Improved *Raoultella planticola* Strains for the Production of 2,3-Butanediol from Glycerol

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Abstract: Raw glycerol is an industrial byproduct from biodiesel production and is one of the most promising substrates for 2,3-butanediol (2,3-BD) production; however, 2,3-BD is not yet produced by fermentation from glycerol on a commercial scale due to poor process economics. Class 1 microorganism collections were screened and *Raoultella planticola* strain CECT 843 proved to be the best 2,3-BD producer, achieving (23.3 ± 1.4) g 2,3-BD per L and a yield of 36% (g 2,3-BD per g glycerol). To further increase product concentration and yield, *R. planticola* CEC T843 was subjected to random mutagenesis using ultra-violet (UV) light and ethyl methane sulfonate (EMS). Two mutant strains were found to produce at least 30% more 2,3-BD than the wild type: *R. planticola* IA1 [(30.8 ± 3.9) g 2,3-BD per L and 49% yield] and *R. planticola* IIIA3 [(30.5 ± 0.4) g 2,3-BD per L and 49% yield].

Keywords: 2,3-butanediol; Improved strain; *Raoultella planticola*; Raw glycerol

1. Introduction

The alcohol 2,3-butanediol is naturally found in cacao butter and roots of *Ruta graveolens*. There is rising interest in this compound due to its many industrial applications, mainly for the chemical and energy industry. 2,3-BD (2,3-butanediol), as well as some derivatives, are employed in plastic and solvent production, such as octane booster in fuels, antifreeze agent or analytical reagent in the racemic separation of carbonyl compounds for gas chromatography [1,2]. One of the main applications of 2,3-BD is its conversion to 1,3-butadiene, employed in synthetic rubber production [3]. Dyacetil, the dehydrogenation product of 2,3-BD, is a highly-valued flavor and bacteriostatic agent for the food industry. Moreover, dehydration of 2,3-BD yields methyl-ethyl-ketone (MEK), an additive with high combustion heat for fuels. MEK is also employed as solvent for resins and lacquers. Polyurethane-meliamides (PUMAs), which are useful for cardiovascular applications, are obtained from 2,3-BD esterification with malic acid. Other 2,3-BD esterification products are employed in the cosmetic and pharma industries. Other potential applications of 2,3-BD are the production of moistening and softening agents, elastane, fumigants, plasticizers, perfumes, printing inks and carriers for pharmaceuticals [4,5].

Nowadays, the economical production of many chemicals, including 2,3-BD, has been possible via chemical synthesis from fossil fuels, but it has now become necessary to develop new, environmentally friendly biotechnologies based on renewable resources [1]. The first information on the subject was announced on the Lanzatech company website, www.lanzatech.com (access on 24 August 2010), with a technology that involves *Clostridium* strains and syngas as feedstock. Indeed, 2,3-BD production

could be achieved by various bacterial strains belonging mainly to the genera *Bacillus*, *Enterobacter*, *Klebsiella*, *Raoultella* and *Serratia* [6–8]. The most efficient 2,3-BD producers described so far are *B. polymyxa*, *K. pneumoniae* and *K. oxytoca,* mainly using sugars as substrates [5,9].

Furthermore, there are well-known advantages of using industrial by-products (raw glycerol, whey or agriculture waste) for the production of chemicals. Due to economic and ecological concerns, the industrial production of 2,3-BD should utilize easily available and relatively cheap renewable resources, preferably by-products from the food and biofuel industries [5]. Considering that in biodiesel production approximately 100 kg of raw glycerol is produced per ton of product, it is very interesting to seek alternatives to upgrade this residual substrate. Thus, glycerol is one of the most promising substrates for 2,3-BD production [10].

To date the best 2,3-BD producers using glycerol are *K. pneumoniae* strains [11,12]. This species also produces useful compounds such as lactic acid, 3-hydroypropionic acid, ethanol, 1,3-propanediol and succinic acid, depending on the fermentation conditions [13,14]. However, *K. pneumoniae* is listed as a group risk 2 biological agent. This is a serious drawback as it is a pathogenic agent, which may cause infections in humans, thus posing a hazard for workers in contact with it (European Directive 20009/54/CD of European Parliament and European Council 18 September 2000) and makes it an unsuitable strain for industrial-scale fermentation.

The development of genetic engineering techniques and intensive studying of metabolic potential of microorganisms has allowed designing genetically modified microorganisms (GMOs), including *K. oxytoca*, *K. pneumoniae* or *R. ornithinolytica* strains, with higher yields of 2,3-BD from raw glycerol but they remain pathogenic [15–17]. Some wild type non-pathogenic *Raoultella* species such as *Raoultella planticola* and *Raoultella terrigena* strains (risk group 1) are able to produce 2,3-BD from pure glycerol and raw glycerol as sole carbon source without 1,3-propanediol formation [8,18]. However, the results are still lower than 2,3-BD titers obtained by *K. pneumoniae* possessing the 1,3-propanediol synthesis pathway [12].

The objective of this work is to describe a procedure to generate improved *Raoultella planticola* strains in order to obtain a better risk-1 biocatalyst for 2,3-BD production from glycerol as raw material and to contribute on the development of a safer and sustainable process to the production of a cost-competitive building block for the future bio-economy era [19].

2. Materials and Methods

2.1. Strains, Medium Composition and Culture Conditions

The wild-type strain *Raoultella planticola* CECT 843 was obtained from the Spanish Type Culture Collection (CECT), in Paterna (Valencia, Spain). After receiving the strain, it was recovered in the media recommended by the supplier (nutrient broth, NB) and stored in 20% glycerol for long-term preservation. *Raoultella planticola* strains CECT 8158 and CECT 8159 were obtained in this work by random mutagenesis.

Batch tests of 2,3-BD production were carried out in 50-mL flasks, containing 10 mL of the growth medium MB or MC. Medium MB composition was described by Nakashimada and coworkers [20], with 30 or 60 g glycerol per liter. MC is a simpler medium, whose composition per liter is: 60 g glycerol, 1.5 g yeast extract, 0.42 g citric acid, 2 g NH_4Cl, 6 g KH_2PO_4, 12 g Na_2HPO_4, 1 g NaCl, 246 mg $MgSO_4 \cdot 7H_2O$, 14.7 g $CaCl_2 \cdot 2H_2O$. Inoculum was prepared in NB and added up to 1% (v v^{-1}) of the culture volume. Flasks were prepared in triplicates and incubated under aerobic conditions at 175 rpm shaking, 28 °C and an initial pH of 7. Samples were taken at 24 h and bacterial growth was measured; glycerol and product concentration were analyzed.

Fermentations were performed in Applikon$^{\circledR}$ 1.5 L bioreactors with 0.5 working volume and the optimal conditions found in the experimental design optimization assay, with medium MC, and initial glycerol concentration of 60 g L^{-1}. The fermentation conditions were: 33 °C, 500 rpm, and an oxygen saturation of 5%. Initial pH was set at 6.8 and it was not controlled during fermentation.

2.2. Design of Experiments and Data Interpretation

Taguchi Method was used to enhance 2,3-BD production by using a design of orthogonal arrays, studying the influence of operational temperature and medium composition (glycerol initial concentration and Co^{2+} salt) with a reduced number of runs [21]. Two responses, called target functions, were selected: 2,3-BD production ($y_1 = [2,3\text{-BD}]_{max}$), and 2,3-BD yield ($y_2 = Y_{2,3\text{-BD/Glyc}}$).

In contrast to traditional full or fractional factorial design and response surface methods, the Taguchi Method minimizes experimental work by means of a standard layout array. To examine the influence of three parameters at three levels, a standard L_9 orthogonal array was used, consisting of just nine runs in duplicates (Table 1). All intersections between parameters are assumed negligible. The assayed parameters were: initial glycerol concentration (60, 75, 90 g L^{-1}), $CoCl_2$ concentration (0.012, 0.024, 0.036 g L^{-1}), and temperature (28, 30, 33 °C). The oxygen saturation was kept fixed at 5% with 500 rpm of agitation. The assays were performed in a micro-reactor (Applikon® μ-24 Bioreactor) with 3 mL of working volume using MB medium. Fermentations were finished at 24 h.

According to the Taguchi method, experimental data of response variables (y_1 and y_2) were transformed into a Signal-to-Noise (S/N) ratio for evaluating the performance of the system. For the S/N ratio analysis, the correct function must be chosen as the-smaller-the-better, the-larger-the-better and the-nominal-the-better. In the present work, the highest 2,3-BD yield and glycerol consumption were assayed. Data interpretation requires a comparison of the average values of S/N ratios of three cutting factors at each level. The optimum level of each studied parameter corresponds to the highest S/N ratio.

Table 1. Experimental layout using an L_9 Orthogonal Array. Selected parameters and their levels.

| Exp. | Factors | | | Results | | | |
	T (°C)	[Glyc] (g L^{-1})	[$CoCl_2$] (g L^{-1})	CDW (g L^{-1})	[2,3-BD]$_{max}$ (g L^{-1})	[Glyc]$_{cons}$ (g L^{-1})	$Y_{2,3\text{-BD/Glyc}}$ (g g^{-1})
E1	28 (1)	75 (2)	0.036 (3)	6.0 ± 1.5	18.0 ± 2.1	37.9 ± 2.7	0.24 ± 0.02
E2	30 (2)	60 (1)	0.012 (1)	6.5 ± 0.5	14.2 ± 3.4	34.6 ± 1.6	0.24 ± 0.04
E3	33 (3)	90 (3)	0.024 (2)	3.8 ± 1.0	20.4 ± 1.7	23.3 ± 3.6	0.23 ± 0.03
E4	28 (1)	90 (3)	0.012 (1)	7.7 ± 0.7	15.2 ± 1.6	27.0 ± 2.4	0.17 ± 0.01
E5	30 (2)	75 (2)	0.024 (2)	8.4 ± 0.3	19.1 ± 3.3	40.0 ± 3.3	0.25 ± 0.02
E6	33 (3)	60 (1)	0.036 (3)	10.6 ± 1.3	22.3 ± 4.5	54.4 ± 4.1	0.37 ± 0.05
E7	28 (1)	60 (1)	0.024 (2)	6.0 ± 0.8	15.5 ± 2.4	33.9 ± 1.2	0.26 ± 0.03
E8	30 (2)	90 (3)	0.036 (3)	8.3 ± 0.7	18.0 ± 3.7	26.6 ± 0.9	0.20 ± 0.01
E9	33 (3)	75 (2)	0.012 (1)	8.4 ± 0.2	14.0 ± 2.6	32.3 ± 1.8	0.19 ± 0.03

[2,3-BD]$_{max}$ = maximum 2,3-BD concentration achieved; [Glyc]$_{cons}$ = consumed glycerol concentration; $Y_{2,3\text{-BD/Glyc}}$ = fermentation yield, grams of 2,3-BD obtained with respect to the grams of glycerol consumed.

2.3. Random Mutagenesis and Screening Protocols

2.3.1. Ethyl Methane Sulfonate (EMS) Mutagenesis

For EMS mutagenesis, a *R. planticola* CECT 843 culture grown in medium MB with 30 g L^{-1} of glycerol in exponential phase (2×10^8 cfu mL^{-1}) was employed. Cells were washed with phosphate buffer 0.1 M, and resuspended in the same volume buffer. The cell suspension was divided in 1 mL aliquots in Eppendorf tubes, to which different EMS volumes were added (0, 2, 4, 6, 8, 10, and 20 μL per mL of cell suspension). Incubation with EMS was for 1 h at 30 °C. Afterwards, two washing steps were performed with the phosphate buffer and two more with sodium thiosulfate 10% (w v^{-1}) solution in order to neutralize the mutagenic agent (centrifugation steps during 15 min at 10.000 *g*). Each cell pellet was transferred to a culture tube with 5 mL of MB medium with 30 g L^{-1} of glycerol

and incubated at 30 °C for 16–18 h for cell recovery. Cell count was performed in NB plates to assess the death percentage of the treatment. The dosage chosen for mutant screening was 10 µL per mL of EMS (95% cell death).

The culture suspension (from 10 µL per mL EMS dosage) was plated on selection plates with NaBr/NaBrO$_3$ (100 mM NaBr and 100 mM NaBrO$_3$), 12 g L^{-1} glucose, 4 g L^{-1} peptone, 1.2 g L^{-1} meat extract, 2 g L^{-1} NaCl and 16 g L^{-1} agar), and was incubated at 30 °C for 2–3 days until colonies appeared. Previously, different NaBr/NaBrO$_3$ ratios and pH values had been tested: Br$^-$/BrO$_3^-$ 160/40 mM, 130/32.5 mM, 100/25 mM and 100/100 mM; pH 7.0, 6.5 and 6.0. The combination of 100/100 mM of each salt and pH 6.0 was chosen as it allowed the growth of enough colonies to be tested: proton suicide method of Cueto and Méndez [22]. This method enables mutants with lower acid production to be selected, because those that produce organic acids are killed by the Br^0 formed in the plate. As the production of organic acids competes with 2,3-BD production, lower acid production should result in increased 2,3-BD production.

2.3.2. UV Mutagenesis

For the UV mutagenesis procedure, 1 mL of cell suspension washed with phosphate buffer (2×10^8 cfu mL^{-1}) from an exponential growing culture, was inoculated on plates containing NaBr/NaBrO$_3$ 100 mM at pH 6.0. As controls, serial dilutions of the cell suspension were plated on LB medium plates. Different UV exposure times were applied: 0, 15, 30, 45 and 60 s. The plates were incubated in the dark until colonies appeared. The exposure time of 45 s was chosen (95% death rate).

2.3.3. Mutant Screening

The isolated colonies obtained in the NaBr/NaBrO$_3$ plates from the two mutagenesis procedures were assayed for 2,3-BD and acetoin production in micro-titer plates with culture medium MB with 30 g L^{-1} of glycerol at 28 °C and 500 rpm, adding phenol red at 0.008% w/v. Below pH 6.4 this indicator turns yellow, enabling the rejection of those mutants lowering pH below this value, and the selection of those producing less acids. Colorimetric assays for acetoin and 2,3-BD detection were performed as described below. Following this method, up to 650 mutants were analyzed. Those that gave positive for acetoin and 2,3-BD production (24 mutants) were kept for further fermentation assays.

2.4. Analytical Methods

Cell concentration was determined by means of optical density measurements at 600 nm (Eppendorf Biophotometer) or by cell dry weight (CDW) from freeze-dried samples. Additionally, pH values were measured with a selective pH-meter (Crison pH-meter Basic 20+). Cells were separated from cultures by centrifugation (14,000 × g/10 min, 20 °C) (Eppendorf Centrifuge 5425) in order to analyse supernatant composition by HPLC. Glycerol, 2,3-BD and by-products (succinic acid, lactic acid, acetic acid, acetoin and ethanol) were detected and quantified using a refractive index detector (Waters 2695 HPLC with a Refractive index detector 2414), after separating them in a Rezex ROA-Organic Acid column (Phenomenex). The column was operated at 65 °C, and 0.05 M sulphuric acid was used as mobile phase at a flow rate of 0.5 mL min^{-1}.

Colorimetric tests for the detection of 2,3-BD and acetoin were adapted for micro-titer plates from previous literature reports [23–25]. For the assay of 2,3-BD in micro-titer plates, 150 µL of supernatant was mixed with 32 µL of H$_5$IO$_6$ 0.1M and incubated for 30 min at room temperature. Afterwards, 20 µL of ethylene glycol, 48 µL of piperazine saturated solution (30% w v^{-1}) and 16 µL of sodium nitroprusside solution (4% w v^{-1}) were added. A transient intense blue colour appears in response to the presence of 2,3-BD. The acetoin assay involved adding and mixing 100 µL of culture supernatant, 12 µL of creatine solution (0.5% w v^{-1}), 30 µL of α-naphthol in ethanol (solution at 5% w v^{-1}) and 20 µL of KOH solution (40% w v^{-1}). After 5–10 min, the mixture turns red if the assay is positive for acetoin, and the colour disappears after 30 min.

3. Results and Discussion

3.1. Optimization of Fermentation Conditions for the Wild-Type Strain

The strain *Raoultella planticola* CECT 843 was identified as one of the best producers of 2,3-BD from glycerol in a previous report [8]. 2,3-BD biosynthesis plays a very important physiological role in microorganisms preventing acidification, regulating NADH/NAD$^+$, and storing carbon and energy for growth. In order to assess the optimal environmental parameters and medium composition, a Taguchi experimental design was adopted: the effect of temperature, initial glycerol concentration and initial cobalt concentration were assayed, employing MB medium. The influence of operational temperature is closely connected to enzymatic activity and cellular maintenance; while, in relation to medium composition, Co^{2+} possessed the most important influence among all bivalent ions [11,26]. The dissolved oxygen concentration was set at 5% in all cases because it has been reported that 2,3-BD production with *Raoultella* sp. strains is efficient under oxygen limiting conditions [18]. Statistical analysis of variability associated to each factor indicated that glycerol concentration, temperature and cobalt-salt concentration significantly influenced the objective functions. The pH value was set at 6.8 at the beginning of the assay and was not controlled during fermentation.

The experimental results are given in Table 1 and the optimal levels for each factor are shown in Figure 1. In general, temperature range between 30 and 37 °C is the optimum for cell growth and fermentation; in this case results are better when temperature was increased between 28–33 °C because it resulted in a substantial reduction in ethanol synthesis in favor of 2,3-BD formation [8], but under applied conditions a temperature of 33 °C appeared to be optimal. Changes in temperature had little effect on 2,3-BD formation [4]. On the other hand, the excess substrate can cause inhibition of the strain, which apparently occurs at high concentrations of glycerol while the cobalt ion seems to play an important role in the synthesis of 2,3-BD. Therefore, to increase 2,3-BD production, yield and glycerol consumption, the best conditions were 33 °C, 60 g L^{-1} of initial glycerol concentration and 0.036 g L^{-1} of $CoCl_2$.

A batch-fermentation assay in 1.5-L bioreactor was performed with the wild-type strain at the optimal conditions determined in the variable optimization assay. All the glycerol was consumed during the cultivation, and a 2,3-BD concentration of 22 g L^{-1} was obtained, with a yield of 0.34 gram of 2,3-BD per gram of glycerol, which is 55% of the theoretical yield, 0.62 g of 2,3-BD per gram of glycerol obtained from an electron balance [8].
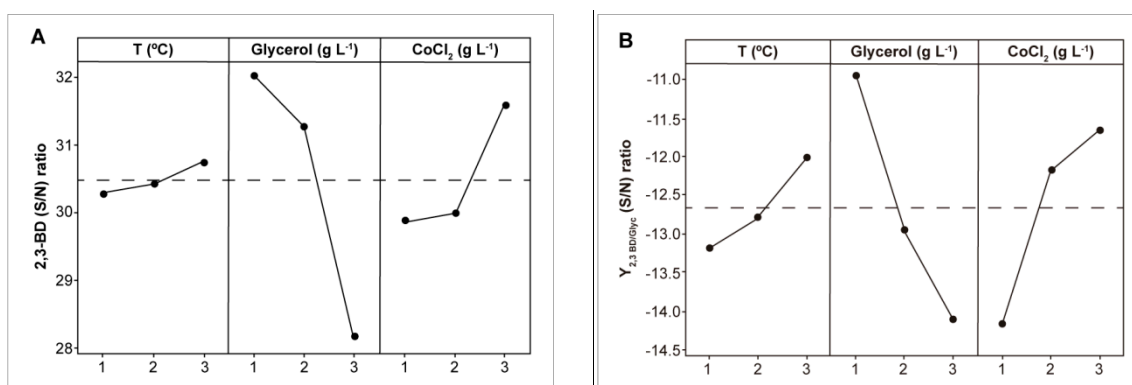

Figure 1. Effect of the variables on the production of 2,3-BD by *Raoultella planticola* CECT843 (S/N ratio). (**A**) Effect of the variables on the 2,3-BD production. (**B**) Effect of the variables on the yield for the 2,3-BD production. These variables were tested in a micro-reactor with medium MB and 3 mL of work volume (see Materials and Methods section). Results were analyzed by Taguchi method.

3.2. Mutation of Raoultella Planticola CECT843 and Selection of the 2,3-BD Overproducing Strains

A procedure to obtain overproducing 2,3-BD strains was employed with *R. planticola* CECT 843. Cells were exposed to UV radiation or EMS followed by selection in plates with bromated/bromide (proton suicide method). In total, 650 mutants were analyzed, of which 24 were selected after micro-titer plate assays: those with low pH decrease (red phenol in the culture medium) and the production of acetoin and 2,3-BD (by colorimetric tests). These 24 selected strains were assayed in a micro-reactor device with the optimal fermentation conditions found for the wild-type strain. Glycerol consumption, 2,3-BD production and cell growth were analyzed. The results are shown in Table S1.

Seven of these strains (A7, H7, IA1, IA12, IIC12, IIIA1 and IIIA3) were selected according to 2,3-BD concentration and yield (Figure 2). Mutants IA1, IA12, IIC12, IIIA1 and IIIA3 come from the EMS procedure, whereas the H7 mutant was a spontaneous mutant obtained when the wild-type strain was grown in $NaBr/NaBrO_3$ 100 mM plates. The A7 mutant was obtained with the UV mutagenesis procedure.
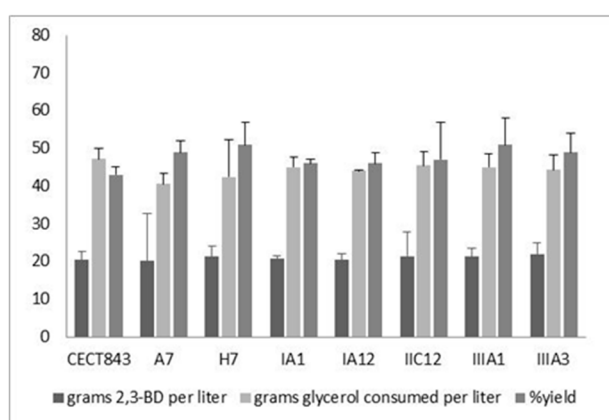

Figure 2. Comparison of wild-type and mutant strains obtained by random mutagenesis. Twenty-four strains were tested in a micro-reactor with medium MB and 3 mL of work volume (see Material and Methods section) but strains A7, H7, IA1, IA12, IIC12, IIIA1 and IIIA3 showed the best yields.

These seven strains were tested again at bioreactor scale with the optimized conditions found previously for the wild type strain by Taguchi method (Table S2). The assays were performed to confirm the previous results at higher scale and to select the best producers. Figure 3 shows that the 2,3-BD concentration obtained for strains IA1 and IIIA3 (both obtained by random mutagenesis with EMS and selection in $NaBr/NaBrO_3$ plates) is higher than that obtained with the other mutants. Mutants IA1 and IIIA3 produced more than 30 g L^{-1} of 2,3-BD, with yields approximating the theoretical maximum for this transformation.

With the mutants IA1 and IIIA3, especially mutant IA1, the yield is very close to the theoretical yield, to the best of our knowledge, this is the best result reported so far. In the case of *K. pneumoniae*, 1,3-propanediol is usually the main fermentation product. Although it is possible to modulate the process by aeration and pH-forced fluctuations [11,12], the maximum yield reported in the fermentations with glycerol was 0.39 (w w^{-1}). Another drawback of *K. pneumoniae* is its pathogenicity. In fact, attempts to remove pathogenicity factors of *K. pneumoniae* are being addressed [27]. Another recent report described the conversion of glycerol to 2,3-BD with *Serratia sp.*, with a yield of 0.43 (w w^{-1}) [28] and 0.42 (w w^{-1}) by a genetically modified *Raoultella ornithinolytica* strain and glycerol [17]. However, unsurpassed yields of 0.50 (w w^{-1}) were achieved with the mutants obtained in this work.
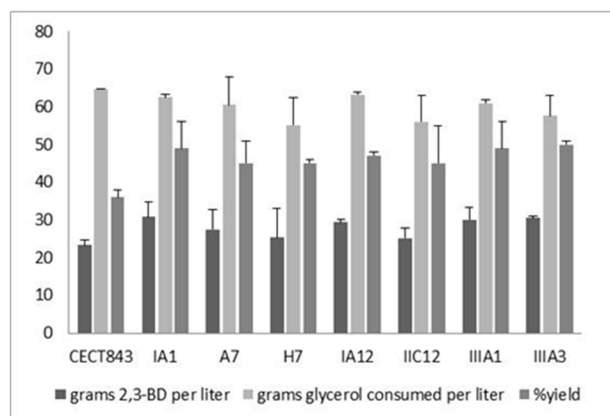
Figure 3. Second round of comparisons between wild type and selected mutants at bioreactor scale. Strains A7, H7, IA1, IA12, IIC12, IIIA1 and IIIA3 were tested in 1.5 L bioreactors with medium MB (see Material and Methods section) to evaluate their performance at the optimum conditions found with Taguchi method.

3.3. Characterization of Fermentation Capacity of the Mutant Strains

In the next fermentation assays, a simpler medium was chosen in order to develop a cheaper fermentation process. Fermentation was performed under the same optimized conditions as in the previous assays, with pure glycerol in 50 mL flasks. The same fermentation procedure was employed for the wild-type and the two selected mutant strains. The results are shown in Table 2. Mutant IA1 produced 56% of 2,3-BD and mutant IIIA3 23.5% more 2,3-BD than the wild-type strain. The results with this medium in flasks are not as good as those obtained with the richer medium MB due to the changes in composition. Another factor to consider is that aeration was not controlled in flask assays and the mass transfer is better in bioreactors. Therefore, the next step was to transfer this fermentation process to bioreactors in which aeration was properly controlled, keeping the simplest medium to make the process more economical.

Table 2. Flask and bioreactor fermentation results of *R. planticola* CECT 843 and mutants CECT 8158 (IA1) and CECT 8159 (IIIA3) with MC culture medium and the optimal conditions selected.

Strain	Device	$[\text{2,3-BD}]_{max}$ (g L^{-1})	$[\text{Glyc}]_{cons}$ (g L^{-1})	$Y_{\text{2,3-BD/Glyc}}$ (g g^{-1})	$[\text{Acet}]_{max}$ (g L^{-1})	$[\text{EtOH}]_{max}$ (g L^{-1})
CECT 843	Flask	15.0 ± 1.2	60.2 ± 4.3	0.25 ± 0.02	6.6 ± 0.5	2.4 ± 0.1
	Bioreactor	22.0 ± 4.1	64.2 ± 3.5	0.34 ± 0.05	6.8 ± 0.3	0.9 ± 0.1
CECT 8158	Flask	23.4 ± 2.3	61.5 ± 6.4	0.38 ± 0.01	3.7 ± 0.3	1.9 ± 0.2
	Bioreactor	33.6 ± 1.5	61.8 ± 1.6	0.54 ± 0.06	4.3 ± 0.2	0.5 ± 0.1
CECT 8159	Flask	18.5 ± 2.1	60.8 ± 2.3	0.30 ± 0.04	3.8 ± 0.7	1.2 ± 0.3
	Bioreactor	30.7 ± 3.5	61.3 ± 4.6	0.50 ± 0.02	3.5 ± 0.8	0.8 ± 0.2

$[\text{2,3-BD}]_{max}$ = maximum 2,3-BD concentration achieved; $[\text{Glyc}]_{cons}$ = consumed glycerol concentration; $[\text{Acet}]_{max}$ = maximum acetoin concentration produced; $[\text{EtOH}]_{max}$ = maximum ethanol concentration produced; $Y_{\text{2,3-BD/Glyc}}$ = fermentation yield, grams of 2,3-BD obtained with respect to the grams of glycerol consumed.

For the fermentation scale up in 1.5-L Applikon® bioreactors, the same MC medium and the optimized conditions for the wild-type strain were employed. Variables such as oxygen saturation and pH were established from previous 2,3-BD production studies [29]. Although 2,3-BD is a product of anaerobic metabolism, aeration has been shown to enhance its production. Low oxygen availability maximizes 2,3-BD yield, but minimizes cell growth, therefore conversion rates are slow. By increasing oxygen supply, higher cell densities and higher production rates are obtained. However, too much aeration, exceeding the microbial oxygen demand, leads to biomass and carbon dioxide as products [18].

Therefore, fine-tuning of oxygen availability is required in order to optimize 2,3-BD production, so the oxygen saturation was controlled at 5%.

The optimum pH depends on the microorganism and the substrate employed [4]. For 2,3-BD production in *K. oxytoca* the optimum pH ranges from 5-6. For *K. pneumoniae*, according to Bielb and coworkers [13] the lower the pH, the higher the 2,3-BD final concentration. However, the best values were obtained when pH was not controlled (starting from pH 7). These authors suggested it is the presence of acetic acid rather than pH that triggers the shift to 2,3-BD synthesis, however acetic acid is a by-product that reduces the yield of 2,3-BD. Petrov and Petrova [12] achieved the best 2,3-BD concentrations by forcing pH fluctuations. In this case, pH was left to develop without restraint.

The results of bioreactor assays are summarized in Table 2. Mutant strains produce 52.7% and 39.5% (IA1 and IIIA3 respectively) more 2,3-BD than the wild-type strain, reaching the maximum yield so far. These mutants have been deposited in the CECT collection under accession numbers CECT 8158 (mutant strain IA1) and CECT 8159 (mutant strain IIIA3). Finally, the optimal fermentation conditions were established for the wild-type strain, and these conditions are suitable for mutants, as the maximum yield is achieved. Concentration of organic acids such as succinic acid, lactic acid and acetic acid are very low or not detected under the conditions of the assays, particularly in bioreactor tests. Only acetoin and ethanol were detected by HPLC method (see Materials and Methods section), then considering metabolic pathways of 2,3-BD production from glycerol, the fermentation conditions were properly optimized [18,21]. The potential that lies in the improvement of biocatalysts and intensified fermentation methods, coupled with optimization procedures and making worthy fermentation systems, will enable the development of bioprocesses to achieve 2,3-BD production efficiently [29].

4. Conclusions

As a conclusion, it is possible to obtain improved biocatalysts for the conversion of glycerol to 2,3-BD with a high yield. These biocatalysts are improved mutants from a risk-1 *R. planticola* CECT 843 strain, obtained by random mutagenesis procedures; therefore, the new biocatalysts, *R. planticola* CECT 8158 and CECT 8159, are not genetically modified organisms. All these factors contribute to providing an alternative for biorefinery processes using glycerol as a potential substrate for valorization and establishing the first steps in the development of safer and more sustainable processes. Experiments are underway to understand the genetic changes related with this hyperproducer phenotype and the industrial suitability of the strains. These results exceed the best data previously reported for 2,3-BD from glycerol.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/5/1/11/s1, Table S1. Comparison of wild-type and mutant strains obtained by random mutagenesis. Table S2. Comparison of wild-type and mutant strains obtained by random mutagenesis in bioreactor

Author Contributions: D.R. and M.T. conceived the research; A.R. designed the experiments; D.B., S.S., and A.M. performed the experiments and analyzed the samples; D.B. developed the DOE and statistical analysis; A.R. evaluated the results; A.R. and D.B. wrote the paper. All the authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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