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# Novel biocatalysts for glycerol conversion into 2,3-butanediol

Vanessa Ripoll[c], Gonzalo de Vicente[d], Bruno Morán[a], Antonia Rojas[b], Silvia Segarra[b], Alejandro Montesinos[b], Marta Tortajada[b], Daniel Ramón[b], Miguel Ladero[a], Victoria E. Santos[a,*]

[a] Department of Chemical Engineering, Faculty of Chemistry, Universidad Complutense de Madrid, 28040 Madrid, Spain
[b] BIOPOLIS, S.L., Parc Científic Universitat de València, 46980 Paterna, Valencia, Spain
[c] Engineering Department, Universidad de Almería, 04120 Almeria, Spain
[d] Fakultät II—Mathematik und Naturwissenschaften, Technische Universität Berlin, 10623 Berlin, Germany

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ABSTRACT

Bioconversion of biodiesel-derived waste glycerol into high-value products is proposed as a solution to improve economic viability of biorefineries. Several microorganisms are able to metabolize glycerol into 2,3-butanediol (2,3-BD), a promising bulk chemical with wide applications: solvent, fuel additive, and feedstock for synthetic rubber production, among them. In the present work, a wide screening of microorganisms present both into the waste water treatment system in a biodiesel industry and in culture collections was carried out in order to evaluate their potential as new 2,3-BD producer biocatalysts. Two microorganisms for 2,3-BD production from glycerol as sole carbon source have been selected, namely *Raoultella planticola* CECT 843 and *Raoultella terrigena* CECT 4519. *Raoultella* strains belong to the non-pathogenic bacteria class (biosafety level 1). This genus has not been previously described as biocatalyst for the studied process. The influence of operational temperature, organic acid addition, and yeast extract concentration on 2,3-BD yield and productivity have been studied through Taguchi design methodology as well as initial glycerol concentration. Based on these results, the feasibility of the process employing pure glycerol and different samples of raw glycerol has been demonstrated.



## 1. Introduction

The increasing worldwide energy demand has led to rely on petroleum and natural gas in modern societies; however, these non-renewable resources are progressively been exhausted. This fact and the fluctuating price of fossil fuels, together with environmental problems derived from their use, have spurred on the development of alternative fuels made from biomass, such as bioethanol and biodiesel [1]. Current European Union policy is focused on increasing the use of biofuels and other renewable fuels for transport. The main objective of European Directives 2003/30/EC and 2009/27/EC was to ensure that at least 2% of the market of diesel or petrol for transport purposes was replaced by biofuels in each Member State by the end of 2005. Since the end of 2010, the lower limit content of biofuels was set on 5.75%.

Biodiesel, the most common biofuel in Europe, is composed of a mixture of fatty acid alkyl monoesters, whose similar physical and chemical properties to diesel allow its use for conventional diesel engines as sole energy source or blended with fossil diesel fuel [2–4]. In spite of the environmental advantages, the economic viability of this biofuel is the main concern. Thus, the implementation of biorefineries that co-produce higher-value products from the resulting waste could improve its economic feasibility [5].

Industrial synthetic biodiesel pathway generates also glycerol as by-product in a large extension: 10% (w/w) [6,7]. Downstream processing operations involving the separation of glycerol from the biodiesel phase through different techniques, such as filtration, centrifugation or decantation are needed. However, raw glycerol contains several impurities, such as soaps, remaining catalyst, alcohol, water and salts and the final content of glycerol is, as usual, between 60 and 90%, depending on the feedstock composition and the purification methods [1,8–11].

Conventional glycerol applications related to pharmaceutical, food and cosmetic uses require high refining of raw glycerol. Thus,

* Corresponding author.
*E-mail addresses:* vripoll@ual.es (V. Ripoll), g.devicentelucas@tu-berlin.de (G. de Vicente), bjusdado2@hotmail.com (B. Morán), antonia.rojas@biopolis.es (A. Rojas), silvia.segarra@biopolis.es (S. Segarra), alejandro.montesinos@biopolis.es (A. Montesinos), marta.tortajada@biopolis.es (M. Tortajada), daniel.ramon@biopolis.es (D. Ramón), mladerog@ucm.es (M. Ladero), vesantos@ucm.es (V.E. Santos).

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**Nomenclature**

| | |
|---|---|
| $C_{2,3\text{-BD}}$ | 2,3-BD concentration (g/L) |
| $C_A$ | Acetic acid concentration (g/L) |
| $C_{AC}$ | Acetoin concentration (g/L) |
| $C_L$ | Ethanol concentration (g/L) |
| $C_G$ | Glycerol concentration (g/L) |
| $C_G{}^\circ$ | Initial glycerol concentration (g/L) |
| $C_L$ | Lactic acid concentration (g/L) |
| $C_X$ | Dry biomass concentration (g/L) |
| n | Number of observations, Eq. (3) |
| $P_{2,3\text{-BD}}$ | 2,3-BD productivity (g/L h), Eq. (2) |
| S/N | Signal-to-Noise ratio, Eq. (3) |
| t | Time (h) |
| $Y_{2,3\text{-BD}}$ | 2,3-BD yield (g/g), Eq. (1) |

different chemical and biochemical strategies related to revalue raw glycerol have been developed during the last decades in order to improve the economic feasibility of the overall process [9,11–14].

Since several bacteria are able to metabolize glycerol, many promising applications focused on raw glycerol as carbon source in microbiological processes have been studied, including production of 1,3-propanediol, ethanol, hydrogen, succinic acid and propionic acid [5,9,15–17]. Recently, 2,3 butanediol (2,3-BD) production from glycerol has received attention, due to the wide applicability of this diol as liquid fuel, antifreeze agent, octane booster for petrol, and bulk chemical involved in the synthesis of other interesting molecules such as methyl-ethyl-ketone (MEK) and butadiene [18,19].

In the last decades, a few studies about 2,3-butanediol (2,3-BD) production from glycerol have been published. Promising results have been obtained employing biocatalyst belonging to the genera *Klebsiella*, typified as a pathogenic group (biosafety level 2). This reason makes it unsuitable for industrial-scale fermentation because of the strict safety regulations and increasing costs of the process [20–25].

The present work is focused on the searching of wild type non-pathogenic strains to produce 2,3-BD from glycerol as sole carbon source. Two *Raoultella* sp. strains, belonging to risk group 1 strains (non-pathogenic) are chosen for the study. To our knowledge, this is the first report that describes bioconversion of residual glycerol from biodiesel synthesis to produce 2,3-BD using as biocatalyst *Raoultella planticola* CECT 843 and *Raoultella terrigena* CECT 4519. For each strain, the influence of operational temperature, organic acids addition (citric and/or acetic acid) and yeast extract concentration on 2,3-BD production were analyzed through Taguchi optimization methodology in order to maximize 2,3-BD yield and 2,3-BD productivity. A study regarding the influence of the initial glycerol concentration in a commercial lab-scale bioreactor was also carried out. The aim of this work is to assess the potential of both novel biocatalysts to produce 2,3-BD from raw glycerol derived from biodiesel process industry.

## 2. Material and methods

### 2.1. Identification and isolation of bacterial strains

A strain collection comprising both strains from activated sludge and culture collections was set up. Strains from genera and species described as 2,3-BD producers were obtained from different culture collections in order to increase the possibilities to have a good producer, and to have some benchmarks (the risk class 2 strains).

A screening strategy was set up using activated sludge samples from a water depuration system of a biodiesel production site: 500 μL of depuration mud was incubated with 45 mL of culture medium in 50 mL tubes. The incubation was performed at 30 and 37 °C, with and without agitation (150 rpm), employing two different culture media with 15 g/L of glycerol as the carbon source: medium A (MA, adapted from Ref. [20]); and Medium B (MB, adapted from Ref. [26]), being MB richer in nutrients than MA. The composition of MA per liter is: 15 g pure glycerol, 0.75 g KCl, 1.38 g $NH_2PO_4{\cdot}2H_2O$, 5.35 g $(NH_4)_2SO_4$, 0.28 g Na2SO4, 0.26 g $MgSO_4{\cdot}7H_2O$, 0.42 g $HNO_3$, 2 g yeast extract, 0.3 mL trace element solution (per liter: 34.2 g $ZnCl_2$, 2.7 g $FeCl_3{\cdot}6H_2O$, 10 g $MnCl_2{\cdot}4H_2O$, 0.85 g $CuCl_2{\cdot}2H_2O$, 0.3 g $H_3BO_3$, 23.8 g $CoCl_2{\cdot}6H_2O$). Composition of MB per liter is: 15 g pure glycerol, 5.0 g yeast extract, 5.0 tryptone, 7.0 g $KH_2PO_4$, 7.0 g $KH_2PO_4$, 1.0 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.12 g $NaMoO_4{\cdot}7H_2O$, 0.021 g $CaCl_2{\cdot}2H_2O$, 0.029 g $CoCl_2{\cdot}6H_2O$, 0.039 g $Fe(NH_2)_2SO_4{\cdot}6H_2O$, 2.0 mg nicotinic acid, 0.172 mg $Na_2SeO_3$, 0.02 mg $NiCl_2$, and 10 mL of trace element solution (per liter: 0.5 g $Na_2EDTA$, 0.5 g $MnCl_2{\cdot}4H_2O$, 0.1 g $H_3BO_3$, 1.0 mg $CuCl_2{\cdot}2H_2O$). Initial pH was adjusted to neutral value before use both growth media.

After 48 h of incubation, the cultures were transferred to solid plates prepared by adding 2% agar to MA and MB media. After 48 incubation at the same temperature (30 and 37 °C), the isolated colonies were transferred to 96-mL well plates [flat bottom. Thermo Scientific] with the same medium, which were incubated for 16–18 h at 30 °C and 150 rpm, in order to perform massive screening of 2,3-BD production. The same screening procedure was performed with 45 strains from culture collections. Producer strains have been identified and characterized as it has been previously described [27]. Colorimetric tests for detection of 2,3-butanediol and characterized as it has been previously described [26] acetoin were adapted for micro-titer plates from previous literature reports [28–30]. For the assay of 2,3-BD in micro-titer plates, 150 μL of supernatant was mixed with 32 μL of $H_5IO_6$ 0.1 M and incubated for 30 min at room temperature. Afterwards, 20 μL of ethylene glycol, 48 μL of piperazine saturated solution (30% w/w) and 16 μL of sodium nitroprusside solution (4% w/w) 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, μL of creatine solution (0.5% w/w), 30 μL of α-naphtol in ethanol (solution at 5% w/w) and 20 μL of KOH solution (40% w/w). After 5–10 min, the mixture turns red if the assay is positive for acetoin, and disappears after 30 min.

The best 40 strains were checked at higher scale fermentation: assays were carried out in 50-mL shake flasks (without baffles) at 150 rpm and 30 °C with 60 g/L of pure glycerol as carbon source concentration employing MA as broth medium.

### 2.2. Maintenance and inoculum growth conditions

Storage of selected strains took place at −80 °C as concentrated stocks in a 50% (w/w) glycerol-saline solution (0.9% w/v). Inoculum cultures were grown in 250-mL shake flask at 30 °C and 200 rpm at aerobic conditions. 250 mL Erlenmeyer flasks containing 50 mL of the medium (MS; composition per liter is: 2 g $NH_4Cl$; 6 g $KH_2PO_4$; 12 g $Na_2HPO_4$; 1 g NaCl; 0.246 g $MgSO_4{\cdot}7H_2O$; 0.011 g $CaCl_2$; 1.5 g Yeast extract; and 30 g pure glycerol) were employed in the studies carried out. Initial pH was set to neutral value before use the medium. Biocatalyst growth was carried out by a standard method based on two consecutive inoculum steps in order to obtain repetitive results, for 12 and 4 h, respectively. Initial dry biomass concentration was 0.1 g/L in both steps.