage to develop the bioprocess at industrial scale.

The evolution of biomass, glycerol and 2,3-BD concentrations are shown in Figure 1 (A)-(B)-(C) respectively. In this Figure it can be observed that the initial glycerol concentration has a deep effect on biocatalyst growth, substrate consumption, and diol production. Regarding the microbial growth (Figure 1A), the initial glycerol concentration affects not only to the specific growth rate but also the maximal biomass concentration reached. There is a clear substrate inhibition on the growth for initial glycerol concentration higher than 110 g/L. However, the results dealing with glycerol consumption (Figure 1B) showed that the substrate inhibition is more pronounced for higher initial glycerol concentration higher than 140 g/L. Figure 1C shows that for initial glycerol concentration lower than 140 g/L, 2,3-BD production rate seems to be independently of the substrate concentration. As it can be expected, the final 2,3-BD concentration depends on the initial amount of substrate, but, due to substrate inhibition, 50 g/L is the maximal 2,3-BD concentration reached in batch experiments. Substrate inhibition is also showed in productivity and yield values (Table 2 and Figure 2). Maximal values for both variables are obtained in the range of glycerol concentration between 110 and 140 g/L.

The substrate inhibition in the production of 2,3-BD employing glucose as carbon source has been reported by other authors (Jurchescu et al. 2013; Kim et al. 2016). Nevertheless, this phenomenon has not previously described for the glycerol metabolism. Fed-batch operation is recommended in order to avoid substrate inhibition and increase the concentration of the target product,. In this way, the conclusions of the batch fermentations are the key for developing a successful fed-batch fermentation. Glycerol consumption and 2,3-BD production rates are directly relating to the slope of the evolution of glycerol and 2,3-BD concentration. The influence of the increase of the initial substrate concentration on glycerol consumption and diol production rates are shown in Figure 2A. The trends observed for these parameters follow the behaviour previously described. Both rates remain almost constant (1.15 g/L·h and 0.45 g/L·h, respectively) whereas glycerol concentration is lower than 125 g/L. The substrate inhibition is more pronounced for substrate consumption rate than for 2,3-BD production rate.

The 2,3-BD yield is expressed as the ratio between the yield (2,3-BD concentration / initial glycerol concentration) and the theoretical maximum yield, which is calculated applying the electron balance (Doran, 2013). The value of the theoretical maximum yield of 2,3-BD from glycerol is 0.62 g/g. The influence of initial glycerol concentration on 2,3-BD yield respect to the maximum theoretical yield is shown in Figure 2B. According to the results, the yield ratio reaches a maximum value around70 % for glycerol concentration between 100 and 140 g/L.

Based on these results, 140 g/L is established as the maximum glycerol concentration in broth during the fermentation,. This concentration will be assumed as a biological constriction for the development of the bioprocess in fed-batch operational mode.

3.2 *2,3-butanediol production enhancement by means of a fed-batch strategy*

In order to increase the final 2,3-BD concentration, different fed-batch experiments were carried out according to the conclusions from the batch runs. Firstly, an experiment employing pure glycerol with a feeding profile of 100 g/L as initial concentration and two subsequent pulses of 60 g/L was performed. Both feeding pulses were supplied before the glycerol concentration in

188 the broth was lower than 30 g/L. The evolution of biomass and broth composition is shown in
189 Figure 3A: glycerol consumption and 2,3-BD production rates remain constant during the first 6
190 days of fermentation. From that moment, the microorganism metabolism seems to slow down
191 and 2,3-BD concentration does not change any more. It could be due to the large time length
192 needed for the fermentation. However, the total glycerol concentration employed in this run
193 (190-200 g/L) yields a higher 2,3-BD concentration (93.3 g/L) compared to the results in batch
194 mode (29.85 g/L) from the same glycerol concentration.

195 Right afterwards, a new glycerol feeding profile was proposed in order to achieve the complete
196 glycerol consumption. In this case, the initial glycerol concentration was 60 g/L and several
197 pulses (5) of 30 g/L were carried out. The results are shown in Figure 3B. In this case, glycerol
198 is complete exhausted at 180 h. At that point, the final broth composition is formed mainly by
199 2,3-BD, acetoin and biomass. Similar 2,3-BD concentration is achieved (90.5 g/L) with a
200 constant rate during the fermentation (0.52 g/L·h).

201 Finally, biodiesel-derived raw glycerol was employed as sole carbon source in order to produce
202 2,3-BD by means of a fed-batch mode operation. The feeding profile employed was similar to
203 that one used for the best results for pure glycerol (initial glycerol concentration of 60 g/L and 5
204 pulses of 30 g/L). The results are shown in Figure 3C. Similar trends are observed for the
205 evolution of glycerol and the fermentation metabolites. For raw glycerol, the complete
206 consumption of the carbon source lasts 200 h. 2,3-BD production taken place with a constant
207 rate of 0.40 g/L·h. This rate is slower than the rate for the pure substrate. However, industrial
208 waste has been employed as only carbon source. Therefore, the obtained results are also quite
209 interesting since the point of view of increase the value of the waste as well as the overall profits
210 in biodiesel industry. The reached 2,3-BD titre from raw glycerol is 80.5 g/L.

211 Final broth composition in fed-batch runs is also included in table 2. It must be pointed out that
212 both is principally composed by 2,3-BD and biomass at the end of fermentation. This is a main
213 advantage for the recovery and purification of the product at industrial scale.

214 Regarding the 2,3-BD production from glycerol by means of a fed-batch fermentation, a
215 comparison between our results and the previously published studies has been summarized in
216 Table 3. Table 3 presents a summary of literature values of 2,3-BD concentration, yield and
217 productivity along with values from this reported experimental work, discerning between
218 researches that use pure and biodiesel-derived raw glycerol. It should be pointed out that, in the
219 present work 2,3-BD yield reached 90 % respect to the maximal theoretic yield. Regarding these
220 previously published results, the highest 2,3-BD concentrations produced from glycerol are
221 achieved employing *K. oxytoca* as biocatalyst. This strain belongs to risk group 2, not
222 recommended for industrial-scale fermentations. To date, the results presented in this paper
223 show the highest 2,3-BD concentration employing glycerol as sole carbon source and a
224 biocatalyst belonging to risk group 1.

225 **CONCLUSIONS**

226 2,3-butanediol production by means of *R. terrigena* is clearly affected by initial glycerol
227 concentration. Substrate concentration presents an inhibitory effect not only on the final diol
228 concentration but also on the production rate. The highest yield (68 %) and concentration (49.4
229 g/L) in batch runs were achieved employing 140 g/L of pure glycerol. Different feeding
230 strategies in fed-batch runs were carried out in order to avoid inhibition of the process.
231 Successful 2,3-butanediol production results were obtained employing pure glycerol (final titre:
232 90.5 g/L) as carbon source as well as raw glycerol (80.5 g/L).

233

NOMENCLATURE

AA	Acetic acid concentration (g/L)
Ac	Acetoin concentration (g/L)
CDM	Cell dry mass concentration (g/L)
Et	Ethanol concentration (g/L)
G	Glycerol concentration (g/L)
G_C	Consumed glycerol concentration (g/L)
G_T	Total fed glycerol concentration (g/L)
G_0	Initial glycerol concentration (g/L)
LA	Lactic acid concentration (g/L)
P	2,3-butanediol productivity (g/L·h)
t	time (h)
Y	2,3-butanediol yield respect to the maximal theoretic yield (%)
2,3-BD	2,3-butanediol concentration (g/L)

TABLE TITLES

Table 1 Summary of published studies on 2,3-BD production employing different industrial wastes as renewables carbon source.

Table 2 Broth composition at the end of the batch and fed-batch experiments

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

FIGURE CAPTIONS

Figure 1 Influence of initial pure glycerol concentration on **(A)** *R. terrigena* growth, **(B)** glycerol consumption, and **(C)** 2,3-BD production. **Symbols:** runs dealing with different initial glycerol concentration: ■ 35 g/L; ● 80 g/L; ▲ 110 g/L; ▼ 140 g/L; ◆ 190 g/L, and ★ 210 g/L.

Figure 2 Influence of initial pure glycerol in batch fermentation **(A)** on 2,3-BD production rate and glycerol consumption rate **(B)** on 2,3-BD yield respect to the maximal theoretic yield. **Symbols:** Rate dealing with ■ glycerol consumption, ● 2,3-butanediol production, and ▲ yield respect to theoretical maximum yield.

Figure 3 Evolution of biomass glycerol and metabolites concentration in fed-batch experiments employing **(A)** -**(B)** pure glycerol and **(C)** raw glycerol. **Symbols:** concentration of ▼ glycerol; ■ cell dry mass concentration; ◆ 2,3-butanediol; ▶ acetoin; ▲ lactic acid; ◀ acetic acid, and★ ethanol.

TABLES

Table 1 Summary of published studies on 2,3-BD production employing different industrial wastes as renewables carbon source. Risk class classification of the microorganisms was made according to the available information by the Spanish Culture Collection (www.cect.org)

Substrate	Microorganism	Risk class	2,3-BD titre (g/L)	Reference
Jerusalem artichoke	*Klebsiella pneumoniae*	2	80.5	Li et al. 2010
	Paenibacillus polymyxa	1	67.9 77.1	Cao et al. 2017 Gao et al. 2010
	Bacillus sp.	1	28.6	Park et al. 2017
Sugarcane molasses	*Enterobacter aerogenes*	2	98.7 140.0	Jung et al. 2013 Jung et al. 2015
	Klebsiella oxytoca	2	99.5	Dai et al. 2015
	Bacillus subtilis	1	17.4	Deshmukh et al. 2016
Cheese whey powder	*Klebsiella pneumoniae*	2	57.6	Guo et al. 2017
Switchgrass biomass	*Klebsiella oxytoca*	2	79.4	Guragain and Vadlani 2017
Oil palm	*Enterbacter cloacae*	2	7.7	Hazeena et al. 2017
Lignocellulosic hydrolysates	*Enterobacter aerogenes*	2	14.3	Joo et al. 2016
	Zymomonas mobilis	1	10.0	Yang et al. 2016
Glycerol	*Enterbacter cloacae*	2	41.4	Priya and Lal 2019
	Klebsiella pneumoniae	2	11.0 70.0	Huang et al. 2013 Petrov and Petrova 2009
	Klebsiella oxytoca	2	131.5 30.1	Cho et al. 2015 Yen et al. 2014
	Enterobacter aerogenes	2	32.0	Metsoviti at al. 2012
	Raoultella ornithinolytica	1	78.1	Kim et al. 2017
	Raoultella planticola	1	30.8 27.5	Bustamante et al. 2019 Ripoll et al. 2016
	Raoultella terrigena	1	33.6 80.5	Ripoll et al. 2016 This work
Beet Molasses + Glycerol	*Bacillus amyloliquefaciens*	1	83.3 102.3	Yang at al. 2013 Yang et al. 2015

Table 2 Broth composition at the end of the batch and fed-batch experiments

			Glycerol			Biomass	2,3-butanediol			By-products			
Run	**Mode**	**Time (h)**	**Type**	**Initial (g/L)**	**Final (g/L)**	**CDM (g/L)**	**Titre (g/L)**	**P (g/L·h)**	**Y (%)**	**Ac (g/L)**	**LA (g/L)**	**AA (g/L)**	**Et (g/L)**
1	Batch	25	Pure	34.88 ± 1.89	2.60 ± 0.43	11.52 ± 0.58	5.95 ± 0.82	0.24	30	0.57 ± 0.11	0 ± 0.01	0.37 ± 0.16	0.95 ± 0.20
2	Batch	67	Pure	82.67 ± 3.12	2.27 ± 0.55	12.54 ± 0.79	22.01 ± 1.56	0.33	46	1.60 ± 0.33	0 ± 0.01	0.65 ± 0.21	2.94 ± 0.35
3	Batch	92	Pure	110.73 ± 6.14	0 ± 0.01	13.84 ± 0.62	33.11 ± 2.41	0.36	64	3.35 ± 0.24	0 ± 0.01	0.52 ± 0.08	2.41 ± 0.42
4	Batch	128	Pure	142.97 ± 5.95	12.43 ± 1.34	12.40 ± 0.81	49.42 ± 3.45	0.39	68	4.45 ± 0.08	0 ± 0.01	0.77 ± 0.17	0.88 ± 0.28
5	Batch	128	Pure	194.20 ± 7.95	135.58 ± 6.24	11.77 ± 0.59	29.85 ± 2.02	0.22	25	1.59 ± 0.12	0 ± 0.01	0.93 ± 0.14	0 ± 0.01
6	Batch	128	Pure	211.54 ± 8.20	174.55 ± 8.35	9.13 ± 0.46	21.79 ± 1.95	0.17	17	1.89 ± 0.24	0 ± 0.01	0 ± 0.01	0 ± 0.01
7	Fed-batch	192	Pure	100+2·(60)	32.68 ± 3.95	17.89 ± 0.72	93.25 ± 4.65	0.49	96	8.30 ± 0.54	0 ± 0.01	1.23 ± 0.37	0.26 ± 0.08
8	Fed-batch	175	Pure	60+5·(30)	0 ± 0.01	15.01 ± 0.89	90.50 ± 5.05	0.52	88	7.21 ± 0.14	0 ± 0.01	2.25 ± 0.31	0 ± 0.01
9	Fed-batch	200	Raw	60+5·(30)	0 ± 0.01	13.65 ± 0.66	80.54 ± 3.95	0.40	90	10.83 ± 0.36	0 ± 0.01	0 ± 0.01	0.35 ± 0.06

281

282 **Table 3** Summary of published studies on 2,3-BD production from pure and raw glycerol as sole carbon source using different strains in fed-batch mode
283 operation.

Microorganism	Risk group	Glycerol	G_T (g/L)	G_C (g/L)	2,3-BD (g/L)	P (g/L·h)	Y (%)	Reference
Klebsiella pneumoniae	2	Pure	-	180	70.0	0.47	78	Petrov and Petrova 2010
Klebsiella oxytoca	2	Pure	310	297	115.0	1.01	82	Cho et al. 2015
		Raw	345	300	131.5	0.84	90	
Bacillus amyloliquefaciens	1	Raw	-	113	43.1	0.45	78	Yang et al. 2013
Raoultella ornitholytica	1	Raw	200	175	78.1	0.87	72	Kim et al. 2017
Raoultella terrigena	1	Pure	210	210	90.5	0.52	88	This work
		Raw	182	182	80.5	0.40	90	

FIGURES

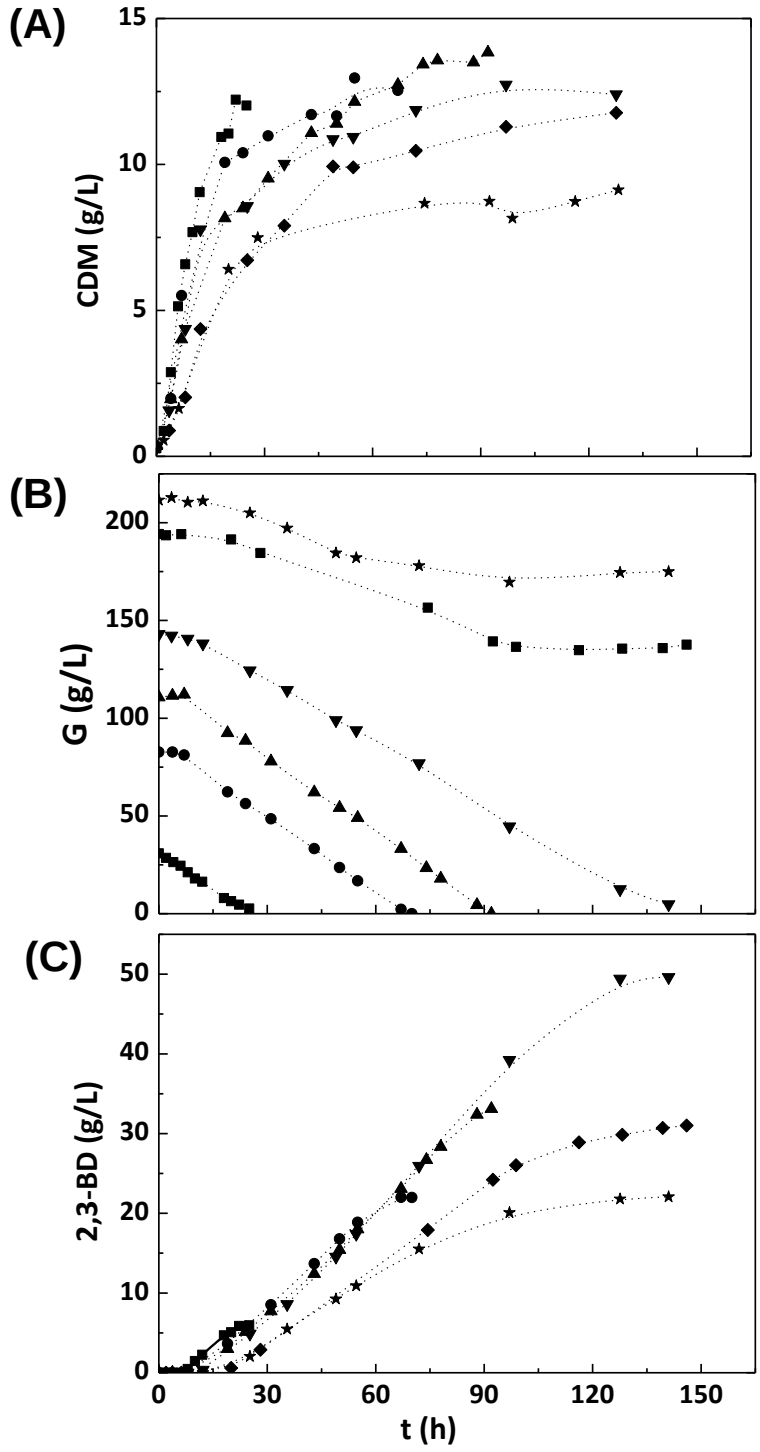

Figure 1 Influence of initial pure glycerol concentration on **(A)** *R. terrigena* growth, **(B)** glycerol consumption, and **(C)** 2,3-BD production. **Symbols:** runs dealing with different initial glycerol concentration: ■ 35 g/L; ● 80 g/L; ▲ 110 g/L; ▼ 140 g/L; ◆ 190 g/L, and ★ 210 g/L.

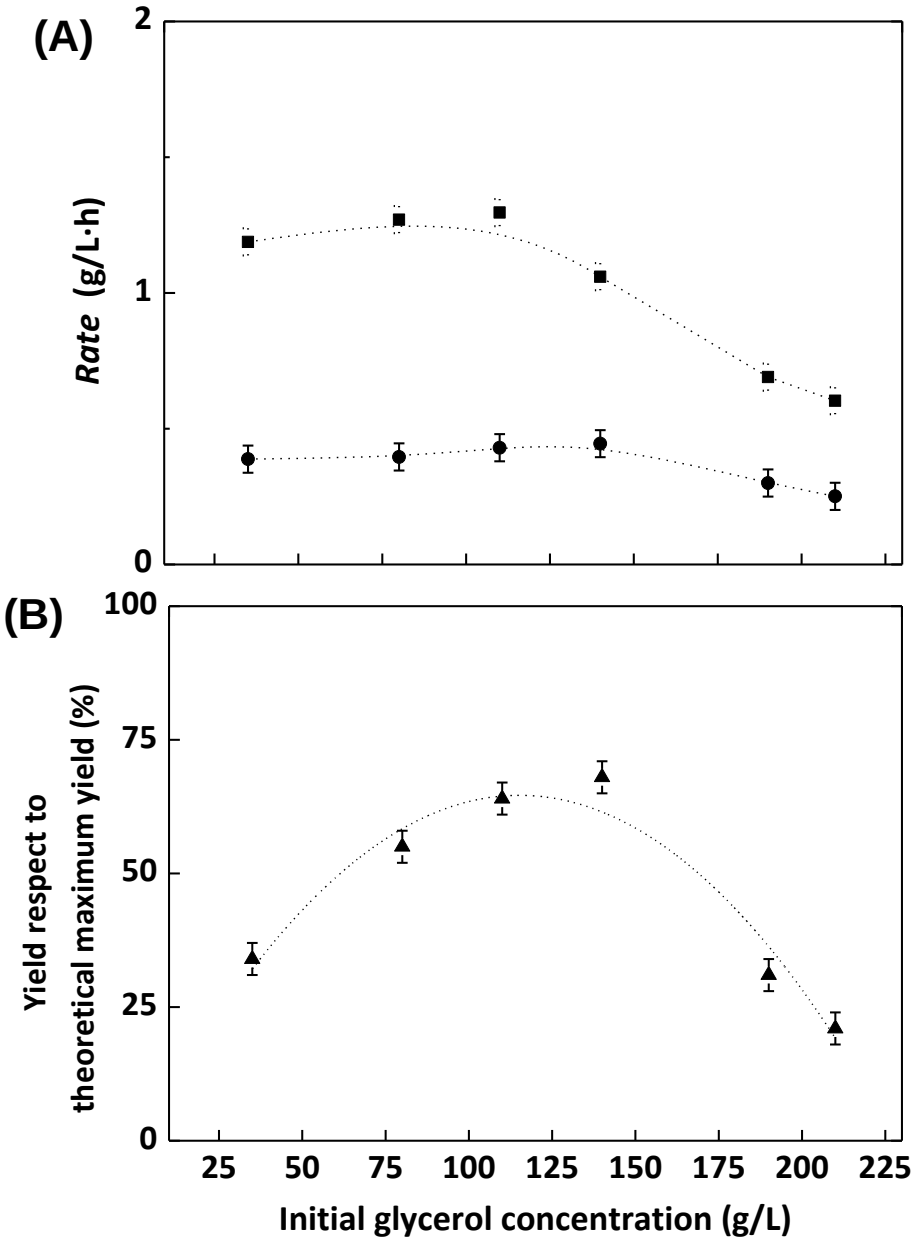

Figure 2 Influence of initial pure glycerol in batch fermentation **(A)** on 2,3-BD production rate and glycerol consumption rate **(B)** on 2,3-BD yield respect to the maximal theoretic yield. **Symbols:** Rate dealing with ■ glycerol consumption, ● 2,3-butanediol production, and ▲ yield respect to theoretical maximum yield.

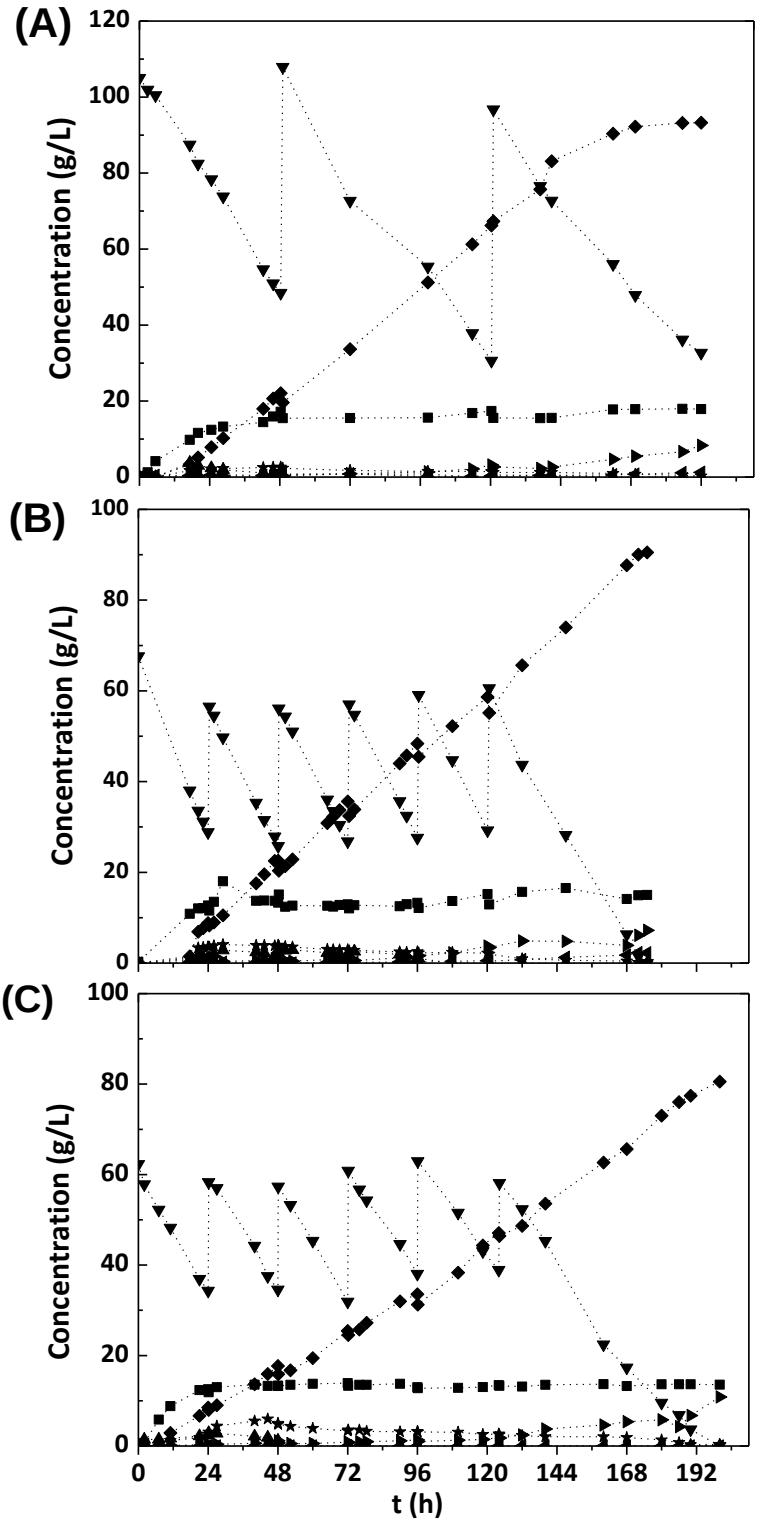

Figure 3 Evolution of biomass glycerol and metabolites concentration in fed-batch experiments employing **(A)** -**(B)** pure glycerol and **(C)** raw glycerol. **Symbols:** concentration of ▼ glycerol; ■ cell dry mass concentration; ◆ 2,3-butanediol; ▶ acetoin; ▲ lactic acid; ◀ acetic acid, and★ ethanol.

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