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# Production and characterization of *Escherichia coli* glycerol dehydrogenase as a tool for glycerol recycling

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ABSTRACT

NAD$^+$-dependent glycerol (Gro) dehydrogenase (GroDHase) catalyzes the conversion of Gro into dihydroxyacetone (DHA), the first step for fermentative Gro metabolism in *Escherichia coli*. In this work, we cloned the *gldA* gene that codes for the *E. coli* GroDHase and homologously expressed, purified, and kinetically characterized the recombinant protein. To achieve this, the enzyme was over-produced using Gro supplemented growth medium and lactose as the inducer. The enzyme was highly purified using either pseudo-affinity chromatography or a simple heat-shock treatment, which is potentially valuable for industrial production of GroDHase. We detected efficient oxidation of Gro derived from biodiesel production to DHA by gas chromatography. The results presented in this work support recombinant GroDHase production in a biorefinery setting as a relevant tool for converting Gro into DHA for future biotechnological applications.



## 1. Introduction

Glycerol (Gro) dehydrogenase (GroDHase, EC 1.1.1.6) is a widely distributed enzyme amongst bacteria and fungi that belongs to the medium-chain dehydrogenase/reductase (MDRase) superfamily [1] and catalyzes the reversible NAD$^+$-dependent oxidation of Gro into dihydroxyacetone (DHA) [2]. GroDHase is mainly involved in Gro utilization as a carbon and energy source. In *Escherichia coli*, the enzyme (*Eco*GroDHase) catalyzes the first step in fermentative Gro metabolism to produce DHA. This product is then phosphorylated by a specific kinase (EC 2.7.1.29) and enters into the glycolytic pathway [3]. *Eco*GroDHase is encoded by the *gldA* gene (1104 bp), is a polypeptide of ~40 kDa, and in solution it forms homodimeric and homooctameric structures [4]. Nearly 60 years ago, Asnis and Brodie [5] purified this enzyme directly from the bacterium using a simple heat-shock protocol. In the last decade, the *E. coli gldA* gene was recombinantly expressed in order to improve either the capacity of redox cofactor regeneration in reverse micelles [6] or to reach a superior electrocatalytic performance of *E. coli* cells [7]. More recently [8], overexpression of GroDHase and DHA kinase in *E. coli* cells increased fermentative production of ethanol from crude Gro. Despite these relevant applications, the kinetic and biochemical properties of the purified recombinant enzyme have been scarcely explored.

Worldwide petroleum reserves are limited and petroleum-derived fuels contribute to the greenhouse gas effect by increasing atmospheric $CO_2$ levels. Thus, it is imperative to find new processes for fuel production from raw materials. Bioethanol and biodiesel production is attractive because it is eco-friendly, its products are biodegradable and renewable, and it gives us the potential to manage the $CO_2$ balance in the atmosphere [9,10]. Biodiesel, the alternative to petroleum-derived diesel, is produced from vegetable oils or animal fats through trans-esterification with ethanol or methanol (alcoholysis) [9]. This process generates ethyl or methyl esters of fatty acids and ~10% (v/v) of Gro as a major byproduct [11]. As biodiesel production increased, Gro production increased as well and currently exceeds its industrial demands. The cost of Gro has decreased because it is being generated in huge quantities from the biodiesel industry and it is not easily disposed of due to its environmental toxicity. Nonetheless, Gro constitutes an attractive and competitive carbon source because the reduced state of its carbon atoms has potential for the conversion into other useful chemicals such as 1,3-propanediol, DHA, ethanol, and organic acids (like succinic, propionic, and citric acids) (Fig. 1) [12,13]. These

*Abbreviations:* DHA, dihydroxyacetone; *Eco*, *Escherichia coli*; FID, flame ionization detector; GC, gas chromatography; Gro, glycerol; GroDHase, Gro dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside.

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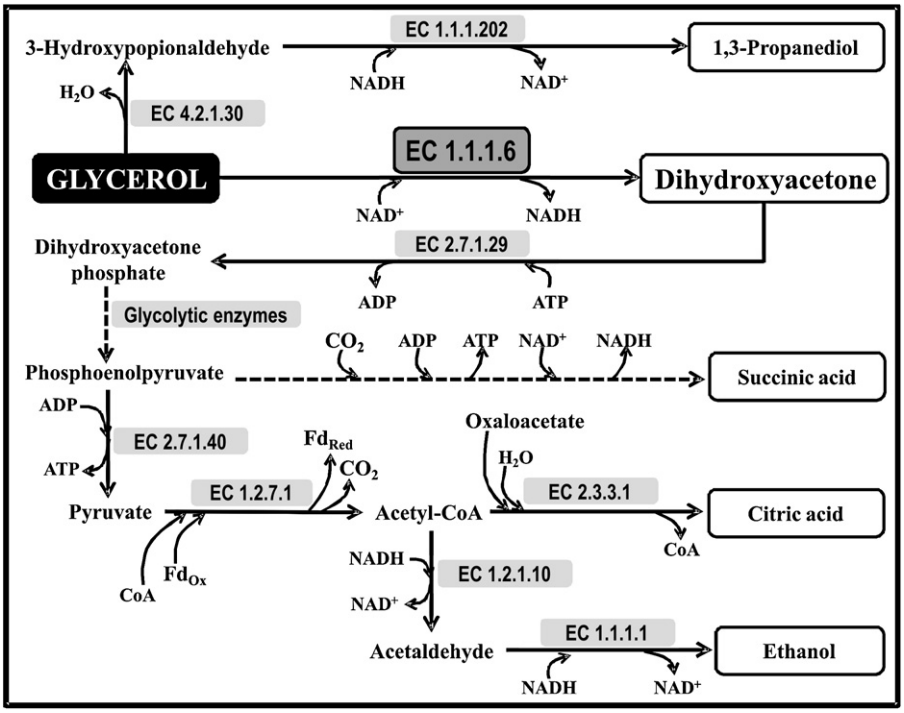


**Fig. 1.** Schematic diagram of the enzymes (EC number) and pathways involved in the biochemical transformation of Gro. EC 1.1.1.1, alcohol dehydrogenase; EC 1.1.1.6, GroDHase; EC 1.1.1.202, 1,3-propanediol oxidorreductase; EC 1.2.1.10, acetaldehyde dehydrogenase; EC 1.2.7.1, pyruvate synthase; EC 2.3.3.1, citrate synthase; EC 2.7.1.29, DHA kinase; EC 2.7.1.40, pyruvate kinase; EC 4.2.1.30, Gro dehydratase; $Fd_{ox}$ and $Fd_{red}$, oxidized and reduced ferredoxin, respectively. Dashed arrows mean that more than one enzyme is involved in the conversion step.

compounds are the main precursors for economically valuable products in organic synthesis, biocatalysis, and the cosmetics industry [14]. This potential use of Gro in biotechnology establishes the need for developing affordable tools that will improve and help implement effective biorefinery recycling strategies [15].

Recently, several authors highlighted the potential of enzymes to be used as efficient, economical, and environmentally friendly biocatalysts [16,17], particularly in the biodiesel industry [18]. For instance, Luna et al. [19] successfully used covalently immobilized pig pancreatic lipase for obtaining a second generation biodiesel. In this context, we report the molecular cloning and homologous recombinant over-expression of the gene encoding for *Eco*GroDHase. We optimized the production of a highly pure state of the enzyme and determined its kinetic properties and capacity for using Gro derived from the biodiesel industry. Results are analyzed in the framework of the importance of the potential use of the enzyme in the biorefinery processes to convert the byproduct Gro into more valuable chemicals, primarily DHA.

## 2. Materials and methods

### 2.1. Bacterial strains and chemicals

*E. coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA) were used for cloning procedures and plasmid maintenance. *E. coli* BL21 (DE3) cells (Invitrogen) were used for protein expression. Enzymes used for molecular biology procedures were from Fermentas (Glen Burnie, MD, USA). Gro and 1-butanol standards were acquired from Merck (Darmstadt, Germany) and BDH Laboratory Reagents (Poole Dorset, UK), respectively. DHA and NAD(H) were from Sigma (St. Louis, MO, USA). All the other chemicals were of the highest quality available.

### 2.2. Molecular cloning and subcloning of the E. coli gldA gene

The *gldA* gene was amplified by PCR from genomic DNA of the *E. coli* JM109 strain (New England BioLabs, Ipswich, MA, USA) using the specific oligonucleotides: 5′-CATATGGACCGCATTATTCAATCACCAGG-3′ (forward, *Nde*I site is underlined) and 5′-CTCGAGTTCCCACTCTTGCAGGAAACGCTG-3′ (reverse, *Xho*I site is underlined). Amplification conditions were: 95 °C for 5 min; 29 cycles at 94 °C 1 min, 55 °C for 49 s, and 72 °C for 2 min; and a final step of 72 °C for 10 min. The amplified gene (*EcogldA*) was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and *E. coli* TOP10 cells were transformed with this construct. The identity of the cloned gene was confirmed by DNA sequencing (Macrogen, Seoul, Korea). The *EcogldA* gene was subcloned into the pET22b (Merck, Darmstadt, Germany) plasmid, obtaining the construct [pET22b/*EcogldA*] that expresses a recombinant *Eco*GroDHase with a $His_6$-tag at the C-term with no addition of extra amino acid.

### 2.3. Protein expression, culture conditions and media

Unless otherwise stated, *E. coli* BL21 (DE3) cells transformed with [pET22b/*EcogldA*] were grown at 30 °C with shaking at 200 rpm in LB medium supplemented with 100 μg/ml ampicillin. Over-expression of the recombinant enzyme was induced at $OD_{600}$ of 0.4–0.6 with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) or 2.5 g/l lactose for 5 h. Cell cultures were centrifuged at 5000 × *g* for 10 min and cells were stored at −20 °C until use. For optimization of recombinant GroDHase expression, several media were assayed under different concentrations of lactose, temperature and induction time. The different media used were: LB (5 g/l yeast extract, 10 g/l peptone, 10 g/l NaCl); GB (30 g/l yeast extract, 27.5 g/l peptone, 1 g/l NaCl, 1.4 g/l Gro); and A1 (43 g/l yeast extract, 20 g/l peptone, 18 g/l NaCl, 0.3 g/l Gro).

### 2.4. Enzyme purification

Harvested cells were resuspended in *Buffer A* (25 mM Tris–HCl pH 7.5, 300 mM NaCl, 10 mM imidazole), disrupted by sonication and centrifuged to separate soluble and insoluble fractions. The soluble sample was loaded onto a metal affinity resin column (HisTrap, GE Healthcare Life Sciences, Piscataway, NJ, USA) charged with $Ni^{2+}$ and pre-equilibrated with *Buffer A*. After extensive washing, the column was eluted with a linear gradient of 10 to 300 mM imidazole in *Buffer A*. As an alternative to column chromatography, the recombinant *Eco*GroDHase was purified by heat-shock treatment. Briefly, the soluble fraction was heated for 10 min at 60 °C followed by incubation on an ice-cold bath [5]. After 30 min, precipitated proteins were separated by centrifuging 10 min at 4 °C and 30,000 × *g*. Active fractions were pooled, fractionated and stored at −80 °C until use. Under these conditions the enzyme remained fully active for at least three months.

### 2.5. Protein methods

Protein concentration was determined by the Bradford method [20], with BSA as a standard. Protein electrophoresis under denaturing conditions (SDS-PAGE) was carried out on discontinuous 12% polyacrylamide gels as described by Laemmli [21]. Molecular mass standards for SDS-PAGE (GE Healthcare Life Sciences) included

phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (30 kDa).

### 2.6. Native molecular mass determination

To determine the molecular mass of the native recombinant protein, the purified enzyme was subjected to gel filtration chromatography. Typically, 50 μl of sample were loaded onto a 4 ml Superdex 200 HR resin contained in a Tricorn 5/200 column (GE Healthcare Life Sciences) previously equilibrated with *Buffer G* (25 mM Tris–HCl pH 8.0, 100 mM NaCl). The molecular mass was calculated using the calibration curve constructed with standard proteins (GE Healthcare Life Sciences), including thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (44 kDa). The column void volume was determined using Blue Dextran loading solution (Promega).

### 2.7. Activity assay

GroDHase activity was assayed as described previously [5], with minor modifications. In the direction of Gro oxidation, the standard medium contained 100 mM CAPS-NaOH pH 10.5, 5 mM $NAD^+$, and 500 mM Gro. In the direction of DHA reduction, the standard medium was composed of 100 mM MES-NaOH pH 6.5, 0.2 mM NADH, and 200 mM DHA. Alternatively, 50 mM phosphate/carbonate pH 10.3 (Gro oxidation) and 6.5 (DHA reduction) were used as buffers. Both assays were initiated by the addition of an appropriate amount of enzyme in a final volume of 50 μl. Reactions were performed at 30 °C and the reduction/oxidation of $NAD^+$/NADH was followed at 340 nm using a Multiskan Ascent 384-microplate reader (Thermo Electron Corporation, Waltham, MA, USA). One unit of enzyme activity (U) is defined as the amount of enzyme catalyzing the formation of 1 μmol of product (NADH or $NAD^+$) in 1 min under the specified assay conditions.

### 2.8. Kinetic studies

Experimental data of enzyme activity were plotted against substrate concentration and fitted to the Hill equation: $v = {V_{max}}^{nH} \times S^{nH}/({S_{0.5}}^{nH} + S^{nH})$, using the program Origin 7.0 (OriginLab Corporation). The term $S_{0.5}$ is the concentration of substrate (S) producing 50% of the maximal activity ($V_{max}$), and $n_H$ is the Hill coefficient. For activation studies, data were adjusted to a modified Hill equation: $v = (V_{max} - v_0)^{nH} \times A^{nH}/({A_{0.5}}^{nH} + A^{nH})$, where $v_0$ is the initial velocity, and $A_{0.5}$ is the concentration of activator (A) producing 50% of the maximal activation ($V_{max}$). Kinetic constants are means of at least two independent sets of data, which were reproducible within ±10%.

### 2.9. Enzyme stability and activity as a function of pH and temperature

The behavior of *Eco*GroDHase was evaluated at different values of pH and temperature. Data of enzyme activity measured from 4 °C to 75 °C were fitted to the Arrhenius plot [22] to calculate the activation energy ($E_a$). Activity of the recombinant protein in both directions of the reaction, Gro oxidation and DHA reduction, was measured at pH values ranging from 3 to 12, using 50 mM phosphate/carbonate adjusted to the desired pH. The stability of *Eco*GroDHase was evaluated at 30 °C and 60 °C using 50 mM phosphate/carbonate pH 8.0 and 10.3. In both assays, aliquots of the mixture were taken at regular time intervals and used to evaluate enzyme activity in the direction of Gro oxidation using 50 mM phosphate/carbonate pH 10.3, as previously described in Section 2.7.

### 2.10. Gas chromatography

Reactant and reaction products of *Eco*GroDHase were analyzed by gas chromatography (GC) using a Shimadzu GC-2014 chromatograph provided with a flame ionization detector (FID), and a DB-20 column of 25 m length and 0.33 mm internal diameter. Analyses were made using the following temperature program: 3 min at 150 °C, heating up to 210 °C at 20 °C/min, and maintaining this temperature during 25 min. Because some of the acids that can produce the chemical reaction of Gro oxidation are not detected by FID, the internal standard method was used to quantify Gro and DHA. Calibration curves for Gro and DHA were made using 1-butanol as the standard. Then, samples were analyzed adding 1-butanol as the internal standard, determining Gro conversion into DHA.

## 3. Results and discussion

### 3.1. Expression and purification of EcoGroDHase

Although the *E. coli gldA* gene was successfully expressed as a recombinant polypeptide in its homologous host to improve the bioconversion capacity of bacterial cells [7,8], quantification and purification of the recombinant enzyme, as well as a complete characterization of its kinetic properties are still lacking. It has been shown that optimization of physicochemical parameters (like medium composition, inducer, induction conditions, pH, time, and temperature) plays an important role in enzyme production [23]. We considered various alternative expression procedures to maximize the yield of the His-tagged *Eco*GroDHase using *E. coli* BL21 (DE3) cells transformed with the [pET22b/*EcogldA*] construct. First, we evaluated GB and A1 as alternative culture media to standard LB (Fig. 2A), both containing higher amounts of nutrients (yeast extract and peptone), supplemented with Gro and with different NaCl concentrations (see Section 2 for further details). The A1 medium has advantages over LB and has been used previously in our laboratory for producing other recombinant proteins [24]. However, A1 was not optimal for the expression of *Eco*GroDHase, as it rendered a 4-fold lower yield than LB (Fig. 2A). Conversely, the use of GB medium provided the highest recovery of recombinant *Eco*GroDHase in crude extracts, with values nearly 3- and 10-fold higher than LB and A1 media, respectively (Fig. 2A).

Based on these results, we selected GB as an effective culture medium for use with the *E. coli* BL21 (DE3) [pET22b/*EcogldA*] expression system. To further improve the conditions of protein expression, we evaluated the use of an alternative inducer as well as time and temperature conditions after induction (Fig. 2B–D). For instance, replacement of IPTG by lactose is a valuable alternative for industrial protein production because it is an economical substitute for induction of recombinant proteins. We found that IPTG could be replaced by lactose for successful expression of *Eco*GroDHase, with a maximum over-expression at 20 g/l of the disaccharide (Fig. 2B). Interestingly, the recombinant enzyme was similarly overproduced when protein expression was triggered by addition of 0.5 mM IPTG (Fig. 2C, lane 2) or 2.5 g/l lactose (Fig. 2C, lane 3). Thereafter, we analyzed *Eco*GroDHase expression as a function of temperature and induction time (Fig. 2D). Following optimization of media and induction conditions, and considering economic effectiveness, we found that higher yields of *Eco*GroDHase can be achieved with our recombinant expression system using GB medium and inducing with 2.5 g/l lactose at 37 °C for 5 h. Compared to the initial conditions (LB medium and induction with 0.5 mM IPTG at 30 °C for 5 h), the yield of *Eco*GroDHase was increased 10-fold.

Two different and highly effective protocols were developed and compared for the purification of *Eco*GroDHase (Fig. 3). The His-tagged enzyme was purified to near homogeneity through immobilized metal affinity chromatography (IMAC) (Fig. 3, lane 2), and almost the same purity was achieved using a simple heat-shock treatment (Fig. 3, lane 3). Interestingly, the heat-treated enzyme retained more than 90% of its catalytic capacity and reached a specific activity of 25 U/mg in the direction of Gro oxidation, a value similar to that obtained with the IMAC purified enzyme. Fig. 3 also illustrates that the major protein band obtained after purification of recombinant *Eco*GroDHase is a polypeptide of ~40 kDa, which is consistent with the mass of 39.8 kDa calculated from the enzyme's primary sequence. The purified enzyme was loaded onto a Superdex 200 column and eluted as a protein of 320 kDa (data not shown), indicating that the native structure of *Eco*GroDHase is an octamer. This result is in good agreement with data reported for GroDHases purified from their natural sources, including those from *E. coli* [4], *Cellulomonas* spp. [25,26], and *Schizosaccharomyces pombe* [27].

Our system and procedure developed for the production and purification of the recombinant *Eco*GroDHase has significant advantages for obtaining an enzyme to be used in industrial processes. The high protein expression achieved with lactose is particularly relevant, as the natural disaccharide is a byproduct of milk industry. This constitutes a relatively inexpensive and non-toxic inducer that is by far an economically convenient alternative to IPTG [28]. In the same way, the possibility to employ heat-treatment as a successful purification procedure makes it highly

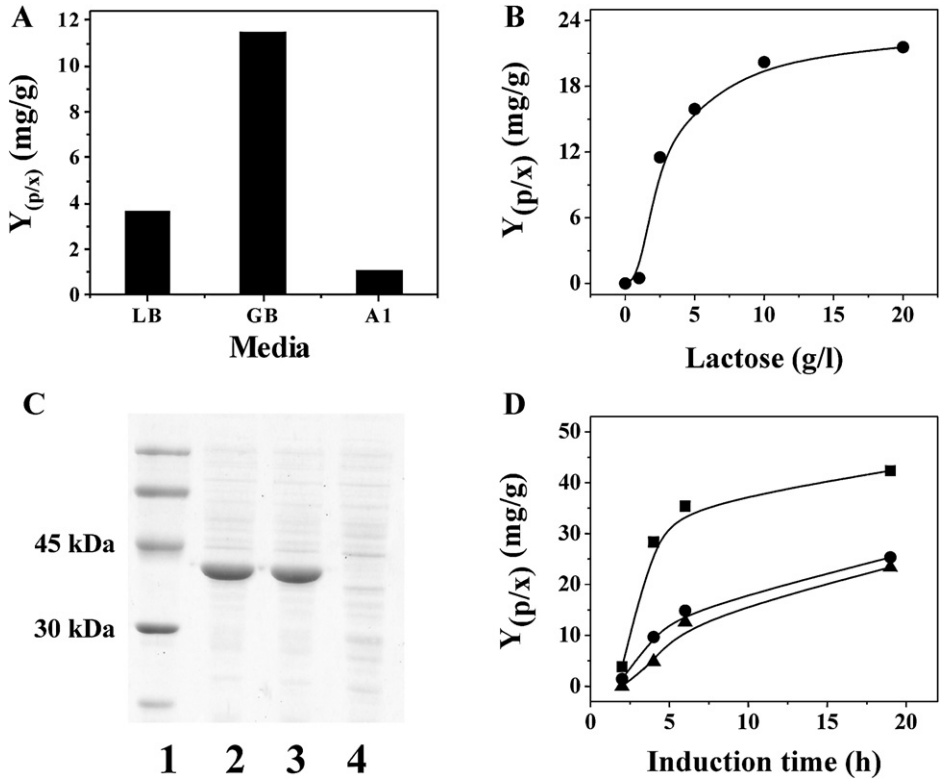


**Fig. 2.** Optimization of recombinant *Eco*GroDHase expression in different culture media and induction conditions with lactose. (A) Yield [mg enzyme/g wet biomass] ($Y_{p/x}$) of recombinant GroDHase achieved in cultures of *E. coli* BL21 (DE3) [pET22b/*EcogldA*] with different culture media (LB, GB or A1) induced with IPTG 0.5 mM for 5 h. (B) Yield of *Eco*GroDHase from cells grown in GB medium induced with increasing lactose concentrations. (C) Analysis of *Eco*GroDHase expression by 12% SDS-PAGE followed by Coomassie Blue staining. Molecular mass markers (lane 1); soluble fraction from *E. coli* BL21 (DE3) [pET22b/*EcogldA*] after 5 h induction with 0.5 mM IPTG (lane 2); or 2.5 g/l lactose (lane 3); soluble fraction from *E. coli* BL21 (DE3) [pET22b] after induction with 0.5 mM IPTG (lane 4). (D) Yield of *Eco*GroDHase as a function of induction time. Transformed cells were grown in GB medium, induced with 2.5 g/l lactose at 37 °C (■), 30 °C (●), 20 °C (▲), and harvested at different times.

feasible for large-scale production, practically simple and remarkably low-priced. In addition, increased enzyme production when recombinant *E. coli* cells were grown in a culture medium supplemented with Gro reinforces the utility of the procedure.

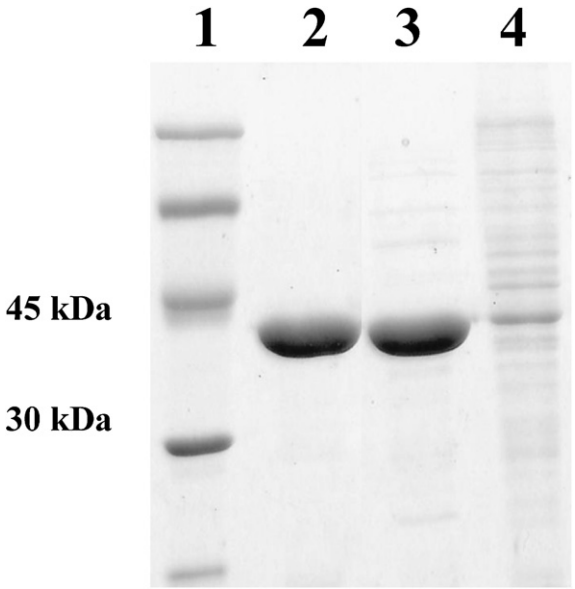


**Fig. 3.** Purification of recombinant *Eco*GroDHase analyzed by 12% SDS-PAGE followed by Coomassie Blue staining. Molecular mass markers (lane 1); *Eco*GroDHase purified by IMAC (lane 2); soluble (lane 3); and insoluble (lane 4) fractions obtained after heat-shock treatment of crude extracts containing recombinant *Eco*GroDHase.

### 3.2. Kinetic characterization and enzyme stability

Although *Eco*GroDHase has been previously purified [4,5], its kinetic properties were poorly analyzed. It is highly relevant to establish the most convenient pH and temperature conditions if the intention is to use this enzyme in industrial processes. We first studied the activity of the recombinant enzyme in a wide pH range using phosphate/carbonate (Fig. 4A). As it has been reported for the enzyme purified from *E. coli* [4] and other bacteria [26,27,29], activity of recombinant *Eco*GroDHase in the direction of Gro oxidation was higher at pH values between 10.0 and 11.0, whereas the highest activity in the direction of DHA reduction was observed at a pH ranging from 7.0 to 8.0 (Fig. 4A). It is clear from Fig. 4A that at the respective optimum pH in one sense the opposite reaction is more than one order of magnitude lower.

Next, we analyzed the influence of temperature on the activity and stability of *Eco*GroDHase (Fig. 4B and C). The activity of *Eco*GroDHase in the direction of Gro oxidation showed a maximum at 60 °C and further temperature increases caused a loss of enzyme activity (Fig. 4B). With this data we constructed the Arrhenius plot (Fig. 4B, inset) to calculate the $E_a$ for *Eco*GroDHase, which was 50.3 kJ/mol. As previously shown [5], *Eco*GroDHase is relatively stable at 60 °C, which was conveniently utilized for enzyme purification. However, we found that thermal stability of the enzyme is critically affected by the pH. Fig. 4C shows that *Eco*GroDHase was stable when incubated at 60 °C and pH 8.0 and quickly inactivated at pH 10.3. Conversely, when incubated at 30 °C the enzyme was relatively stable at both pH values, losing only 20% and 40% of activity after 4 h incubation at pH 8.0 or 10.3, respectively (Fig. 4C). These results are critical for designing heat-shock treatment

protein purification procedures and maintenance protocols, as well as to establish conditions to measure enzyme activity.

It has been reported that GroDHases can be activated by monovalent cations such as $NH_4^+$ and $K^+$ [4,26,27,29], a characteristic that was also found for *Eco*GroDHase. Fig. 5 shows that in the Gro oxidation direction the recombinant enzyme is activated nearly 4-fold by $NH_4Cl$, whereas it is activated only 2-fold by KCl. In the DHA reduction direction, almost 2-fold activation was observed for either $NH_4Cl$ or KCl. In the direction of Gro oxidation, the $A_{0.5}$ for $NH_4Cl$ was $1.14 \pm 0.08$ mM ($n_H$ of 1.4), while in the direction of DHA reduction the $A_{0.5}$ was $0.67 \pm 0.09$ mM ($n_H$ of 1.0) (values determined from Fig. 5). When *Eco*GroDHase was assayed in the presence of 30 mM $NH_4Cl$ or 20 mM KCl, no significant changes were observed in the profiles of activity/stability versus temperature or pH (data not shown).

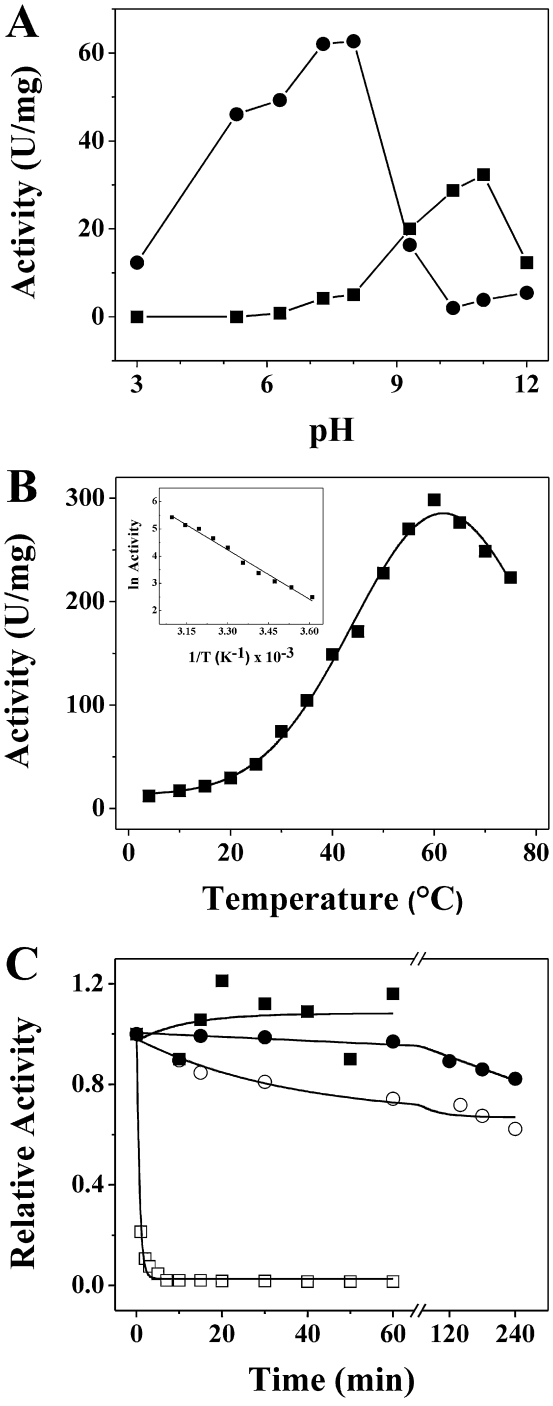


**Fig. 4.** Analysis of *Eco*GroDHase activity and stability as a function of pH and temperature. (A) Assays were performed in the direction of Gro oxidation (■) or DHA reduction (●) in phosphate/carbonate adjusted to the desired pH. (B) Activity of the recombinant enzyme was determined in the direction of Gro oxidation in phosphate/carbonate pH 10.3 at increasing temperatures. The inset shows the Arrhenius plot used to determine the $E_a$ of *Eco*GroDHase. (C) The enzyme was incubated at 30 (circles) and 60 °C (squares) in phosphate/carbonate pH 8.0 (filled symbols) or pH 10.3 (open symbols). Assays were performed as described under Section 2.

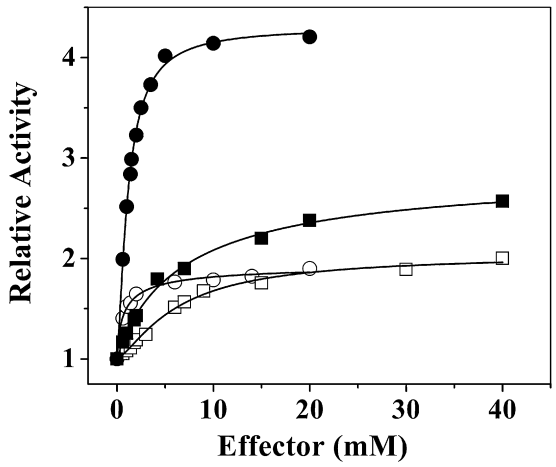


**Fig. 5.** Activation of *Eco*GroDHase by monovalent cations. Activity of the recombinant enzyme was measured with increasing concentrations of $NH_4Cl$ (circles) and KCl (squares). Assays were performed in the direction of Gro oxidation (filled symbols) or DHA reduction (open symbols) with CAPS-NaOH (pH 10.5) and MES-NaOH (pH 6.5), respectively.

Subsequently, we determined the kinetic parameters for substrates in both directions of the reaction at the respective pH values for maximal activity, in the absence of effectors or in the presence of 30 mM $NH_4Cl$ or 20 mM KCl. Saturation curves for the substrates of *Eco*GroDHase were fitted to the Hill equation (see Fig. S1), as described under Section 2. Kinetic parameters for *Eco*GroDHase reported in Table 1 are similar to those described for GroDHase from *Aerobacter aerogenes* [29]. Noteworthy is the 4-fold increase in $V_{max}$ produced by $NH_4Cl$ in the direction of Gro oxidation (Fig. 5 and Table 1). This is a relatively inexpensive salt and its addition to the reaction could efficiently increase the conversion of Gro into DHA. Also striking is the 70-fold reduction in the $S_{0.5}$ for DHA and the 2-fold increase in the $V_{max}$ when 30 mM $NH_4Cl$ was added to the reaction mixture (Table 1). These results are in good agreement with those reported for the enzyme purified from *A. aerogenes* [29]. The addition of 20 mM KCl produced a similar decrease in the $S_{0.5}$ for DHA but the $V_{max}$ did not change significantly (Table 1).

**Table 1**
Kinetic parameters for *Eco*GroDHase obtained with commercial Gro. Data were calculated in absence and presence of 30 mM $NH_4Cl$ or 20 mM KCl. Reactions were performed in CAPS-NaOH pH 10.5 (Gro oxidation) and MES-NaOH pH 6.5 (DHA reduction), as described under Section 2.

| Substrate | Effector | $S_{0.5}$ (mM) | $V_{max}$ (U/mg) | $n_H$ |
|---|---|---|---|---|
| Gro | None | 76 ± 6 | 33 ± 1 | 0.9 ± 0.1 |
| | $NH_4Cl$ | 81 ± 24 | 142 ± 7 | 0.9 ± 0.1 |
| | KCl | 47 ± 3 | 72 ± 5 | 1.0 ± 0.1 |
| $NAD^+$ | None | 0.81 ± 0.09 | 33 ± 1 | 0.9 ± 0.1 |
| | $NH_4Cl$ | 1.0 ± 0.1 | 142 ± 7 | 1.0 ± 0.1 |
| | KCl | 3.2 ± 0.5 | 72 ± 5 | 1.0 ± 0.1 |
| DHA | None | 15 ± 3 | 58 ± 4 | 0.7 ± 0.1 |
| | $NH_4Cl$ | 0.22 ± 0.03 | 125 ± 4 | 0.9 ± 0.1 |
| | KCl | 0.24 ± 0.02 | 47 ± 2 | 1.0 ± 0.1 |
| NADH | None | 0.05 ± 0.01 | 58 ± 4 | 0.9 ± 0.1 |
| | $NH_4Cl$ | 0.08 ± 0.01 | 125 ± 4 | 0.9 ± 0.1 |
| | KCl | 0.07 ± 0.01 | 47 ± 2 | 1.8 ± 0.1 |

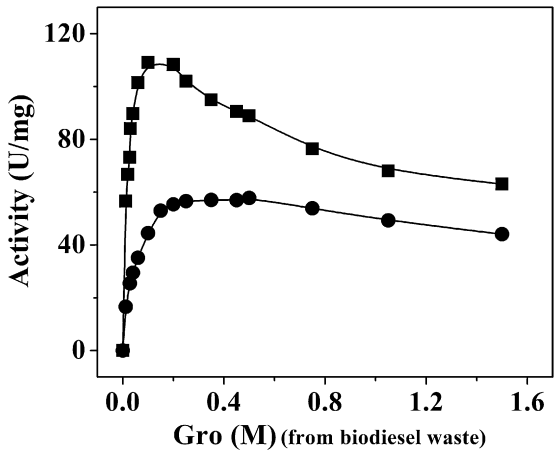


**Fig. 6.** Gro from biodiesel waste is efficiently used by recombinant *Eco*GroDHase. Assays were performed in the absence (●) or presence (■) of 30 mM $NH_4Cl$ in phosphate/carbonate pH 10.3, as described under Section 2.

It has been previously described that DHA inhibits Gro oxidation [26,29,30]. Thus, we conducted product inhibition studies for *Eco*GroDHase in the presence of 30 mM $NH_4Cl$. We found that the inhibitory effect of DHA was more significant when analyzed with variable concentrations of Gro rather than $NAD^+$. Curves of Gro with varying concentrations of DHA at 5 mM $NAD^+$ showed a competitive inhibition pattern (Fig. S2). According to Cornish-Bowden [31], a $K_{ic}$ of 0.31 mM was calculated for DHA from the secondary plot of the slopes ($K_m^{app}/V_{max}^{app}$) versus DHA concentration (Fig. S2, inset). This result is consistent with those reported for GroDHase from *A. aerogenes* [29] and *Candida valida* [30], which were inhibited by DHA. Although this is a relatively small $K_{ic}$, it should be noted that the inhibition is competitive. If *Eco*GroDHase would be used in a biorefinery there are at least two different ways to overcame DHA inhibition: (i) Gro concentration should be always maintained high enough and (ii) design a continuous system to remove the product and maintaining it below an inhibitory level.

### 3.3. Kinetic characterization with biodiesel wasted Gro

To further explore the utility of *Eco*GroDHase for industrial applications, we evaluated the ability of the enzyme to use Gro from biodiesel waste as substrate. The biodiesel waste was generated by an industrial chemical process in a plant utilizing triglycerides from *Glycine max* (producing 30,000 tons of biodiesel per year). We determined the concentration of Gro in the biodiesel waste solution by GC as 57.5% (w/v), which is equivalent to 7.8 M. This solution also contained water (30%), methanol (2.5%), and other organic compounds commonly found in biodiesel waste [32]. *Eco*GroDHase effectively used Gro from biodiesel waste as a substrate (Fig. 6), and the curves of activity versus Gro concentration were similar to those obtained with commercial Gro (data not shown). We also determined the kinetic parameters of recombinant *Eco*GroDHase using Gro from biodiesel waste. Table 2 shows that in phosphate/carbonate buffer, which is considerably cheaper than other commercially available buffers, the calculated parameters are similar to those obtained using CAPS-NaOH and MES-NaOH (see Table 1). Interestingly, a considerably higher $V_{max}$ was observed with Gro from biodiesel waste when the activity was measured without addition of effectors (Table 2). This behavior could be due to the high ionic strength present in Gro from biodiesel waste, which could contain a high concentration of KCl, commonly used as a catalyst for transesterification of triglycerides with methanol [9].

**Table 2**
Kinetic parameters for *Eco*GroDHase obtained with Gro from biodiesel waste. Reactions were performed in phosphate/carbonate buffer pH 10.3, in absence and presence of 30 mM $NH_4Cl$ or 20 mM KCl, as described under Section 2.

| Substrate | Effector | $S_{0.5}$ (mM) | $V_{max}$ (U/mg) | $n_H$ |
|---|---|---|---|---|
| Gro | None | 46 ± 6 | 65 ± 4 | 1.0 ± 0.1 |
| | $NH_4Cl$ | 13 ± 2 | 111 ± 6 | 1.1 ± 0.1 |
| | KCl | 24 ± 2 | 63 ± 3 | 1.0 ± 0.1 |
| $NAD^+$ | None | 0.8 ± 0.1 | 65 ± 4 | 1.0 ± 0.1 |
| | $NH_4Cl$ | 1.4 ± 0.2 | 111 ± 6 | 0.9 ± 0.1 |
| | KCl | 1.1 ± 0.1 | 63 ± 3 | 1.0 ± 0.1 |

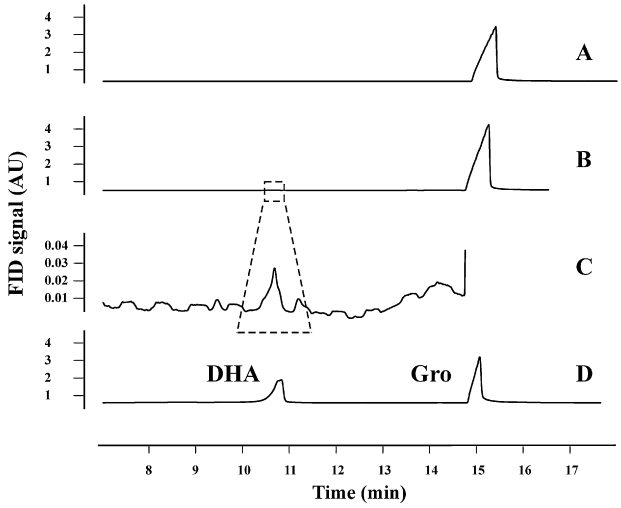


**Fig. 7.** *Eco*GroDHase converts Gro into DHA. The product of the reaction catalyzed by the recombinant enzyme was analyzed by GC, as described under Section 2. (A) Chromatogram obtained for Gro from biodiesel waste. (B) Chromatogram obtained for the product of the reaction catalyzed by *Eco*GroDHase using Gro from biodiesel waste as a substrate. (C) The same chromatogram shown in (B) was magnified 100-fold. (D) Control chromatogram obtained for a mixture of commercial DHA and Gro.

To determine if *Eco*GroDHase produced DHA by bioconversion of Gro from biodiesel waste, we analyzed the product of the reaction by GC. The enzymatic reaction was performed in phosphate/carbonate pH 10.3 with 0.78 M Gro from biodiesel waste (commercial Gro was used as control in a separate reaction), 20 mM $NAD^+$, and 0.4 mg/ml *Eco*GroDHase at 30 °C. The reaction progress was followed by measuring the absorbance of NADH at 340 nm until it was constant. Next, the enzyme was removed by using a Microcon-30 kDa centrifugal filter unit with Ultracel-30 membrane (Millipore, Billerica, MA, USA) and the filtrate was conveniently diluted and assayed by GC. The product was DHA and the estimated conversion of Gro into DHA was calculated as 2.2% (Fig. 7), which is equivalent to 17 mM. This value was calculated using calibration curves for Gro and DHA made using 1-butanol as the internal standard. This quantity of DHA produced is reasonable because it stoichiometrically agrees with the expected value since the initial concentration of the limiting reactant ($NAD^+$) was 20 mM. We should consider using coupled assays in the future to enzymatically regenerate $NAD^+$ since it is one of the most expensive reagents to enzymatically convert Gro into DHA.

## 4. Conclusions

Production of biodiesel involves generation of relatively huge amounts of Gro as a byproduct, which makes critical developing tools for its recycling. Gro has a great potential to produce other chemicals that could be used in different industrial processes. We found that recombinant *Eco*GroDHase is a promising biocatalyst to

convert Gro from biodiesel waste into DHA as a biorefinery strategy. In this work we report a thorough kinetic analysis of recombinant EcoGroDHase. Our study includes convenient production of the recombinant enzyme at a relatively low cost. The growth of transformed cells producing *Eco*GroDHase was efficiently achieved with a medium containing Gro, thus representing an extra use of the byproduct. Also, induction of recombinant protein expression was optimized with lactose, an inexpensive chemical. Development of biorefineries is a key step to reach integrated production of biofuels, chemicals, and materials, making the process sustainable, environment-friendly and also economically more feasible.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2013.01.011.

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