Valorization of Waste Glycerol to Dihydroxyacetone with Biocatalysts Obtained from *Gluconobacter oxydans*

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Abstract: Waste glycerol is the main by-product generated during biodiesel production, in an amount reaching up to 10% of the produced biofuel. Is there any method which allows changing this waste into industrial valuable compounds? This manuscript describes a method for valorization of crude glycerol via microbial bioconversion. It has been shown that the use of free and immobilized biocatalysts obtained from *Gluconobacter oxydans* can enable beneficial valorization of crude glycerol to industrially valuable dihydroxyacetone. The highest concentration of this compound, reaching over 20 g·L\(^{-1}\), was obtained after 72 h of biotransformation with free *G. oxydans* cells, in a medium containing 30 or 50 g·L\(^{-1}\) of waste glycerol. Using a free cell extract resulted in higher concentrations of dihydroxyacetone and a higher valorization efficiency (up to 98%) compared to the reaction with an immobilized cell extract. Increasing waste glycerol concentration to 50 g·L\(^{-1}\) causes neither a faster nor higher increase in product yield and reaction efficiency compared to its initial concentration of 30 g·L\(^{-1}\). The proposed method could be an alternative for utilization of a petrochemical waste into industry applicated chemicals.

Keywords: waste/crude glycerol; dihydroxyacetone; cell extract; immobilization; *Gluconobacter oxydans*; glycerol dehydrogenase

1. Introduction

Biodiesel can be an alternative to environmentally-unfriendly fossil fuels, whose global resources have depleted rapidly over the past several years. On the other hand, replacement of conventional fuels with biofuels (i.e., biodiesel) entails generation of high amounts of waste glycerol (crude glycerol), which is the major by-product from biodiesel production. According to literature data, more than 10 pounds of crude glycerol are generated per every 100 pounds of biodiesel produced [1,2]. Following expert opinions, biodiesel production in European countries and around the globe will be developing very dynamically in the upcoming years, and the amount of crude glycerol will be growing with it [3].

Studies on the utilization of the waste glycerol fraction are carried out in many research centers. Many methods are being developed which foster a hope for economical and environmentally-friendly solutions in this area [4]. Crude glycerol can be used as the starting material for the production of some valuable products to be applied in many sectors of industry [5].

One of the main hurdles in adapting this waste fraction for further use is the high cost of it’s purification to a level that enables it’s application in industry. Purified waste glycerol can be used as an additive to feedstock for animals due to its non-toxicity and high calorific value [6,7]. It can also
be used as an additive to wood pellets [8]. The possibilities of its industrial application also include production of chemicals, fuel additives, hydrogen, ethanol, or methanol [9].

One of the concepts for using unpurified waste glycerol is its microbial bioconversion into dihydroxyacetone (DHA), with the use of free or immobilized biocatalysts. DHA is a ketotriose, produced on the industrial scale with a microbiological method using specific species of free acetic acid bacteria, characterized by a high activity of membrane-bound glycerol dehydrogenase (GlyDH) [10]. Immobilization of microbial cells in fermentation holds several advantages over free cells in terms of higher cell density, easy separation of product from the reaction mixture, repeated use of biocatalyst for several cycles, reduced cell washout problems, less substrate or product inhibition effects, and control over biomass growth. Several studies have reported the use of immobilized cells over various supports for effective utilization of glycerol. The merits and demerits of the fermentation process rely on the choice of immobilization method. Previous studies also reported the use of various carried material for immobilization of microbial cells for DHA production from glycerol. Hekmat et al. [11] reported the use of a novel carrier material (Ralu rings) for immobilization of G. oxydans cells, and the experiments were carried out in a repeated-fed-batch process in a packed-bed bubble-column bioreactor. In another study, Dikshit and Moholkar [12] used commercial reticulated polyurethane foam as a support for G. oxydans, which provides a high porous surface area for increasing cell density. In the present study, we have selected calcium alginate beads as the support for immobilization of G. oxydans cells. The major advantages of using alginate beads in the immobilization process are their low cost, easy operation, and mild operating conditions [13]. Calcium alginate beads are most widely supported for immobilization of cells and enzymes.

Oxidation of glycerol by acetic acid bacteria may follow two pathways (Figure 1). The first pathway (independent from adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD)) proceeds at pH 5.0–5.5 in periplasmic space and is catalyzed by membrane-bound GlyDH. The only product of this pathway is DHA, which is directly released out of the membrane into the outside environment. The second pathway of glycerol transformation proceeds at pH 8.5–10.0 and requires the presence of ATP and Mg\textsuperscript{2+} ions, in addition to an enzyme—glycerol kinase. In a number of subsequent biochemical transformations, the phosphorylated DHA is transformed into other compounds (such as fructose-1,6-diphosphate, glucose-6-phosphate) and used for biomass production [14] (Figure 1).

![Figure 1](image_url)

**Figure 1.** A scheme of two pathways of glycerol metabolism in bacteria cells depending on pH.

Dihydroxyacetone is presently applied in the food and pharmaceutical sectors, but it still finds new applications in other branches of industry [15]. It may play a beneficial role in the treatment of hypotension and cardiogenic shock by restoring blood volume and cellular respiration.
The newest findings suggest the promising therapeutic potential of DHA against poisoning by aluminum phosphide, which is a pesticide widely used to protect agricultural products from pests [16]. This compound releases phosphine gas upon contact with water, moisture, or gastric acids, and ensures 70–100% lethality [16]. DHA is also the main active compound in all types of self-tanning cosmetics [17–19].

*Gluconacetobacter oxydans* ATCC 621 (*G. oxydans*) belongs to the *Acetobacteriaceae* family, and is capable of oxidizing glycerol to DHA in a reaction catalyzed by GlyDH. It has been demonstrated that GlyDH strongly adheres to the cytoplasmic membrane of this bacteria cell [20–22]. The enzymatic activity of GlyDH changes depending on conditions (for example temperature, pH, aeration). Staying bound with the membrane ensures its higher enzymatic activity. Purification of GlyDH was demonstrated to strongly decrease its activity and stability. The optimal temperature for GlyDH ranges from 23 °C to 25 °C, and optimal pH from 7.0 to 7.5 [22–24].

Glycerol is a renewable feedstock in biorefineries to synthesize fuel, chemicals, and energy [25]. There is a constant and unmet need to develop new methods that can be implemented on the industrial scale. The goal of this study was to evaluate the possibility of converting waste glycerol to DHA depending on the initial concentration of the substrate and the type of biocatalysts used.

The novelty of the presented method is the possibility of utilization of waste glycerol into valuable, industrially used dihydroxyacetone. Replacement of whole cells of *G. oxydans* by cell extract provides many benefits for the proposed method. For example, it enables the matching of optimal factors for GlyDH (not for whole cells) and enables elimination of the inhibition effect at high substrate or product concentrations, which can be observed with reactions using whole cells. Moreover, the immobilization of biocatalysts allows them to be easily separated from the reaction mixture, which can facilitate the acquisition of the final product from the reaction medium.

2. Materials and Methods

2.1. Biological Material

The experiments were conducted with the *Gluconobacter oxydans* ATCC 621 strain from American Type Culture Collection, Manassas NY. Waste glycerol fraction from biodiesel production originated from the Factory of biodiesel (BIOAGRA-OIL S.A., Tychy, Poland), and it was characterized by 24°Blg, a pH of 6.12, concentration of glycerol of 659.5 g·L⁻¹, dry substance content of 69.76%, and the following medium content of individual elements in mg·g⁻¹: Ca (0.33), K (0.07), Mg (0.04), Na (19.06), and P (0.08).

2.2. Media Composition and Culture Conditions

Medium for refreshing and storage of *G. oxydans* (g·L⁻¹): Yeast extract, 5; peptone, 3; mannitol, 25; and agar, 15. Incubation was on agar slants for 48 h at 28 °C.

Medium for proliferation of *G. oxydans* cells (g·L⁻¹): Yeast extract, 30; ethanol, 20; and pH 5.0. Fifty cubic centimeters of medium was inoculated on pure culture of *G. oxydans*. Proliferation was conducted in a 500 cm³ shake flask for about 48 h at 28 °C with shaking (200 rpm).

Medium for GlyDH activation (g·L⁻¹): Yeast extract, 5; glycerol, 20; (NH₄)₂SO₄, 5; and pH 5.0. Activation was conducted in 150 cm³ of medium in a 500 cm³ shake flask for about 48 h (the end of stationary phase) at 28 °C with shaking (200 rpm).

Medium for glycerol biotransformation included crude glycerol with a final concentration of 30 g·L⁻¹ or 50 g·L⁻¹, and pH of 5.0 (for variants with free or immobilized cells) or pH of 7.5 (for variants with free or immobilized cell extract).

Reagents were obtained from Avantor Performance Materials, Poland.

All the culture media were sterilized at a temperature of 121 °C for 20 min.
2.3. Preparation of Biocatalysts

There were four biocatalysts used in this study: Free cells, immobilized cells, free cell extract, and immobilized cell extract.

Preparation of free cells: The culture of *G. oxydans* from the medium for GlyDH activation was centrifuged (10 min, 10,000 rpm) and rinsed twice with sterile distilled water. The final weight of the wet biomass was 1 g.

Preparation of immobilized cells: Wet biomass obtained by the above procedure was suspended in 60 cm$^3$ of sterile water and mixed (1:1, v/v) with sodium alginate 40 g·L$^{-1}$ (Fluka, Steinheim, Germany). The mixture was further added dropwise to 0.2 M CaCl$_2$ solution using a syringe with a needle of 1.2 mm diameter for formation of beads. The beads were incubated in the same CaCl$_2$ solution for 3 h at 4 °C.

Preparation of free extract: The wet *G. oxydans* biomass (1 g) prepared by the above procedure was suspended in 60 cm$^3$ of sterile distilled water and subjected to ultrasonic disintegration for a total duration of 7 min (1 min sonication and 30 s resting cycle for cooling), at 210 W, 18 kHz, and 4 °C in an Omni Ruptor 4000 (OMNI International, Kennesaw, GA, USA) with a Titanium 3/8” DiaSolid tip. The obtained cell extract was centrifuged (as above) to remove cell debris, and the supernatant that contains a mixture of cell enzymes was recovered for further use.

Preparation of immobilized extract: The recovered supernatant in the above procedure (1 g biomass, disruption, centrifugation) was suspended with 60 cm$^3$ of sterile water and mixed (1:1, v/v) with sodium alginate (40 g·L$^{-1}$, Fluka). The mixture was added to a 0.2 M solution of CaCl$_2$ using a syringe with a needle of 1.2 mm diameter and incubated in CaCl$_2$ at 4 °C for 3 h.

Every variant of the biocatalysts was prepared in three independent replicates.

2.4. Utilization of a Waste G·Lycerol

Utilization of a waste glycerol fraction was conducted in 150 cm$^3$ of each solution in 500 cm$^3$ shake flasks on a reciprocating shaker (200 rpm) in conditions dependent on the use of an appropriate biocatalyst (optimal for acetic acid bacteria activity or for GlyDH activity). When biocatalyzers were free or immobilized cells, waste glycerol concentration was 30 or 50 g·L$^{-1}$, pH 5.0 and temperature was 28 °C. In utilization variant with free or immobilized cell extract, the initial waste glycerol concentration was 30 or 50 g·L$^{-1}$ and pH and temperature of this process were 7.5 and 28 °C, respectively. Utilization was carried out for 96 hours for each used variant of biocatalyst.

All variants were conducted in three independent replicates.

2.5. Determination of the Concentration of Glycerol and Dihydroxyacetone by Gas Chromatography

The analysis of the samples was carried out using a Thermo SCIENTIFIC Trace 1300 Gas Chromatograph (Waltham, US) equipped with a Thermo SCIENTIFIC AI 1310 autosampler. The capillary compatible with water column ZB-Wax Plus (resistant to water) with the dimensions 30 m × 0.2 mm × 0.22 µm was used. A flame ionization detector was also used. The samples of the utilization medium were diluted with a water solution of acetonitrile (1:1 v/v). Injection of a 2 µL sample using a 50:1 split ratio to the dispenser insert (liner) was completed with an internal diameter of 4 mm with constriction, and an isothermal of 260 °C.

The initial sample temperature was 80 °C for 1 min. The temperature was increased by 10 °C per minute to a final (injection) temperature of 260 °C, which was maintained for 2 min.

Gas flow: Air, 400 mL·min$^{-1}$; hydrogen, 40 mL·min$^{-1}$; makeup gas, 25 mL·min$^{-1}$; and carrier gas, 1.5 mL·min$^{-1}$

The internal standard used in this study was alpha-chlorohydrin. The samples for determination of glycerol and DHA concentration were taken at the beginning of biotransformation (time 0) and after 24, 48, 72, and 96 h intervals. All detections of single samples were measured in three independent replications.
2.6. Analysis

The rate of glycerol consumption and DHA production was calculated and the results were expressed in g·L⁻¹·h⁻¹ units. The results of DHA yield were expressed in g of obtained DHA on each g of glycerol consumed. The efficiency of the reaction was calculated according to the stoichiometric equation of a chemical oxidation of glycerol into DHA. The standard deviation was calculated for all experimental results.

3. Results

3.1. Conversion of Waste Glycerol with Free and Immobilized Cells of G. oxydans ATCC 621

Time profiles of glycerol conversion and DHA formation during biotransformation of 30 g·L⁻¹ of a waste glycerol fraction with the use of free or immobilized G. oxydans cells are depicted in Figures 2 and 3.

![Figure 2](image2.png)

**Figure 2.** Time profiles of glycerol conversion and dihydroxyacetone (DHA) formation during biotransformation of 30 g·L⁻¹ waste glycerol with free *G. oxydans* cells.

![Figure 3](image3.png)

**Figure 3.** Time profiles of glycerol conversion and DHA formation during biotransformation of 30 g·L⁻¹ waste glycerol with immobilized *G. oxydans* cells.

Conversion of the waste fraction of glycerol (initial concentration 30 g·L⁻¹) with the use of free cells caused a rapid depletion of substrate concentration by approximately 19 g·L⁻¹ within 24 h.
After another day of the process, the substrate concentration decreased to the final value of 0.9 g·L\(^{-1}\) (Figure 2). Extending the conversion time to 96 h had no significant effect on glycerol concentration in the reaction medium (Figure 2). In the reaction with free \textit{G. oxydans} cells and an initial crude glycerol content of 3\%, the concentration of DHA was about 9 g·L\(^{-1}\) and 18 g·L\(^{-1}\) after 24 and 48 h of biotransformation, respectively (Figure 2). The highest concentration of the expected product was observed after 72 h and was approximately 20 g·L\(^{-1}\). Elongation of the bioconversion process to 96 h resulted in a decrease in DHA concentration in the reaction media (Figure 2). During conversion of 30 g·L\(^{-1}\) of a crude glycerol fraction with immobilized cells as biocatalysts, the glycerol concentration decrease was slower compared with the same reaction conducted using free cells (Figures 2 and 3). The lowest substrate concentration (0.9 g·L\(^{-1}\)) was determined after 72 h of the bioconversion process and it was sustained at the same level until the end of the process (Figure 3). In the medium with 3\% initial glycerol concentration and immobilized cells, DHA production was lower than in the same medium but with a different biocatalyst (free cells), regardless of process duration. The maximum concentration of the final product reached after 72 h was about 14.6 g·L\(^{-1}\) (Figure 3).

Time profiles of glycerol conversion and DHA formation during bioconversion of 50 g·L\(^{-1}\) of a waste glycerol fraction with free or immobilized \textit{G. oxydans} cells are presented in Figures 4 and 5.

![Figure 4](image1.png)

**Figure 4.** Time profiles of glycerol conversion and DHA formation during conversion of 50 g·L\(^{-1}\) of a waste glycerol fraction with free \textit{G. oxydans} cells.

![Figure 5](image2.png)

**Figure 5.** Time profiles of glycerol conversion and DHA formation during conversion of 50 g·L\(^{-1}\) of a waste glycerol fraction with immobilized \textit{G. oxydans} cells.
In the medium with 50 g·L⁻¹ of waste glycerol and free cells, substrate utilization was observed only for the first 48 h of the bioconversion process (Figure 4). Subsequently, the concentration of glycerol remained steady at about 22.5 g·L⁻¹ (Figure 4). Concurrently, the DHA concentration in the reaction mixture increased to about 9 g·L⁻¹ (after 24), 19 g·L⁻¹ (after 48 h), and 21 g·L⁻¹ at the end of the process (Figure 4). During the first two days of bioconversion with 5% of the initial waste content, glycerol concentration was lower than in the process with free cells (about 3 and 4 g·L⁻¹ after 24, 51 g·L⁻¹ after 48 h, 61 g·L⁻¹ after 72 and 96 h, respectively) (Figures 4 and 5). The highest concentration of the expected product was approximately 16 g·L⁻¹ after 96 h of the bioconversion process (Figure 5).

3.2. Conversion of Waste Glycerol with Free and Immobilized Cell Extract Obtained from G. oxydans ATCC 621

Time profiles of glycerol conversion and DHA formation during bioconversion of 30 g·L⁻¹ of a waste glycerol fraction with the use of free or immobilized cell extract from G. oxydans cells are presented in Figures 6 and 7.

![Figure 6](image1.png)

**Figure 6.** Time profiles of glycerol conversion and DHA formation during bioconversion of 30 g·L⁻¹ of a waste glycerol fraction with a free cell extract obtained from G. oxydans.

![Figure 7](image2.png)

**Figure 7.** Time profiles of glycerol conversion and DHA formation during bioconversion of 30 g·L⁻¹ of a waste glycerol fraction with an immobilized cell extract obtained from G. oxydans.
In the first day of the process with the use of a free cell extract as a biocatalyst, only 5 g·L⁻¹ of waste glycerol was consumed (Figure 6). The substrate consumption rate increased on the next day to 14.6 g·L⁻¹. After 72 and 96 h, 13 g·L⁻¹ of unutilized glycerol remained in the medium (Figure 6). A significant increase in DHA concentration was recorded after 48 h, and it was about 13 g·L⁻¹. On the next day, its concentration increased by 3 g·L⁻¹, but after 48 h it decreased by 0.5 g·L⁻¹ (Figure 6). The degree of substrate consumption in the bioconversion process conducted with an immobilized cell extract and 3% of crude extract from G. oxydans (ca. 20 g·L⁻¹) still remained unutilized in the reaction medium after 96 h (Figure 7). The concentration of the expected product was also insufficient. After 24 h, only 2 g·L⁻¹ of DHA was produced, after 48 h its production was at around 6 g·L⁻¹, while in the further reaction DHA concentration did not exceed 8 g·L⁻¹ (Figure 7).

Changes in glycerol and DHA concentrations during bioconversion of 50 g·L⁻¹ of a waste glycerol fraction with the use of free or immobilized cell extract from G. oxydans cells are presented in Figures 8 and 9.

**Figure 8.** Time profiles of glycerol conversion and DHA formation during bioconversion of 50 g·L⁻¹ of a waste glycerol fraction with free cell extract obtained from G. oxydans.

**Figure 9.** Time profiles of glycerol conversion and DHA formation during bioconversion of 50 g·L⁻¹ of a waste glycerol fraction with immobilized cell extract obtained from G. oxydans.
Reaction with 50 g·L⁻¹ initial waste glycerol concentration and free cell extract resulted in low consumption of glycerol (only 5 g·L⁻¹ and 7.5 g·L⁻¹ after 24 and 48 h, respectively) (Figure 8). The final concentration of DHA successively increased throughout the reaction from about 4 g·L⁻¹ (after 24 h), through 9 g·L⁻¹ (after 72 h), to the maximum value of 15 g·L⁻¹ (after 96 h) (Figure 8). The course of utilization of 5% of waste glycerol with the immobilized cell extract as a biocatalyst showed a low rate of substrate consumption. After 96 h of biotransformation, a substantial portion of the glycerol remained unutilized in the medium (Figure 9). The concentration of the expected product increased successively until 72 h, and decreased further from 6 to about 5 g·L⁻¹ after 96 h (Figure 9).

3.3. Substrate Consumption Rate and DHA Production Yield during Bioconversion of Waste Glycerol by G. oxydans ATCC 621 Cells and Their Extract

The rates of glycerol consumption, DHA production, and DHA yield during bioconversion of a waste glycerol fraction conducted using all tested variants are presented in Tables 1 and 2.

Table 1. Rates of glycerol consumption, DHA productivity, and DHA yield during bioconversion of a waste glycerol fraction (30 or 50 g·L⁻¹) with free and immobilized cells of G. oxydans.

| Initial Glycerol (g·L⁻¹) | Type of Biocatalysts | Time       |
|-------------------------|----------------------|------------|
|                         |                      | 24 h | 48 h | 72 h | 96 h |
| 30                      | Free cells           | 0.78  | 0.61 | 0.40 | 0.30 |
|                         |                      | 0.39  | 0.38 | 0.28 | 0.20 |
|                         |                      | 0.50  | 0.63 | 0.70 | 0.66 |
|                         | Immobilized cells    | 0.58  | 0.47 | 0.40 | 0.30 |
|                         |                      | 0.12  | 0.19 | 0.20 | 0.13 |
|                         |                      | 0.22  | 0.40 | 0.50 | 0.44 |
| 50                      | Free cells           | 0.21  | 0.30 | 0.24 | 0.18 |
|                         |                      | 0.39  | 0.27 | 0.22 | 0.16 |
|                         |                      | 0.50  | 0.88 | 0.94 | 0.91 |
|                         | Immobilized cells    | 0.21  | 0.17 | 0.12 | 0.11 |
|                         |                      | 0.10  | 0.12 | 0.10 | 0.08 |
|                         |                      | 0.47  | 0.71 | 0.83 | 0.74 |

Table 2. Rates of glycerol consumption, DHA production, and DHA yield during bioconversion of a waste glycerol fraction (30 or 50 g·L⁻¹) with free and immobilized cell extracts.

| Initial Glycerol (g·L⁻¹) | Type of Biocatalysts | Time       |
|-------------------------|----------------------|------------|
|                         |                      | 24 h | 48 h | 72 h | 96 h |
| 30                      | Free cell extract    | 0.21  | 0.30 | 0.24 | 0.18 |
|                         |                      | 0.18  | 0.27 | 0.22 | 0.16 |
|                         |                      | 0.81  | 0.88 | 0.94 | 0.91 |
|                         | Immobilized cell extract | 0.21  | 0.17 | 0.12 | 0.11 |
|                         |                      | 0.10  | 0.12 | 0.10 | 0.08 |
|                         |                      | 0.47  | 0.71 | 0.83 | 0.74 |
| 50                      | Free cell extract    | 0.21  | 0.16 | 0.21 | 0.16 |
|                         |                      | 0.18  | 0.14 | 0.12 | 0.16 |
|                         |                      | 0.88  | 0.89 | 0.58 | 0.97 |
|                         | Immobilized cell extract | 0.29  | 0.23 | 0.31 | 0.23 |
|                         |                      | 0.05  | 0.05 | 0.09 | 0.05 |
|                         |                      | 0.17  | 0.23 | 0.28 | 0.23 |
During bioconversion of a waste glycerol fraction with free cells, the glycerol consumption rate was on average 0.75 g·L⁻¹·h⁻¹ after 24 h, and after the next 24 h it was on average 0.6 g·L⁻¹·h⁻¹, irrespective of the initial concentration (30 or 50 g·L⁻¹) (Table 1). The further course of the process clearly showed that the glycerol consumption rate was much higher in the reaction proceeding in a solution with a lower initial substrate content (30 g·L⁻¹). In these experimental variants (free cells and 30 or 50 g·L⁻¹ initial content of glycerol), the rate of DHA production was very similar at each time of measurement (Table 1). The immobilization of G. oxydans cells, regardless of the initial concentration of substrate, resulted in a similar glycerol consumption rate except for the first day, in which this rate was nearly 3-fold higher in the medium with 3% of the waste (0.58 g·L⁻¹·h⁻¹ for 3% of waste and 0.21 g·L⁻¹·h⁻¹ for 5% of waste) (Table 1). The DHA production rate also developed almost identically in the process with the immobilized cells in the media with the initial concentration of glycerol at both 3% and 5%, and was on average 0.12, 0.20, and 0.15 at the individual measuring points (Table 1). Using free cells allowed us to obtain higher values of DHA yield (independent of initial glycerol concentration) compared to immobilized cells (Table 1).

Regardless of the initial concentration of waste glycerol in the reaction medium, the rate of glycerol consumption in the reaction with a free cell extract was similar, and reached around 0.21 g·L⁻¹·h⁻¹ after 24 h, 0.23 g·L⁻¹·h⁻¹ after 72 h, and about 0.17 g·L⁻¹·h⁻¹ after 96 h (Table 2). The only significant difference in these values was observed after 24 h of the process, when the glycerol consumption rate was 0.3 g·L⁻¹·h⁻¹ in the medium with the lower initial waste content (30 g·L⁻¹), and almost 2-fold lower in the medium with the higher initial substrate concentration (50 g·L⁻¹) (Table 2). DHA production yield in the discussed variants was the same at the beginning and at the end of the bioconversion process (0.18 g·L⁻¹·h⁻¹ after 24 h and 0.16 g·L⁻¹·h⁻¹ after 96 h) (Table 2). However, after 48 and 72 h, significantly higher productivity was observed for the free cell extract in the medium with 3% of the waste, and it was 0.27 and 0.22 g·L⁻¹·h⁻¹, respectively (while for 5% of the waste it was equal to 0.14 and 0.12 g·L⁻¹·h⁻¹, respectively) (Table 2). Analyzing the obtained results, it can be noticed that the initial concentration of waste glycerol affected the glycerol consumption rate in the reaction with an immobilized cell extract. The obtained results clearly suggest that the consumption rates of the substrate (for each variant of biocatalyst) were lower in the solution initially containing 50 g·L⁻¹ of glycerol compared to that with 30 g·L⁻¹ of glycerol (Tables 1 and 2). Whereas, the DHA productivity rate was low independent of the initial concentration (30 or 50 g·L⁻¹), and ranged from 0.05 (Table 2) to 0.4 g·L⁻¹·h⁻¹ (Table 1), regardless of the measurement time or of the initial composition of the reaction medium (Table 2). In the case of obtaining DHA with the use of a free cell extract (initial glycerol of 30 or 50 g·L⁻¹), the DHA yield was higher compared to data obtained in variants with free cells. The lowest DHA yield was observed in the medium with 50 g·L⁻¹ of glycerol and immobilized cell extract as a biocatalyst (Table 2).

### 3.4. Efficiency of Waste Glycerol Bioconversion by G. oxydans ATCC 621 Cells and Their Extracts

The bioconversion efficiency of a waste glycerol fraction with free or immobilized G. oxydans ATCC 621 cells, and cell extracts obtained from this strain, is presented in Figures 10 and 11.
Table 2. Bioconversion of waste glycerol by free or immobilized cells of \textit{G. oxydans} in the medium with different initial contents of the substrate. [30 g L$^{-1}$].

| Time [h] | Free cells [30 g L$^{-1}$] | Free cells [50 g L$^{-1}$] | Immobilized cells [30 g L$^{-1}$] | Immobilized cells [50 g L$^{-1}$] |
|----------|-----------------------------|-----------------------------|-----------------------------------|-----------------------------------|
| 24       | 0.23                        | 0.14                        | 0.27                              | 0.22                              |
| 48       | 0.58                        | 0.46                        | 0.58                              | 0.46                              |
| 72       | 0.85                        | 0.72                        | 0.85                              | 0.72                              |
| 96       | 1.00                        | 0.87                        | 1.00                              | 0.87                              |

**Figure 10.** Efficiency of the bioconversion of waste glycerol by free or immobilized cells of \textit{G. oxydans} in the medium with different initial contents of the substrate. [30 g L$^{-1}$].

**Figure 11.** Efficiency of the bioconversion of waste glycerol by free or immobilized cell extracts from \textit{G. oxydans} in the medium with different initial contents of the substrate.

Analysis of the obtained results allowed an observation that the highest efficiency of the process carried out with free cells of \textit{G. oxydans} was 77\% in the medium with 5\% waste glycerol (after 72 h of utilization). Whereas in the process with immobilized cells, it was 58\% after 24 h, also in the solution with a higher initial content of the substrate (Figure 10).

In the case of the process in which free cell extract served as the biocatalyst, the highest efficiency reached was almost 100\% after 72 h at an initial waste concentration of 30 g L$^{-1}$, and 98\% after 96 h of the reaction in the medium with an initial substrate concentration of 50 g L$^{-1}$ (Figure 11).

4. Discussion

With each passing year, the generation and accumulation of by-products from various industrial processes poses a greater hazard to the natural environment. Waste glycerol generation during biodiesel production has become an inspiration for many scientists to develop methods for it’s effective
utilization. Planning and conducting processes for the valorization of industrial wastes, including glycerol, could bring many benefits. It not only enables removing wastes from the ecosystem, but also creates the possibility of obtaining new, industrially valuable compounds, such as dihydroxyacetone.

Production of DHA using live whole cells requires their early preparation (including refreshing, proliferation, enzyme activation, and biomass washing, etc.) and maintaining the metabolic activity of cells at a high level under optimal conditions for their growth (pH 5.0, 28 °C). The disadvantage in using whole cells is in providing complex fermentation media, which is essential for DHA production. Replacement of whole cells by a cell extract allows the process to be run under optimal conditions for glycerol dehydrogenase (pH 7.5, 23 °C), which catalyzes the oxidation of glycerol to DHA. Immobilization of the biocatalysts facilitates its easy separation from the reaction medium, allows the reaction to become independent from the presence of free, whole, live cells of *G. oxydans*, and also creates an opportunity to reuse the catalyst (gel beads) in subsequent production cycles, which could reduce the cost of the whole process in the future [26].

This study describes microbial conversion of a waste glycerol fraction to DHA with biocatalysts from *G. oxydans*. The highest DHA concentration of 20 g·L⁻¹ was obtained after 72 h of biotransformation with free *G. oxydans* cells in the medium containing an initial waste glycerol concentration of 30 and 50 g·L⁻¹. In this case, process efficiency ranged from 70% to 77%, whereas DHA productivity was about 0.3 g·L⁻¹·h⁻¹. Immobilization of whole, live cells of *G. oxydans* had a significant impact on the course of the bioconversion process, as it significantly reduced DHA productivity and reaction efficiency.

The use of free cell extract resulted in a higher DHA production than in the reaction with the immobilized ones. However, DHA concentrations obtained in these cell extracts were still lower as compared to the reactions with live cells. Reaction efficiency was highest in the medium containing free cell extract as the biocatalyst (up to 98%).

The presented data showed that immobilization could, to some extent, reduce DHA production. Presumably, the weight of wet biomass used in this study (1 g of wet biomass per each variant of utilization media) could have an impact on the course of the reaction. At this condensation of the biological material, substrate competition for active sites of the enzyme was so high that it inhibited the reaction. It may also be hypothesized that the number of available active sites was too low to enable conversion of the whole amount of glycerol present in the production media. In turn, immobilization could hinder the gas exchange between the substrate and the cells entrapped in the carrier (or between the substrate and a cell extract). The spatial structure of the alginate carrier and size of alginate beads could also, to some extent, reduce the rate of product diffusion to the reaction medium. The decrease in final DHA concentration for the immobilized system could also be due to limitations in mass transfer, which hinders easy transport of substrate to active sites of the enzyme present inside the immobilized matrix. This further decreases the actual substrate concentration inside alginate beads in comparison to bulk concentration in media. Whereas, the free cell system was able to access the total available substrate in the bulk medium and resulted in higher DHA production in comparison to immobilized systems. The type of carrier used could also affect the obtained results. The size of alginate beads may also be an important issue. A needle with a diameter of 1.20 mm was used to prepare the alginate beads [27]. It is supposed that the smaller diameter of the gel beads could increase the surface area of the enzyme–substrate contact and facilitate firstly glycerol oxidation and later DHA release into the reaction medium. Orrego et al. [28] reported that mass transfer of substrate from a bulk medium into a immobilized matrix is greatly affected by the size of the alginate bead in ethanol fermentation by *S. cerevisiae*. Reduction in bead size increased the mass transfer of substrates from the liquid to the immobilized cells, which further accelerated sugar consumption and ethanol production. In another study, Gutenwik et al. [29] examined competing effects of mass transfer hindrance and kinetics in a glucose to ethanol fermentation system by *S. cerevisiae*. An increase in diffusion resistance with the substrate decreases the availability of the substrate for microbial cells inside the immobilized matrix.
Based on the obtained results, it can be concluded that an increase in initial waste glycerol concentration to 50 g·L⁻¹ did not cause a faster and greater increase in DHA productivity and reaction efficiency as compared to an initial concentration of 30 g·L⁻¹. Increasing the initial crude glycerol concentration was accompanied by an increase in associated contaminants like methanol, salts, soaps, and residual fatty acids, which could further have an inhibiting effect on the course of the bioconversion reaction [30]. It has also been reported that higher substrate (i.e., glycerol) concentration inhibits growth as well as DHA production in *G. oxydans* cells [4]. Therefore, *G. oxydans* cells were more prone to substrate inhibition in the reaction containing 50 g·L⁻¹ waste glycerol as compared to the reaction with 30 g·L⁻¹ concentration, which subsequently influences the DHA productivity, as well as the reaction efficiency.

It was also observed that running the process for 96 h does not ensure a general increase in the final product concentration. Data analysis indicates, that in most of the conducted reactions, a maximum DHA concentration was witnessed after 72 h of biotransformation. Extending the reaction time to 96 h resulted in a reduction in DHA productivity. The reason for the decreasing DHA concentration could be its further phosphorylation catalyzed by dihydroxyacetone kinase, which probably also occurs in cell extract [14,31].

GlyDH, the key enzyme responsible for glycerol oxidation, exhibits the highest activity at the end of the exponential phase of bacterial growth, which could be approximately 24 h [32–34]. After more than 48 h, it may still convert the substrate, but with a significantly lower efficiency. This membrane-bound GlyDH enzyme is dependent on the pyrroloquinoline quinone (PQQ) cofactor, which transfers oxygen from glycerol to a final acceptor (oxygen) via an electron transport chain [24,35]. In live bacterial cells, resources of PQQ are supplemented in the course of the reaction. When cells are replaced with a cell extract, the initial content of PQQ decreases successively, and if the reaction medium is not supplemented with this cofactor, it is totally consumed after a few hours, and the activity of the enzyme is lower than within the first 24–48 h [35]. It needs to be emphasized that the genome of *G. oxydans* contains genes encoding eight different dehydrogenases associated with the cytoplasmic membrane of bacteria [20]. Glycerol may serve as an alternative source of carbon for the production of various compounds (e.g., sodium glycerate), as well as these enzymes [14,34,36,37]. Some of these enzymes present in the matrix or in the membranes of *G. oxydans* may also catalyze further conversions of dihydroxyacetone (e.g., its phosphorylation). The presence of enzymes other than GlyDH may be the reason for the low efficiency of glycerol conversion to DHA, and of the DHA concentration decrease after 72 h of the reaction [38].

The efficiency of the described method can be improved by transferring the reaction to a bioreactor, which ensures the proper maintenance of parameters, such as pH and aeration, at stable levels throughout the reaction time.

Dikshit and Moholkar [12] conducted research with immobilized *G. oxydans* MTCC 904 cells and achieved approximately 14 g·L⁻¹ of DHA production from biodiesel derived crude glycerol (initial concentration of 20 g·L⁻¹). In their study, the experimental medium was supplemented with MgSO₄·7H₂O, (NH₄)₂SO₄, and KH₂PO₄ as significant components, and also with yeast extract as a supporting component. The obtained results suggest a positive role of salts on the course of the reaction. In the presented study, we have used water solutions of waste glycerol as the reaction media without addition of any other medium supplements.

Another idea to increase the efficiency of glycerol conversion could be sonication [39]. In this method, fermentation was carried out in the presence of sonication (20% duty cycle) by free or immobilized (over polyurethane foam) *G. oxydans* cells. Enhancement in glycerol consumption by 60–84% was noticed with no significant change in cell morphology. Higher reaction efficiency was observed when experiments were carried out with immobilized cells than with free cells [39].

The reported results indicate the feasibility of the bioconversion of waste glycerol with the use of microbiological biocatalysts. Future studies should be aimed at DHA recovery from the post-reaction mixture, and at determination of its purity and properties.
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References

1. Binhayeeding, N.; Klomklao, S.; Sangkharak, K. Utilization of Waste G-Lycerol from Biodiesel Process as a Substrate for Mono-, Di-, and Triacylg-Lycerol Production. In Proceedings of the International Conference on Alternative Energy in Developing Countries and Emerging Economies, Bangkok, Thailand, 25–26 May 2017; Volume 138, pp. 895–900. [CrossRef]

2. Manara, P.; Zabaniotou, A. Co-valorization of crude g-Lycerol waste streams with conventional and/or renewable fuels for power generation and industrial symbiosis perspectives. Waste Biomass Valoriz. 2016, 7, 135–150. [CrossRef]

3. Vasudevan, P.T.; Fu, B. Environmentally sustainable biofuels: Advances in biodiesel research. Waste Biomass Valoriz. 2010, 1, 47–63. [CrossRef]

4. Stasiak-Różańska, L.; Blażejak, S.; Gientka, I.; Bzducha-Wróbel, A.; Lipińska, E. Utilization of a waste g-Lycerol fraction using and reusing immobilized G-Luconobacter oxydans ATCC 621 cell extract. Electron. J. Biotechnol. 2017, 27, 44–48. [CrossRef]

5. Mostafa, N.A.; Maher, A.; Abdelmoez, W. Production of mono-, di-, and trig Lycerides from waste fatty acids through esterification with g-Lycerol. Adv. Biosci. Biotechnol. 2013, 4, 900–907. [CrossRef]

6. Quispe, C.A.G.; Coronado, C.J.R.; Carvalho, J.A., Jr. G-Lycerol: production, consumption, prices, characterization and new trends in combustion. Renew. Sustain. Energy Rev. 2013, 27, 475–493. [CrossRef]

7. Nitayavardhana, S.; Khanal, S.K. Biodiesel-derived crude g-Lycerol bioconversion to animal feed: A sustainable option for a biodiesel refinery. Bioresour. Technol. 2011, 102, 5808–5814. [CrossRef]

8. Bala-Litwiniak, A.; Radomiak, H. Pissibility of the utilization of waste g-Lycerol as an addition to wood pellets. Waste Biomass Valoriz. 2018, 9, 1–7. [CrossRef]

9. Leonetti, A.B.; Aragão-Leoneti, V.; de Oliveira, S.V.W.B. G-Lycerol as a by-product of biodiesel production in Brazil: Alternatives for the use of unrefined g-Lycerol. Renew. Energ. 2012, 45, 138–145. [CrossRef]

10. Poljungreed, I.; Boonyarattanakalin, S. Low-cost biotransformation of g-Lycerol to 1,3-dihydroxyacetone through G-Luconobacter frateurii in medium with inorganic salts only. Lett. Appl. Microbiol. 2011, 53, 377–384. [CrossRef] [PubMed]

11. Zhou, Z.; Li, G.; Li, Y. Immobilization of Saccharomyces cerevisiae alcohol dehydrogenase on hybrid alginate-chitosan beads. Int. J. Biol. Macromol. 2010, 3, 21–26. [CrossRef] [PubMed]

12. Mamlouk, D.; Gullo, M. Acetic Acid Bacteria: Physiology and Carbon Sources Oxidation. Indian J Microbiol. 2013, 53, 377–384. [CrossRef] [PubMed]

13. Stasiak-Różańska, L.; Blażejak, S. Dihydroxyacetone—Characteristic, application and recive. Acta Sci. Pol. Biotechnol. 2011, 11, 17–28.

14. Ahmadi, I.; Joukar, S.; Anani, H.; Karami-Mohajeri, S. Dihydroxyacetone as a definitive treatment for aluminium phosphide poisoning in rats. Arch. Ind. Hyg. Toxicol. 2018, 69, 169–177. [CrossRef]

15. Braunberger, T.L.; Nahhas, A.F.; Katz, L.M.; Sadrie, N.; Lim, H.W. Dihydroxyacetone: A review. J. Drugs Dermatol. 2018, 17, 387–391. Available online: https://www.ncbi.nlm.nih.gov/pubmed/29601614 (accessed on 8 August 2018).
18. Ciriminna, R.; Fidalgo, A.; Ilharco, L.M.; Pag-Liaro, M. Dihydroxyacetone: An Updated Insight into an Important Biopродuct. *Chem. Open* 2018, 7, 233–236. [CrossRef]

19. Stasiak-Różańska, L.; Ploska, J. Study on the use of microbial cellulose as a biocarrier for 1,3-dihydroxy-2-propanone and its potential application in industry. *Polymers* 2018, 10, 438. [CrossRef]

20. Peters, B.; Mientus, M.; Kostner, D.; Junker, A.; Liebl, W.; Ehrenreich, A. Characterization of membrane-bound dehydrogenases from G-Luconobacter oxydans 621H via whole-cell activity assays using multideletion strains. *Appl. Microbiol. Biotechnol.* 2013, 97, 6397–6412. [CrossRef]

21. Habe, H.; Fukuoka, T.; Morita, T.; Kitamoto, D.; Yakushi, T.; Matsushita, K.; Sakaki, K. Disruption of the membrane-bound alcohol dehydrogenase-encoding gene improved g-Lycerol use and dihydroxyacetone productivity in G-Luconobacter oxydans. *BioSci. Biotechnol. Biochem.* 2010, 74, 1391–1395. [CrossRef]

22. De Muynck, C.; Pereira, C.S.S.; Naessens, M.; Parmentier, S.; Soetaert, W.; Vandamme, E.J. The Genus Luconobacter oxydans: Comprehensive Overview of Biochemistry and Biotechnological Applications. *Crit. Rev. Biotechnol.* 2007, 27, 147–171. [CrossRef]

23. Ameyama, M.; Shinagawa, E.; Matsushita, K.; Adachi, O. Solubilization, Purification and Properties of Membrane-bound G-Lycerol Dehydrogenase from G-Luconobacter industrius. *Agric. Biol. Chem.* 1985, 49, 1001–1010. [CrossRef]

24. Lapenaiite, I.; Kurtinaitiene, B.; Razumiene, J.; Laurinavicius, V.; Marcinkeviciene, L.; Bachmatova, I.; Meskys, R.; Ramanavicius, A. Properties and analytical application of PQQ-dependent g-Lycerol dehydrogenase from G-Luconobacter sp. 33. *Anal. Chim. Acta* 2005, 549, 140–150. [CrossRef]

25. Anitha, M.; Kamarudinab, S.K.; Koflia, N.T. The potential of g-Lycerol as a value-added commodity. *Chem. Eng. J.* 2016, 295, 119–130. [CrossRef]

26. Stasiak-Różańska, L.; Blażejak, S. Production of dihydroxyacetone from an aqueous solution of g-Lycerol in the reaction catalyzed by an immobilized cell preparation of acetic acid bacteria G-Luconobacter oxydans ATCC 621. *Eur. Food Res. Technol.* 2012, 235, 1125–1132. [CrossRef]

27. Ye, Z.; Song, J.; Zhu, E.; Song, X.; Chen, X.; Xiaoting, H. Alginic Acid Immobilization Technique Promotes Biobutanol Production by Clostridium acetobutylicum Under Extreme Condition of High Concentration of Organic Solvent. *Front. Microbiol.* 2018, 9, 1071. [CrossRef] [PubMed]

28. Orrego, D.; Zapata-Zapata, A.D.; Kim, D. Ethanol production from coffee mucilage fermentation by S. cerevisiae immobilized in calcium-alginate beads. *Bioresour. Technol. Rep.* 2018, 9, 445–456. Available online: https://www.ncbi.nlm.nih.gov/pubmed/29511361077 (accessed on 10 August 2018). [PubMed]

29. Gutenwik, J.; Nilsson, B.; Axelsson, A. Mass transfer effects on the reaction rate for heterogeneously distributed immobilized yeast cells. *Biotechnol. Bioeng.* 2003, 79, 664–673. [CrossRef] [PubMed]

30. Chatzifragkou, A.; Papanikolaou, S. Effect of impurities in biodiesel-derived waste g-Lycerol on the performance and feasibility of biotechnological processes. *Appl. Microbiol. Biotechnol.* 2012, 95, 13–27. [CrossRef]

31. Prust, C.; Hoffmeister, M.; Liesegang, H.; Wiezer, A.; Fricke, W.F.; Ehrenreich, A.; Gottschalk, G.; Deppenmeier, U. Complete genome sequence of the acetic acid bacterium G-Luconobacter oxydans. *Nat. Biotechnol.* 2005, 23, 195–200. [CrossRef] [PubMed]

32. VanLare, I.J.; Claus, G.W. Purification and properties of NAD(P)-independent polyol dehydrogenase complex from the plasma membrane of G-Luconobacter oxydans. *Can. J. Microbiol.* 2007, 53, 504–508. [CrossRef] [PubMed]

33. Matsushita, K.; Fujii, Y.; Ano, Y.; Toyama, H.; Shinjoh, M.; Tomiyama, N.; Miyazaki, T.; Sugisawa, T.; Hoshino, T.; Adachi, O. 5-Keto-D-Luconate production is catalyzed by a quinoprotein g-Lycerol dehydrogenase, major polyol dehydrogenase, in G-Luconobacter species. *Appl. Environ. Microbiol.* 2003, 69, 1999–1966. [CrossRef] [PubMed]

34. Mientus, M.; Kostner, D.; Peters, B.; Liebl, W.; Ehrenreich, A. Characterization of membrane-bound dehydrogenases of G-Luconobacter oxydans 621H using a new system for their functional expression. *Appl. Microbiol. Biotechnol.* 2017, 101, 3189–3200. [CrossRef]

35. Ikemoto, K.; Mori, S.; Mukai, K. Synthesis and crystal structure of pyrroloquinoline quinol (PQQH2) and pyrroloquinoline quinone (PQQ). *Acta Crystallogr. B Struct. Sci. Cryst. Eng. Mater.* 2017, 73, 489–497. [CrossRef] [PubMed]

36. Gupta, A.; Singh, V.K.; Qazi, G.N.; Kumar, A. G-Luconobacter oxydans: Its biotechnological applications. *J. Mol. Microbiol. Biotechnol.* 2001, 3, 445–456. Available online: https://www.ncbi.nlm.nih.gov/pubmed/11361077 (accessed on 10 August 2018). [PubMed]
37. La China, S.; Zanichelli, G.; De Vero, L.; Gullo, M. Oxidative fermentations and exopolysaccharides production by acetic acid bacteria: A mini review. Biotechnol. Lett. 2018, 40, 1–7. [CrossRef] [PubMed]

38. Erni, B.; Siebold, C.; Christen, S.; Srinivas, A.; Oberholzer, A.; Baumann, U. Small substrate, big surprise: Fold, function and phylogeny of dihydroxyacetone kinases. Cell. Mol. Life Sci. 2006, 63, 890–900. [CrossRef]

39. Dikshit, P.K.; Kharmawlong, G.J.; Moholkar, V.S. Investigations in sonication-induced intensification of crude g-Lycerol fermentation to dihydroxyacetone by free and immobilized G-Luconobacter oxydans. Bioreour. Technol. 2018, 256, 302–311. [CrossRef]

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