

ORIGINAL ARTICLE

Efficient glycerol transformation by resting *Gluconobacter* cellsErienne Jackson | Magdalena Ripoll | Lorena Betancor 

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Abstract

In the present work, glycerol biotransformation using *Gluconobacter* strains was studied with a process intensification perspective that facilitated the development of a cleaner and more efficient technology from those previously reported. Starting from the industrial by-product, crude glycerol, resting cells of *Gluconobacter frateurii* and *Gluconobacter oxydans* were able to convert glycerol under batch reactor conditions in water with no other additive but for the substrate. The study of strains, biomass:solution ratio, pH, growth stage, and simplification of media composition in crude glycerol bioconversions facilitated productivities of glyceric acid of 0.03 g/L.h and 2.07 g/L.h (71.5 g/g % pure by NMR) of dihydroxyacetone (DHA). Productivities surmounted recent reported fermentative bioconversions of crude glycerol and were unprecedented for the use of cell suspended solely in water. This work proposes a novel approach that allows higher productivities, cleaner production, and reduction in water and energy consumption, and demonstrates the applicability of the proposed approach.

KEYWORDS

biotransformation, dihydroxyacetone, *Gluconobacter*, glyceric acid, glycerol

1 | INTRODUCTION

Glycerol is a side product of fat and oil transesterification with alcohols in the production of biodiesel. This feedstock by-product contains varying amounts of contaminants such as salts, ashes, methanol, and fats with low levels of purity (60%–80%) that compromise its market value (Ayoub & Abdullah, 2012; Yang, Hanna, & Sun, 2012). The current glycerol production will exceed the demand in 2020 in more than 400% (Ayoub & Abdullah, 2012). Therefore, new processes able to convert glycerol into novel value-added products need to be developed to sustain the viability of the biofuel economy (Ayoub & Abdullah, 2012; Yang et al., 2012; Zhou, Shen, Wang, Sun, & Xiu, 2018). In this context, glycerol has been converted into high value-added products by both homogeneous and heterogeneous catalysis or through more sustainable methods using biocatalysts (Habe, Fukuoka, Kitamoto, Fukuoka, Kitamoto, & Sakaki, 2009a; Khanna, Goyal, & Moholkar, 2012; Yang et al., 2018). The vast majority of biocatalytic processes involve the microbial transformation

of glycerol under growing conditions using culture media in the reaction mixture (Andreeßen & Steinbüchel, 2012; Szymanowska-Powatońska & Biała, 2014; Yang et al., 2018). Therefore, products are generated in complex media that complicates the downstream purification of the target chemical and therefore augment the economic demands of the biotransformation. In contrast, the use of nongrowing resuspended cells or resting cells could provide a number of operational advantages such as facilitating product separation (Julsing, Kuhn, Schmid, & Bühler, 2012) or avoid secondary reactions and undesired growth metabolites that could contaminate the system. However, for applied purposes the use of resting cells should counterbalance the loss in productivities that are often observed when compared with growing cells conversions. Therefore, the design of new strategies for the microbiological conversion of glycerol that address these limitations is not only appropriate but also necessary, and of fundamental and applied value.

The oxidative metabolism of glycerol generates glyceric acid (GA) and dihydroxyacetone (DHA) in *Gluconobacter* and *Acetobacter*

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species (Habe et al., 2009a; Khanna et al., 2012). Particularly, *Gluconobacter* catalyzes the oxidation of sugars and sugar alcohols, being large producers of both DHA and GA. DHA is a marketable chemical worth more than 250-fold the price of crude glycerol produced around the globe via biotechnological processes. It is broadly used as a precursor for the synthesis of fine chemicals in the chemical and pharmaceutical industry, and in cosmetic industry, DHA is an active component in sunless tanning products (Ciriminna, Fidalgo, Ilharco, & Pagliaro, 2018; Dikshit & Moholkar, 2016; Yang et al., 2012). The alternate oxidation product of glycerol, GA, has potential as a building block for many chemicals, and although it has a lesser demand in the industrial market, its market value is more than 1,000 times that of the DHA (Habe et al., 2009). Traditionally obtained by metal catalytic oxidation of glycerol, its biocatalytic production has recently emerged and literature is still piling up on process improvements that provide economic advantages of the biotechnological over the chemical production process.

In this work, the biological transformation of glycerol to GA and DHA was studied using *Gluconobacter frateurii* NBRC13465 (*G. frateurii*) and *Gluconobacter oxydans* NBRC 14819 (*G. oxydans*). Our work demonstrates that active nongrowing cells suspended solely in water are able to catalyze the transformation of glycerol to target compounds with unprecedented productivities under the studied conditions.

2 | RESULTS AND DISCUSSION

2.1 | Conditions for pure glycerol conversions using resting cells

Bacterial metabolism changes throughout the different growth stages and therefore the stage at which cells are collected during growth for resting cell bioconversions might affect the rate of substrate transformation. As primary metabolites involved in glycolysis, DHA and GA production were likely to increase during

the exponential phase (De Vuyst, Callewaert, & Crabbé, 1996). However, *Gluconobacter* differentiated cells in the stationary phase have been reported to produce twice as much DHA than undifferentiated exponential phase cells in bioconversions (Khanna et al., 2012). The temperature selected for all assays was 30°C, as previously reported for the growth of species of the genus *Gluconobacter* (Black & Nair, 2013; Deppenmeier, Hoffmeister, & Prust, 2003; Habe, Fukuoka, Kitamoto, Fukuoka, Kitamoto, & Sakaki, 2009b; Habe et al., 2009). The reaction was supplemented with KH_2PO_4 , K_2HPO_4 , and MgSO_4 as it has been described that these salts have a significant impact in the amount of DHA formed in fermentative conversions of *G. frateurii* (Poljungreed & Boonyarattanakalin, 2017). After a period of 20 hr, results showed that higher conversions of glycerol were obtained when resting cells from both strains were collected during the mid-exponential phase (Figure 1a and b). Under these conditions, conversion by *G. frateurii* was $1.7 \pm 0.1\%$ to GA and $18.3 \pm 0.9\%$ to DHA, resulting in a total conversion of $20.0 \pm 0.1\%$ of glycerol, and *G. oxydans* showed a conversion of $36.8 \pm 3.9\%$ of DHA with no production of GA (Figure 1a and b). Reaction kinetics under these conditions (Figure 1c and d) showed *G. frateurii* reached $52.2 \pm 2.7\%$ conversion of glycerol after 50 hr ($47.35 \pm 0.7\%$ for DHA and $4.1 \pm 0.1\%$ for GA). In the case of *G. oxydans*, a conversion of $46.6 \pm 14.9\%$ to DHA free of GA was achieved after the same period. The lower glycerol conversion shown by *G. oxydans* could be attributed to inhibition by DHA as it has been reported that high concentrations of this product inhibit the pentose phosphate pathway as well as the enzyme glycerol dehydrogenase, causing cell damage (Khanna et al., 2012).

Overall, the results obtained for pure glycerol conversions with resting cells correlate with those of Khanna et al., 2012 and Sato, Morita, Fukuoka, Kitamoto, & Habe, 2015 that, differently from this work, used growing cells for pure glycerol transformation. Therefore, under the studied conditions, the bacterial behavior in

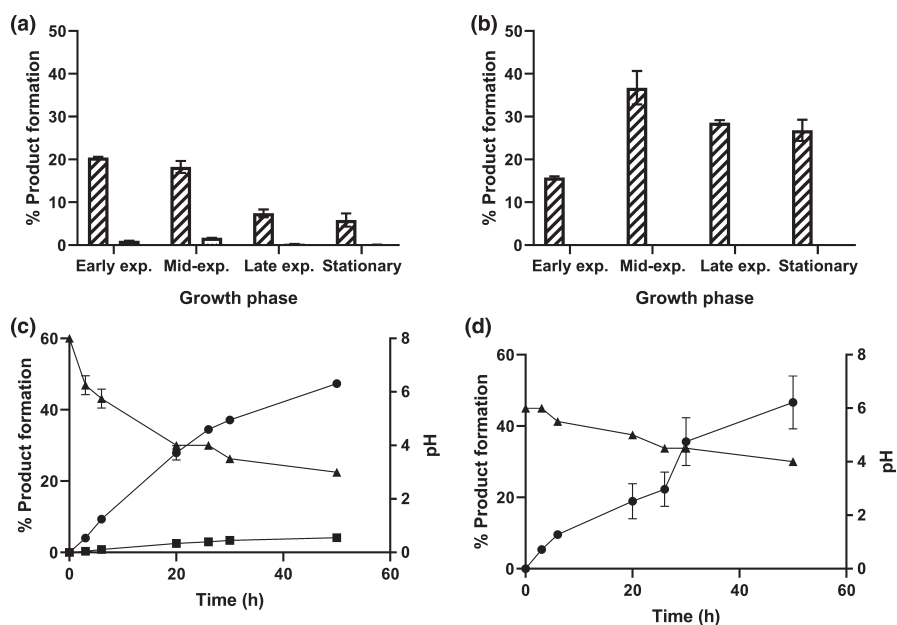


FIGURE 1 Pure glycerol bioconversion with *Gluconobacter* strains. GA (dots) and DHA (stripes) production after 20 hr by resting cells of *Gluconobacter frateurii* (a) and *Gluconobacter oxydans* (b) collected during different growth stages. Kinetics of DHA and GA production from pure glycerol with resting cells of *G. frateurii* (c) and *G. oxydans* (d). DHA (●), GA (■), pH (▲). Reactions were performed in 30 ml total volume at 30°C using 2 mg dry cell weight and 50 g/L initial glycerol concentration. Further conditions are described in Methods

the transformation did not change in the absence of nutrients and additives.

pH is an important parameter to take into account in biotransformations as it directly affects the activity of the enzymes and consequently the microbial growth and metabolism (Shuler & Fikret, 2002). Therefore, the impact of the initial pH of the reaction medium was investigated using resting cells of *G. frateurii* and *G. oxydans* collected in the mid-exponential phase (Figure A1). Variations in the initial pH proved to have an impact in the production of DHA and GA by *G. frateurii*. The production of DHA and GA increased with initial pH, reaching a maximum of almost 50% conversion at 50 hr at pH 8.0. For *G. oxydans*, however, it was observed that at an initial pH 6.0 led to a higher conversion of DHA $46.6 \pm 7.4\%$ after 50 hr of reaction with no significant production of GA. pHs of maximum conversions for both strains were selected for further experiments.

A 10-fold increase in the inoculum had a significant impact in the glycerol conversion for both *Gluconobacter* strains. After 50 hr, the production of GA by resting cells of *G. frateurii* increased from $4.1 \pm 0.2\%$ to $9.1 \pm 0.1\%$. However, the production of DHA decreased slightly from $44.7 \pm 4.1\%$ to $38.1 \pm 2.2\%$. For *G. oxydans*, the increase in the production of DHA was significant, as a 100% conversion to this product was achieved after 50 hr.

As previously described, initial glycerol concentration in the reaction media affects the production of DHA and GA (Black & Nair, 2013; Habe et al., 2010, 2009). To test a possible substrate/product inhibition effect, several initial concentrations of pure glycerol were evaluated (Figure 2). For *G. frateurii*, a change in the ratio of products associated with increasing initial concentrations of glycerol was observed. The substrate or product concentrations may affect the rate of conversion of the enzymes involved in the biotransformation. GA production improved significantly at higher initial concentrations of glycerol, while DHA production decreased (Figure 2a).

The increase in glycerol concentration caused a reduction in the conversion for both strains. Considering the latter, for subsequent experiments the initial glycerol concentration selected was the highest with which it was possible to achieve a 100% conversion. Quantitative conversion of glycerol to products after 45 hr was achieved starting from 25 g/L and 50 g/L for *G. frateurii* and *G. oxydans*, respectively (Figure 2).

2.2 | Crude glycerol conversion using resting cells

Conversion kinetics were studied in the selected conditions, using crude glycerol as substrate (Figure 3). A lower conversion to DHA and GA was found for *G. frateurii* using crude glycerol when compared to pure glycerol after 45 hr of reaction. Another notorious difference in production was the lower GA to DHA ratio produced using crude glycerol as a substrate. This low GA productivity may be associated with the presence of methanol from crude glycerol, which has an inhibitory effect on the enzyme mADH, responsible for the production of GA in *Gluconobacter* (Figure 3a). This result correlates

with Sato et al. that reported a decrease in the productivity of GA in the presence of methanol by the same strain of *G. frateurii* used in this study, and improved it by removal of the alcohol from the reaction medium (Habe et al., 2009; Sato et al., 2013).

Interestingly, for *G. oxydans*, the conversion after 45 hr was similar for both types of glycerol (Figure 3b). This indicates that, unlike *G. frateurii*, *G. oxydans* maintains its ability to convert glycerol independently of the crude glycerol contaminants present.

Samples of the biotransformations were analyzed by ^1H NMR spectroscopy in order to confirm and identify the major components in the starting reaction and the final components of the reaction after 24 hr (Figure A2). The analysis of ^1H -1D spectrum of the starting reaction media shows two major components, glycerol and methanol, together with minor amounts of fatty acids. This result confirms the usual components in crude glycerol obtained as a by-product of the biodiesel industry.

The analysis of 1H-1D spectrum of the final reaction points (24 hr) of samples from *G. frateurii* and *G. oxydans* conversions allowed the identification of signals corresponding to DHA (71.5%), glycerol (18.8%), acetate (2.6%), and methanol (7.1%), in the case of *G. oxydans* and an additional signal of GA in reactions with *G. frateurii* (DHA 71.0%, glycerol 13.0%, MeOH 5.4%, acetate 2.1%, glyceric acid 8.4%). No phosphate was identified confirming that glycerol is not being used by the cells as a metabolic precursor at least in identifiable quantities. The ^1H NMR spectroscopy results support those obtained by HPLC analysis where no GA was observed for the *G. oxydans* biotransformations. They also confirm the absence of any other conversion product or metabolite in significant concentrations, highlighting the benefits of using resting cells to produce media contaminant-free compounds in biotransformations.

2.3 | Process intensification for conversion of crude glycerol

The potential reuse of resting cells was evaluated in the conversion of 50 g/L of crude glycerol. *G. frateurii* maintained its capacity to convert glycerol to DHA after 5 uses. However, productivities decreased gradually throughout the experiment reaching 11.7% conversions after the fifth use (Figure 4). DHA production by reused resting cells of *G. oxydans* could not be achieved since the capacity to convert glycerol was completely lost after the first use (Figure 4). The differences in resistance to repeated batch conversions of the strains might be explained better by the ability of the metabolic machinery to withstand cell death or enzyme inactivation, or support cofactor regeneration.

We have also studied the possibility of using the cells in a fed-batch process. A slight increase in the amount of DHA produced was obtained for both *G. frateurii* and *G. oxydans* after the first addition of substrate (24 hr) which were 25 g/L for *G. frateurii* and 50 g/L for *G. oxydans* (Figure 5). Successive additions of the substrate did not translate into accompanying product formation. In the case of *G. frateurii*, product inhibition could be the main cause of this behavior as observed before for growing cells transformation of glycerol

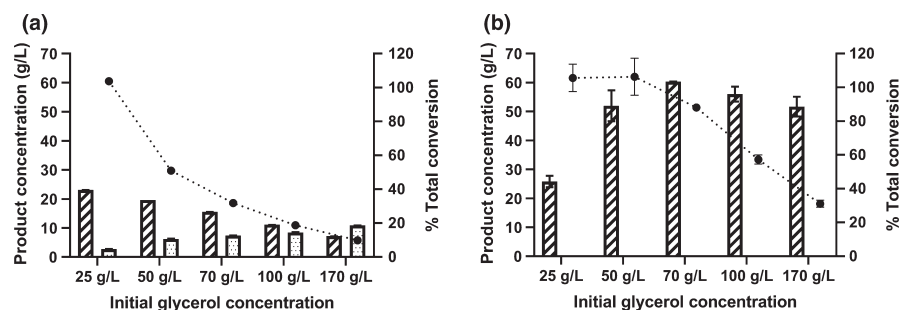


FIGURE 2 DHA and GA production after 45 hr starting from different glycerol concentrations. *Gluconobacter frateurii* (a), *Gluconobacter oxydans* (b). % Conversion (dotted line), DHA (stripes), GA (dots). Reactions were performed in 30 ml total volume at 30°C using 20 mg dry cell weight. Further conditions are described in Methods

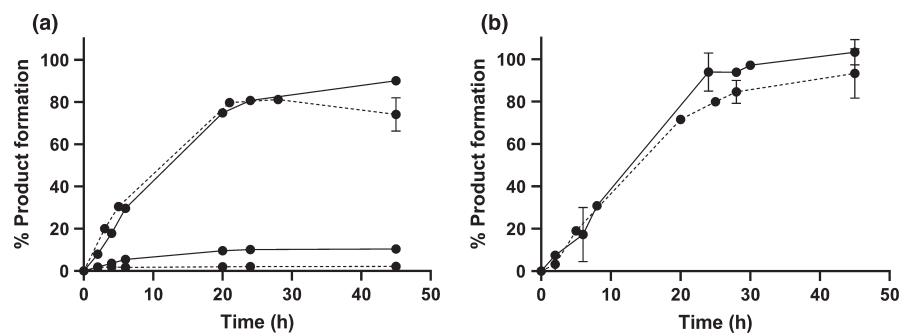


FIGURE 3 DHA and GA production after 45 hr starting from crude and pure glycerol. (a) (*Gluconobacter frateurii*), (b) (*Gluconobacter oxydans*). Products from pure glycerol (full line), products from crude glycerol (dotted line). DHA (upper circles), GA (lower circles) Correction added on 26 November 2019 after first online publication: Figure 3 caption has been corrected]

(Dikshit, Kharmawlong, & Moholkar, 2018). This hypothesis was also supported by results obtained in glycerol conversions initiated with DHA in the reaction media (Figure A3). Product formation in the presence of DHA was significantly decreased for *G. frateurii* strain. For *G. oxydans*, DHA increased from 38.1 ± 0.1 g/L to 52.7 ± 4.2 g/L after the third glycerol spike (Figure 5b). This strain seems to be less sensitive to product inhibition than *G. frateurii* when in suspension.

These results indicate that glycerol conversion with *Gluconobacter* strains could benefit from strategies that include flow-through configurations to continuously remove the produced DHA, minimizing inhibition.

Further strategies were studied to increase product formation while improving or maintaining the costs of the process. The approach involved the permeabilization of bacterial membrane. The biochemical pathway of DHA synthesis is mediated by a FAD and pyrroloquinoline quinone (PQQ)-dependent membrane-bound glycerol dehydrogenase enzyme (GDH) that incompletely oxidizes glycerol to DHA and in turn accumulates it outside the cell. However, the active site of the GDH has been described to be oriented toward the periplasm (Khanna et al., 2012), and therefore, permeabilization strategies might facilitate glycerol and DHA transport to and from the bacterial periplasm. Resting cells of *G. frateurii* and *G. oxydans* were treated with EDTA and PEI, both well-known gram-negative outer membrane permeabilizers (Cánovas, Torroglosa, & Iborra, 2005). EDTA is a chelating agent that removes divalent cations interacting with lipopolysaccharides (LPS) present in the outer membrane, while the polycationic nature of the polymer PEI provokes an interaction with said LPS and causes a disruption of the outer membrane structure (Helander, Alakomi, Latva-Kala, & Koski, 1997; Vaara, 1992). No differences were observed for treated and nontreated cells nor in rates or final productivities in the bioconversions which might imply a poorly

restricted transport of both glycerol and DHA through the outer bacterial membrane of both strains.

Given the promising results obtained for the transformation of glycerol to DHA using resting *G. oxydans* cells, further investigations were centered on process intensification on this strain.

A series of transformations were carried out in which we simplified the composition of the reaction media. Neither the KH_2PO_4 or K_2HPO_4 , nor the MgSO_4 proved necessary for the conversion as even when crude glycerol was offered in water to the cells, the amount of product obtained after 20 hr did not vary (Figure A4). This is the first report on a resting cell transformation of glycerol to DHA performed solely in water.

In order to expand the implementation prospect of crude glycerol bioconversion at large scale, the reaction was studied using regular water and a reduced amount of water without varying the total amount of substrate in the reaction. Results demonstrate that there is no need for the use of distilled water in the reaction and that a 50% decrease in the total reaction volume did not affect the productivity of the conversion (Figure 6). These results are of paramount importance in the light of a possible industrial application of this transformation.

This work is the first report of *G. frateurii* transformation of pure and crude glycerol in media-free conditions using resting cells. The results for GA production showed herein reached lower levels of productivities than others reported in the literature (Habe et al., 2010; Sato, Kitamoto, & Habe, 2014). However, DHA was comparatively produced in a less complex media which would facilitate its further processing (Poljungreed & Boonyarattanakalin, 2018; Tanamool, Hongsachart, & Soemphol, 2018).

Apart from a sole report from Hu et al (Hu, Zheng, & Shen, 2011) where pure glycerol is biotransformed by *G. oxydans* ZJB09113, reports on the use of resting cells of *G. oxydans* starting from crude glycerol have not yet been explored. In the aforementioned work,

FIGURE 4 Reuses of resting cells of *Gluconobacter frateurii* (a) and *Gluconobacter oxydans* (b) in the conversion of crude glycerol to DHA and GA. DHA (stripes), GA (dots). Reactions were performed in 30 ml total volume at 30°C using 20 mg dry cell weight. Each use cycle was after 20 hr. Further conditions are described in Methods

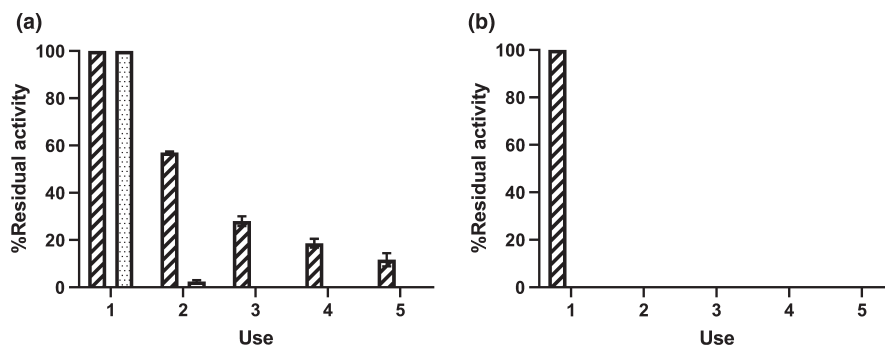
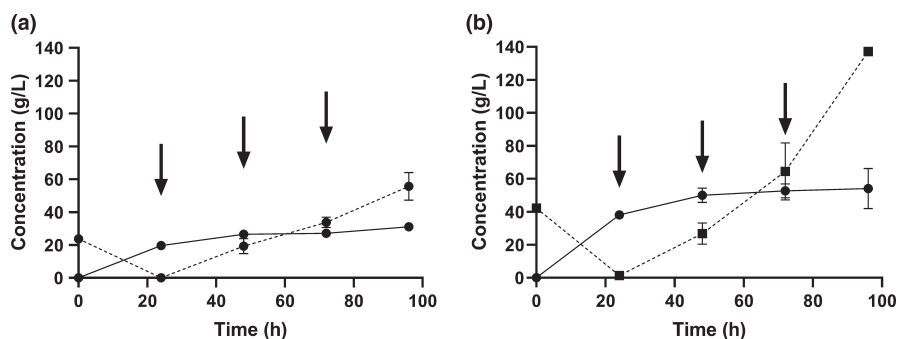


FIGURE 5 Fed-batch conversion of crude glycerol using resting cells of *Gluconobacter frateurii* (a) and *Gluconobacter oxydans* (b). DHA (full line), glycerol (dashed line), and glycerol applications (arrows) were 25 g/L in (a) and 50 g/L in (b)



a productivity of 2.16 g/L.h was achieved for DHA using pure glycerol in a fed-batch airlift reactor. Herein, the productivity reached 1.91 g/L.h for pure glycerol and 2.07 g/L.h for the unprecedented conversion of crude glycerol with resting cells of *G. oxydans* in water. Moreover, an additional advantage of the conversions described in this work lays in the amount of biocatalyst used in the reactions as it was 15 times less than that used by Hu et al (0.67 g/L in this work compared to 10 g/L).

Maximum conversions for glycerol to DHA have been reported by Zhou et al in 2016 reaching productivities of 9.41 g/L.h using *G. oxydans* NL71 but again, using pure glycerol and under growing conditions (Zhou, Zhou, Xu, & Yu, 2016). Future investigations will focus on the use of alternate reactor configurations that may benefit the yields as in (Habe et al., 2009) or (Sato et al., 2013).

In conclusion, efficient conversions of glycerol, both pure and crude, to DHA and GA were demonstrated using resting cells of species of the genus *Gluconobacter* carried out in simple media. Significant results were specially obtained when using biodiesel-derived crude glycerol. Simplification of the reaction media and reduction of the total reaction volume pave the way to an efficient implementation of glycerol transformation, which as any other bioprocess is often limited by the productivity of the conversion and costs of downstream processing. These results demonstrate the feasibility of using media-free biotransformation for a green, cleaner, and more economical revalorization of industrial glycerol.

3 | EXPERIMENTAL PROCEDURES

3.1 | Chemicals

All chemicals used were of analytical grade. Crude glycerol, product of the splitting process (water <12%, ashes <7%, glycerol 70%–80%,

lipids <1%, methanol <5%, chlorides <3%), was donated by Alcoholes del Uruguay (ALUR).

3.2 | Growth curve analysis

Freeze-dried strains of *G. frateurii* NBRC 103,465 and *G. oxydans* NBRC 14819 were obtained from the National Institute of Technology and Evaluation (NITE). 1-L flasks containing 250 ml of glycerol medium (glycerol 100 g/L, peptone 9 g/L, yeast extract 1 g/L, KH_2PO_4 0.9 g/L, K_2HPO_4 0.1 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g/L, pH 6.0) were inoculated with 250 μl of *G. frateurii* NBRC 103465 preculture ($\text{OD}_{600\text{nm}}$ value of 3) or with 2.5 ml of *G. oxydans* NBRC 14819 preculture ($\text{OD}_{600\text{nm}}$ value of 0.6) and incubated for 76 hr at 30°C and 180 rpm. $\text{OD}_{600\text{nm}}$ was measured periodically, and a correlation with dry cell weight (g/L) was established by constructing a graphic model ($\text{OD}_{600\text{nm}}$ vs. dry cell weight). The protocol for dry cell weight measurements was adapted from Black and Nair (Black & Nair, 2013).

3.3 | Inoculum preparation for glycerol conversion

250 ml cultures were incubated at 30°C and 180 rpm. The volume needed to obtain a required amount of dry weight of cells was then centrifuged for 15 min at 2,800g. The bacterial pellet was washed with 30 mM phosphate buffer (NaH_2PO_4 4.14 g/L, K_2HPO_4 5.23 g/L, pH 7.0) to remove the growth medium and was centrifuged at 2,800g for 15 min, discarding the supernatant.

3.4 | Glycerol conversion to GA and DHA

All conversions of glycerol by resting cells were carried out in 30 ml volume of reaction medium supplemented with pure or crude glycerol

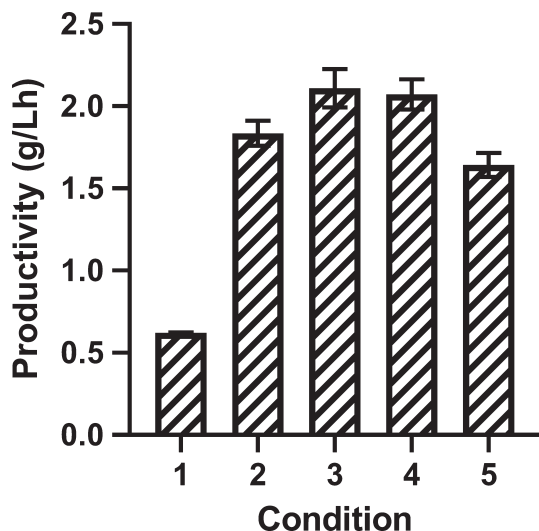


FIGURE 6 DHA production intensification using *Gluconobacter oxydans* as a catalyst and crude glycerol as substrate. (1) Starting conditions (glycerol 50 g/L, KH_2PO_4 0.9 g/L, K_2HPO_4 0.1 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g/L, pH 6 in distilled water, 2 mg of dry cell weight), (2) 10-fold inoculum increase (20 mg of dry cell weight) conditions as in 1, (3) reaction media without salts, (4) use of regular water as reaction media, and (5) decrease in final total volume (20 mg of dry cell weight, 100 g/L glycerol in 15 ml regular water). All the reactions were performed at 30°C

(glycerol 25–170 g/L, KH_2PO_4 0.9 g/L, K_2HPO_4 0.1 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g/L, pH 3–8). Additional experiments were conducted solely in water with no other supplement but for the substrate glycerol. The inoculum for each reaction was 2 mg or 20 mg of dry cell weight. Conversion kinetics was elucidated by taking samples at different time points during the reaction and analyzing them by HPLC.

3.4.1 | Conversions using resting cells from different growth stages

Cells were collected and washed as previously described when the cultures' $\text{OD}_{600\text{ nm}}$ values reached 0.4, 1.0, 2.5, and 3.0 for early exponential, mid-exponential, late exponential, and stationary phases, respectively. The reactions were carried out for 20 hr in duplicates, with 2 mg of dry cell weight and starting 50 g/L of glycerol.

3.4.2 | Effect of initial pH in the conversion

Different initial pH values for the reaction media were evaluated (3.0, 4.0, 6.0, 7.0, and 8.0). Each reaction was inoculated with 2 mg of dry cell weight, obtained from a culture with an $\text{OD}_{600\text{ nm}}$ value of 1. The reactions were carried out for 50 hr in duplicates, starting from 50 g/L of glycerol.

3.4.3 | Reuse of resting cells in the conversion of crude glycerol

Resting cells were reused up to 5 times after 20 hr of reaction. Cells were harvested by centrifugation and washed with 30 mM phosphate buffer before each use.

3.5 | HPLC analysis

Each sample was centrifuged at 13,000g for 15 min to remove the bacterial pellet. The supernatant was dissolved 5 times in mQ water and filtered with a 0.22- μm polyvinylpyrrolidone (PVP)-treated filter for HPLC analysis. Samples were analyzed using a Shimadzu Nexera X2 HPLC, with a diode array detector. An Aminex[®] HPX-87C 300 \times 7.8 mm from Bio-Rad was used with a 4 \times 3.0 mm Carbo-H cartridge precolumn from Phenomenex. 5 mM sulfuric acid was used as the mobile phase. Detection was carried out at 70°C following 210 nm for GA, 271 nm for DHA, and 190 nm for glycerol with a flow rate of 0.6 ml/min for 20 min. Injection volume was 20 μl . Calibration curves were constructed for GA, DHA, and glycerol with solutions of 0.15, 0.30, 0.60, 1.25, 2.50, 5.00, and 10.00 g/L. Samples were analyzed using Shimadzu's LabSolutions software.

3.6 | NMR analysis

NMR data were acquired in a Bruker Avance Neo operating at a 1 H frequency of 400.13 MHz, using a spectral window of 6.25 KHz, a 90° pulse width of 14 μs , acquisition time of 3 s, and a relaxation delay of 2 s. Water suppression was accomplished through presaturation during the relaxation delay. Typically, 16 scans were acquired for each sample. Raw FID data were processed using software package TopSpin 4.0.2 using an exponential window of 0.3 Hz and 65,536 points. Samples were prepared by dissolving 50 μl of sample into 540 μl of D_2O and were transferred to a 5-mm NMR tube.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

EJ investigated the study, contributed to methodology and visualization, and wrote the original draft of the manuscript. MR investigated the study, visualized the data, and wrote the original draft of the manuscript. LB conceived the study, acquired the funding, and wrote, edited, and reviewed the manuscript.

DATA AVAILABILITY STATEMENT

All data are provided in full in the results section of this paper.

ETHICAL APPROVAL

None required.

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REFERENCES

- Andreeßen, B., & Steinbüchel, A. (2012). Biotechnological conversion of glycerol to 2-amino-1,3-propanediol (serinol) in recombinant *Escherichia coli*. *Applied Microbiology and Biotechnology*, *93*, 357–365. <https://doi.org/10.1007/s00253-011-3364-6>
- Ayoub, M., & Abdullah, A. Z. (2012). Critical review on the current scenario and significance of crude glycerol resulting from biodiesel industry towards more sustainable renewable energy industry. *Renewable and Sustainable Energy Reviews*, *16*, 2671–2686. <https://doi.org/10.1016/j.rser.2012.01.054>
- Black, C. S., & Nair, G. R. (2013). Bioconversion of glycerol to dihydroxyacetone by immobilized *Gluconacetobacter xylinus* cells. *International Journal of Chemical Engineering and Applications*, *4*, 310–314. <https://doi.org/10.7763/IJCEA.2013.V4.316>
- Cánovas, M., Torroglosa, T., & Iborra, J. L. (2005). Permeabilization of *Escherichia coli* cells in the biotransformation of trimethylammonium compounds into L-carnitine. *Enzyme and Microbial Technology*, *37*, 300–308. <https://doi.org/10.1016/j.enzmictec.2004.07.023>
- Ciriminna, R., Fidalgo, A., Ilharco, L. M., & Pagliaro, M. (2018). Dihydroxyacetone: An updated insight into an important bioproduct. *ChemistryOpen*, *7*, 233–236. <https://doi.org/10.1002/open.20170201>
- De Vuyst, L., Callewaert, R., & Crabbé, K. (1996). Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology*, *142*, 817–827. <https://doi.org/10.1099/00221287-142-4-817>
- Deppenmeier, U., Hoffmeister, M., & Prust, C. (2003). Biochemistry and biotechnological applications of *Gluconobacter* strains. *Applied Microbiology and Biotechnology*, *60*, 233–242.
- Dikshit, P. K., Kharmawlong, G. J., & Moholkar, V. S. (2018). Investigations in sonication-induced intensification of crude glycerol fermentation to dihydroxyacetone by free and immobilized *Gluconobacter oxydans*. *Bioresource Technology*, *256*, 302–311. <https://doi.org/10.1016/j.biortech.2018.02.024>
- Dikshit, P. K., & Moholkar, V. S. (2016). Optimization of 1,3-dihydroxyacetone production from crude glycerol by immobilized *Gluconobacter oxydans* MTCC 904. *Bioresource Technology*, *216*, 1058–1065. <https://doi.org/10.1016/j.biortech.2016.01.100>
- Habe, H., Fukuoka, T., Kitamoto, D., & Sakaki, K. (2009a). Biotechnological production of D-glyceric acid and its application. *Applied Microbiology and Biotechnology*, *84*, 445–452. <https://doi.org/10.1007/s00253-009-2124-3>
- Habe, H., Fukuoka, T., Kitamoto, D., & Sakaki, K. (2009b). Biotransformation of glycerol to D-glyceric acid by *Acetobacter tropicalis*. *Applied Microbiology and Biotechnology*, *81*, 1033–1039. <https://doi.org/10.1007/s00253-008-1737-2>
- Habe, H., Shimada, Y., Fukuoka, T., Kitamoto, D., Itagaki, M., Watanabe, K., ... Sakaki, K. (2009). Production of glyceric acid by *Gluconobacter* sp. NBRC3259 using raw glycerol. *Bioscience, Biotechnology, and Biochemistry*, *73*, 1799–1805.
- Habe, H., Shimada, Y., Fukuoka, T., Kitamoto, D., Itagaki, M., Watanabe, K., ... Sakaki, K. (2010). Use of a *Gluconobacter frateurii* mutant to prevent dihydroxyacetone accumulation during glyceric acid production from glycerol. *Bioscience, Biotechnology, and Biochemistry*, *74*, 2330–2332.
- Habe, H., Shimada, Y., Yakushi, T., Hattori, H., Ano, Y., Fukuoka, T., ... Sakaki, K. (2009). Microbial production of glyceric acid, an organic acid that can be mass produced from glycerol. *Applied and Environment Microbiology*, *75*, 7760–7766. <https://doi.org/10.1128/AEM.01535-09>
- Helander, I. M., Alakomi, H. L., Latva-Kala, K., & Koski, P. (1997). Polyethyleneimine is an effective permeabilizer of Gram-negative bacteria. *Microbiology*, *143*, 3193–3199. <https://doi.org/10.1099/00221287-143-10-3193>
- Hu, Z. C., Zheng, Y. G., & Shen, Y. C. (2011). Use of glycerol for producing 1,3-dihydroxyacetone by *Gluconobacter oxydans* in an airlift bioreactor. *Bioresource Technology*, *102*, 7177–7182. <https://doi.org/10.1016/j.biortech.2011.04.078>
- Julsing, M. K., Kuhn, D., Schmid, A., & Bühler, B. (2012). Resting cells of recombinant *E. coli* show high epoxidation yields on energy source and high sensitivity to product inhibition. *Biotechnology and Bioengineering*, *109*, 1109–1119. <https://doi.org/10.1002/bit.24404>
- Khanna, S., Goyal, A., & Moholkar, V. S. (2012). Microbial conversion of glycerol: Present status and future prospects. *Critical Reviews in Biotechnology*, *32*, 235–262. <https://doi.org/10.3109/07388551.2011.604839>
- Poljungreed, I., & Boonyarattanakalin, S. (2017). Dihydroxyacetone production by *Gluconobacter frateurii* in a minimum medium using fed-batch fermentation. *Journal of Chemical Technology and Biotechnology*, *92*(10), 2635–2641.
- Poljungreed, I., & Boonyarattanakalin, S. (2018). Low-cost biotransformation of glycerol to 1,3-dihydroxyacetone through *Gluconobacter frateurii* in medium with inorganic salts only. *Letters in Applied Microbiology*, *67*, 39–46.
- Sato, S., Kitamoto, D., & Habe, H. (2014). Chemical mutagenesis of *Gluconobacter frateurii* to construct methanol-resistant mutants showing glyceric acid production from methanol-containing glycerol. *Journal of Bioscience and Bioengineering*, *117*, 197–199. <https://doi.org/10.1016/j.jbiosc.2013.07.004>
- Sato, S., Morita, N., Kitamoto, D., Yakushi, T., Matsushita, K., & Habe, H. (2013). Change in product selectivity during the production of glyceric acid from glycerol by *Gluconobacter* strains in the presence of methanol. *AMB Express*, *3*, 20. <https://doi.org/10.1186/2191-0855-3-20>
- Sato, S., Morita, T., Fukuoka, T., Kitamoto, D., & Habe, H. (2015). Microbial resolution of D-glyceric acid for L-glyceric acid production with newly isolated bacterial strains. *Journal of Bioscience and Bioengineering*, *119*, 554–557. <https://doi.org/10.1016/j.jbiosc.2014.10.016>
- Shuler, M. L., & Fikret, K. (2002). How cells grow. In P. Guerrieri (Ed.), *Bioprocess engineering basic concepts* (2nd edn, pp. 169–170). Upper Saddle River, NJ: Prentice Hall.
- Szymanowska-Powałowska, D., & Biała, W. (2014). Scale-up of anaerobic 1, 3-propanediol production by *Clostridium butyricum* DSP1 from crude glycerol. *BMC Microbiology*, *14*, 1–10. <https://doi.org/10.1186/1471-2180-14-45>
- Tanamool, V., Hongsachart, P., & Soemphol, W. (2018). Bioconversion of biodiesel-derived crude glycerol to 1,3-dihydroxyacetone by a potential acetic acid bacteria. *Sains Malaysiana*, *47*, 481–488. <https://doi.org/10.17576/jsm-2018-4703-07>
- Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiological Reviews*, *56*, 395–411.
- Yang, B., Liang, S., Liu, H., Liu, J., Cui, Z., & Wen, J. (2018). Metabolic engineering of *Escherichia coli* for 1,3-propanediol biosynthesis from glycerol. *Bioresource Technology*, *267*, 599–607. <https://doi.org/10.1016/j.biortech.2018.07.082>
- Yang, F., Hanna, M. A., & Sun, R. (2012). Value-added uses for crude glycerol—a byproduct of biodiesel production. *Biotechnology for Biofuels*, *5*(1), 13. <https://doi.org/10.1186/1754-6834-5-13>
- Zhou, J. J., Shen, J. T., Wang, X. L., Sun, Y. Q., & Xiu, Z. L. (2018). Stability and oscillatory behavior of microbial consortium in continuous conversion of crude glycerol to 1,3-propanediol. *Applied Microbiology and Biotechnology*, *102*, 8291–8305. <https://doi.org/10.1007/s00253-018-9244-6>

Zhou, X., Zhou, X., Xu, Y., & Yu, S. (2016). Improving the production yield and productivity of 1,3-dihydroxyacetone from glycerol fermentation using *Gluconobacter oxydans* NL71 in a compressed oxygen supply-sealed and stirred tank reactor (COS-SSTR). *Bioprocess and Biosystems Engineering*, 39, 1315–1318. <https://doi.org/10.1007/s00449-016-1595-8>

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APPENDIX

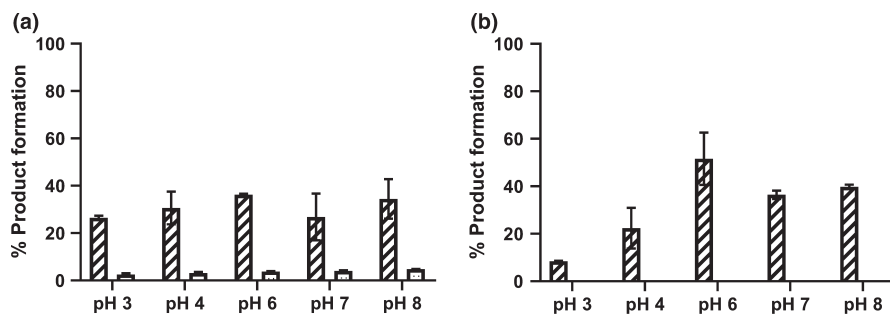


FIGURE A1 Effect of initial pH of the reaction medium in the conversion of glycerol to GA (dots) and DHA (stripes) by resting cells of *Gluconobacter frateurii* (a) and *Gluconobacter oxydans* (b) [Correction added on 26 November 2019 after first online publication: Figure A1 caption has been corrected]

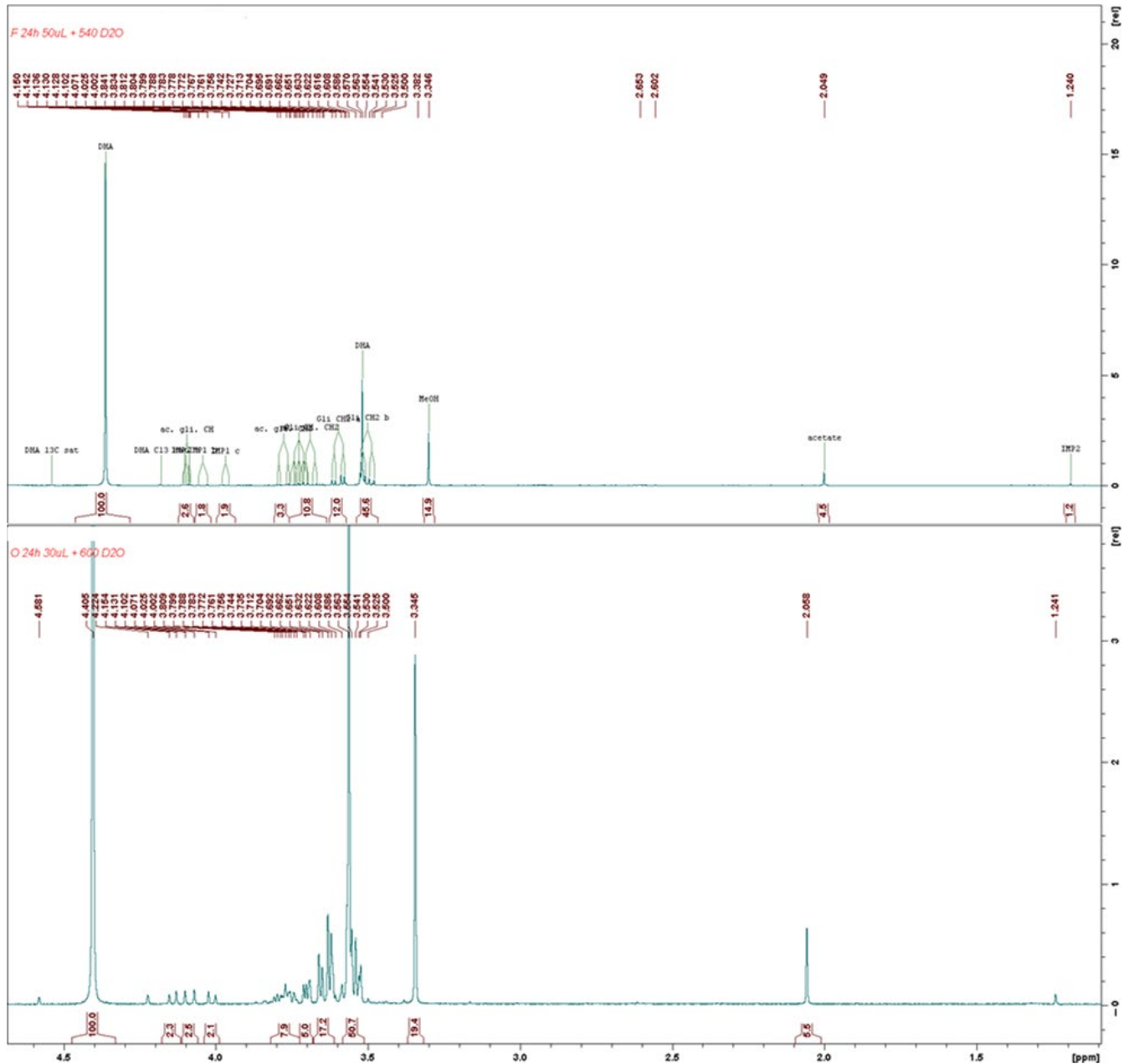


FIGURE A2 ¹H NMR spectroscopy analysis of bioconversion endpoint (24 h). (a) Spectra of glycerol conversion with *Gluconobacter oxydans*. (b) Spectra of glycerol conversion with *Gluconobacter frateurii*. NMR spectral assignments: DHA: 4.405 (s, 1H); 3.563 (s, 4H). Glycerol: 3.763 (tt, 4.4, 9.9Hz, 1H); 3.642 (dd, 4.4, 11.7Hz, 2H); 3.547 (dd, 6.5, 11.7Hz, 2H). Glyceric acid: 4.139 (dd, 3.1, 5.7Hz, 1H); 3.823 (dd, 3.2, 11.9Hz, 1H); 3.734 (dd, 5.5, 11.7Hz, 1H). Methanol: 3.35 (s, 3H). Acetate: 2.05 (s, 3H)

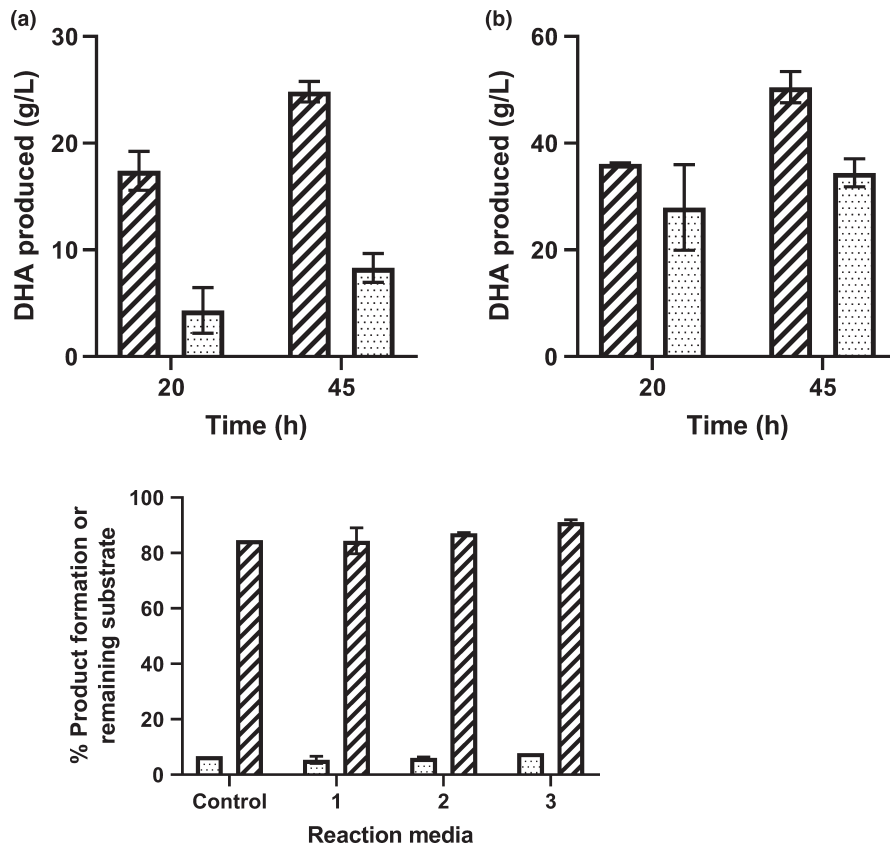


FIGURE A3 DHA production using resting cells of *Gluconobacter frateurii* (a) and *Gluconobacter oxydans* (b), starting from crude glycerol (stripes) and crude glycerol in the presence of DHA (dots). Reactions for *G. frateurii* were started with 25g/L glycerol or 25g/L DHA and 25 g/L de glycerol. In the case of *G. oxydans*, reactions were started with 50 g/L of glycerol or 50 g/L of DHA and 50g/L of glycerol [Correction added on 26 November 2019 after first online publication: Figure A3 caption has been corrected]

FIGURE A4 DHA production using resting cells of *Gluconobacter oxydans* in different reaction media. DHA (stripes), glycerol (dots). Control (distilled water, glycerol, KH_2PO_4 , K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 1 (distilled water and glycerol), 2 (distilled water, glycerol, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 3 (distilled water, glycerol, KH_2PO_4 , K_2HPO_4). Reactions were started with 50 g/L and analyzed after 20 hr [Correction added on XX November 2019 after first online publication: Figure A4 caption has been corrected]