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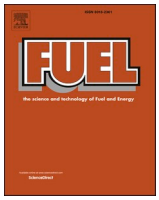

Full Length Article

Elimination of biodiesel contaminants by recombinant glucoside hydrolase produced from crude glycerol

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ABSTRACT

This work presents the direct crude glycerol use by high cell-density fermentation of *Escherichia coli* expressing recombinant glycoside hydrolase that cleaves steryl glucosides, one of the main biodiesel contaminants. Optimized fermentation with crude glycerol reached the biomass concentration of 30 g/l with the 0.285 U/mg$_{DCW}$ specific activity, which is comparable with the pure glycerol production. The expression of the inclusion bodies was eliminated by the increase of the induction temperature to 40 °C. The produced enzyme was able to hydrolyze 300 ppm of steryl glucosides, the major biodiesel contaminants, and reached 100 % conversion. This model approach of waste carbon recycling (crude glycerol) for biorefinery-beneficial recombinant enzyme production is in line with the sustainable circular bioeconomy concept.

1. Introduction

Due to the persisting depletion of fossil fuels as the main source of energy and the imminent climate change crisis, renewable resources and a sustainable bioeconomy concept are essential. Generating up to 28 % of the annual energy consumption and a significant increase in greenhouse gas emissions worldwide, the transportation sector has become one of the major environmental threats [1,2]. To prevent an energy crisis, authorities are forced to set directives for the production and implementation of an eco-friendly, renewable, and biodegradable alternative to petroleum-based fuels [3]. Biofuel manufacturing has been extended globally since 2005 and is expected to reach 41.4 million m3 annually until 2025 [4]. Biodiesel is currently-one of the leading biofuels in the transport sector. There are several ways of biodiesel manufacturing, but the majority is produced by the transesterification of vegetable oils with alcohols, resulting in fatty acid methyl esters (FAME) and glycerol as a side product. Biodiesel production, however, faces certain drawbacks that restrain its application, such as higher viscosity, a higher density, a poor volatility, a poor oxidative stability, and a lower energy content than petroleum-based diesel [5,6]. These limitations can be mitigated by the blending strategy. Currently, the blending of biodiesel with petroleum-based diesel reaches 7 % (v/v) in most of the countries worldwide. However, since the European Union's Renewable Energy Directive sets a 32 % share of renewable energy on the total consumption of energy by 2030 [3], its implementation is expected to rise.

The biodiesel production outbreak brought a large volume of crude glycerol to the global market and a subsequent decrease in its price compared to other carbon sources. Therefore, the application of crude glycerol in biotechnology has become one of the most discussed recent topics [7]. Many technologies of crude glycerol valorization have been developed so far, most of them focused on bacterial fermentation and value-added metabolites production [8]. Glycerol is also a suitable substrate for a wide range of microbes that serve as recombinant protein expression hosts. However, there is a lack of studies discussing the use of crude glycerol for recombinant protein manufacturing. The major drawback is the presence of various impurities that inhibit microbial growth or enzyme expression and the strict purity standards of the pharmaceutical industry, where most of the recombinant biocatalysts are being applied [9]. Since crude glycerol purification is expensive [10,11], its direct use seems to be more advantageous.

One of the technological problems linked with biodiesel manufacturing is the formation of insoluble particles during its storage. These precipitates are composed mainly of steryl glucosides, which are often present in plant crude oils, which are raw materials for biodiesel production [12]. Due to their amphipathic nature, steryl glucosides are insoluble in water and only partially soluble in organic solvent mixtures and biodiesel. During long-term storage, especially at low temperatures,

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steryl glucosides aggregate and form insoluble particles that clog engine filters, resulting in serious damage [13]. The enzymatic hydrolysis of steryl glucosides has been discussed for several years and was proven to be a suitable alternative to the expensive glucoside removal methods that are currently employed [13]. Thermostable glycoside hydrolases (GlyHys) that withstand extreme temperatures, have recently been proven to directly hydrolyze steryl glucosides in biodiesel, and their application in biorefinery seems to be promising [14]. The combination of crude glycerol cost and its application for high value-added enzyme production, the production of recombinant biocatalyst, and its further massive application in biorefineries can enhance the feasibility of the sustainable circular bioeconomy.

Extremophilic microorganisms, including thermophilic bacteria withstand harsh environmental conditions (pH, temperature, pressure, organic solvents, high ionic strength) [15]; therefore, their proteome has become of great interest in the industrial biotechnology. Since the direct cultivation of native extremophiles is technically challenging [16], recombinant expression of genes from thermophilic microorganisms in *E. coli* is massively applied. However, the major drawback of *E. coli*-based expression systems is the production of enzymes in inclusion bodies [17,18]. Interestingly, unlike the recombinant expression of mesophilic genes, the formation of insoluble inclusion bodies of the thermophilic enzyme also occurs at lower induction temperatures [19].

This work focused on the recombinant production of thermophilic GlyHy, which exhibits activity towards steryl glucosides. The effective, high-temperature *E. coli* production of enzyme on crude glycerol was optimized, and the enzyme was applied for hydrolysis of the most abundant steryl glucosides in biodiesel.

2. Material and methods

2.1. Chemicals and media

Crude glycerol samples were kindly provided by Enviral, Inc. (Envien Group, Leopoldov, Slovakia). Kanamycin was purchased from Gibco (Life Technologies, Glasgow, UK), and isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). The steryl glucoside mixture was purchased from Matreya LLC (State College, PA, USA). Analytical standards of β-sitosteryl glucoside, β-sitosterol, and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA), and p-Nitrophenyl-β-D-glucopyranoside (pNPG) was obtained from Alfa Aesar (Haverhill, MA, USA). Heptane, hexane, and ethyl acetate were purchased from Merck (Darmstadt, Germany). Other chemicals were purchased from Sigma-Aldrich.

The M9 medium and the fermentation medium were prepared as described elsewhere [20]. The feeding medium contained 600 g/l glycerol, 36.6 g/l $NaH_2PO_4 \cdot 2H_2O$, 10 g/l $MgSO_4 \cdot 7H_2O$ (sterilized separately, added prior to use).

Partially purified crude glycerol (89,55 % purity; 5,2% ash) was prepared by processing the crude glycerol waste stream by neutralization and concentration by vacuum evaporation. Neutralized crude glycerol (52,66 % purity; 2,6% ash) was prepared by processing the crude glycerol waste stream by neutralization (detailed composition in [43].

2.2. Construction of expression vectors

Codon-optimized genes encoding GlyHy from *Thermococcus litoralis* (WP_004069094.1) were purchased from Generay Biotech Co., ltd. (Shanghai, China). Genes were inserted into a pET28b vector system [13], and the plasmid was transformed into competent cells of *Escherichia coli* BL21 (DE3).

2.3. High cell-density fermentation

Fermentations were performed in the DASBox® Mini Bioreactor System and in 1.3-l New Brunswick BioFlo 115 Bioreactors (Eppendorf, Hamburg, Germany), with an initial fermentation medium volume of 150 and 700 ml, respectively. A single colony of *E. coli* BL21 (DE3) carrying pET28b(+)GlyHy was transferred to 3 ml of M9 medium supplemented with kanamycin (50 μg/ml) and cultivated at 37 °C and 200 rpm overnight. The inoculum was prepared by inoculation of 100 ml of M9 medium supplemented with yeast extract (2 g/l) in a 500-ml shaking flask with 1 % (v/v) of the overnight culture and cultivated at 37 °C, 200 rpm, until the optical density at 600 nm (OD_{600}) reached 2.5. Fermentation was initiated by 1 % (v/v) inoculation, temperature 37 °C, pH 7, maintained by the addition of 26 % (v/v) ammonia and 3.1 M phosphoric acid. Air saturation was maintained at 30 % by 1 vvm aeration and an agitation speed cascade from 400 rpm to the maximum rpm. When OD_{600} reached 30 (150 ml-scale fermentation) or 50 (700 ml-scale fermentation), the temperature was set to 20, 30, 37, 40, 46, and 50 °C, and the expression of GlyHy was initiated by the addition of 0.5 mM IPTG. To determine OD_{600}, glycerol or acetic acid concentration, specific enzyme activity, and the level of GlyHy expression, samples of the fermentation medium were withdrawn regularly. The cells were harvested by centrifugation (16,639 × g, 6 °C, 30 min) and were either processed for the activity assay or analyzed by SDS-PAGE electrophoresis using 12 % (v/v) gel. The supernatant was transferred to a fresh tube, diluted, and analyzed by high-performance liquid chromatography (HPLC). When glycerol was depleted, the culture was supplemented with 70 ml of feeding medium. Fermentation was terminated after complete substrate use. Cells were harvested by centrifugation (16,639 × g, 6 °C, 30 min) and stored at −80 °C.

2.4. Activity assay

Harvested cells were resuspended in 50 mM sodium phosphate buffer pH 7, 20 mM NaCl, to OD_{600} = 10 and disrupted by a high-pressure homogenizer (276 MPa, 4 °C, 2 cycles) (CF Range, Constant Systems ltd., Daventry, UK). The activity of GlyHy was determined by measuring β-glucosidase activity as described elsewhere [21], with the following modifications: 945 μl of sodium phosphate buffer (25 mM, pH 7, 20 mM NaCl) and 50 μl of 20 mM pNPG were added to a UV cuvette and incubated at 80 °C for 30 s; subsequently, 5 μl of crude GlyHy extract was added to start the reaction. The absorbance of p-nitrophenol at 405 nm was measured in 30-s intervals for 6 min against a blank that contained only buffer and substrate. The activity of GlyHy was calculated as a linear increase of p-nitrophenol concentration in time. Each reaction was performed in two parallel measurements. One unit (U) was defined as the amount of enzyme required for the hydrolysis of 1 μmol pNPG per minute. The specific activity of crude GlyHy extracts was calculated as U per mg of dry cell weight (U/mg_{DCW}). With purified GlyHy, the same method was used, but the enzyme was properly diluted prior to the reaction. The specific GlyHy activity was calculated as U per mg of purified GlyHy enzyme (U/mg).

2.5. Purification of GlyHy

Crude extract preparation and purification of GlyHy were performed as described elsewhere [20], with the following modifications: 20 mM sodium phosphate buffer pH 7, 20 mM imidazole, 500 mM NaCl were used as resuspension and binding buffer; 20 mM sodium phosphate buffer pH 7.2, 500 mM imidazole, and 500 mM NaCl were used as elution buffer.

2.6. Characterization of GlyHy

To determine the optimal pH value, the β-glucosidase activity of GlyHy was measured according to 2.4 in the following buffers: 50 mM sodium citrate buffer pH 5, 5.5; 50 mM sodium phosphate buffer pH 5.5, 6, 6.5, 7, 7.5; 50 mM Tris-HCl pH 7.5, 8.

To determine the optimal temperature, the β-glucosidase activity of

GlyHy was measured according to 2.4 at different temperatures: 60, 65, 70, 75, 80, 85, 90 °C.

To determine the kinetic parameters based on the Michaelis-Menten model, the β-glucosidase activity of GlyHy was measured according to 2.4 with different concentrations of pNPG: 0.1, 0.25, 0.5, 1, 2, 3, 4 mM. The V_{MAX} and K_M were calculated by Hanes-Woolf linearization.

2.7. Hydrolysis of steryl glucosides

The stock solution of steryl glucosides was prepared by dissolving 3 mg of the mixture containing 56 % β-sitosteryl glucoside, 25 % β-campesteryl glucoside, 18 % β-stigmasteryl glucoside, and 1 % β-δ-5-avenosteryl glucoside in 1 ml of a chloroform: methanol mixture (1:9) and incubated at 80 °C. Subsequently, 50 mM sodium phosphate buffer pH 7 and 20 mM NaCl were added to a 4-ml screw-neck vial and incubated at 80 °C for 10 min, followed by the addition of 0.05 mg/ml of GlyHy and 100 µl of steryl glucoside stock solution. Hydrolysis was running for 18 h at 80 °C and 250 rpm in three parallels. The reaction mixtures were extracted with the 5-fold volume of heptane two times, and the organic layers were collected and evaporated to dryness. The dry products were dissolved in 100 µl of heptane and analyzed by thin-layer chromatography (TLC).

2.8. Analytics

2.8.1. High-performance liquid chromatography

The HPLC analysis of fermentation media samples was performed as described elsewhere [20].

2.8.2. Thin-layer chromatography

The water phase after extraction and samples after hydrolysis prepared according to 2.7 were loaded to a TLC Silica gel $60F_{254}$ plate with β-sitosterol as a reference. The plate was developed in a mixture of hexane: ethyl acetate (7:3) as eluting solvent and treated with potassium permanganate. Steryl glucosides and free sterols were visualized by heating.

2.8.3. Gas chromatography

Samples containing free sterols prepared according to 2.7 were evaporated to dryness and redissolved in heptane with 0.5 mM of cholesterol as internal standard (IS). To determine the conversion of hydrolysis, samples were analyzed by gas chromatography (GC) as described elsewhere [20], with the following modifications: DB-5 capillary column (Agilent J&W, 30 m × 0.25 mm × 0.25 µm); inlet temperature 350 °C, pressure 80 kPa; temperature gradient 100 °C (2 min), 30 °C/min to 280 °C; 10 °C/min to 325 °C (15 min); detector temperature 350 °C. The conversion of hydrolysis was determined based on substrate depletion and the concentration of β-sitosterol (P) calculated by the following calibration curve, where *A* represents the peak area and *c* represents the concentration:

$$\frac{A_P}{A_{IS}} = a \times \frac{c_P}{c_{IS}} + b$$

To confirm the structure, the samples of sterols were further analyzed by mass spectrometry (GCMS), using an Agilent 6890 N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a HP-5 capillary column (Agilent J&W, 30 m × 0.25 mm × 0.25 µm), a 5975B MS detector, and He_2 as a carrier gas under the following conditions: inlet temperature 290 °C, injection volume 1 µl, split 50:1, temperature gradient 100 °C (2 min), 30 °C/min to 220 °C, 10 °C/min to 325 °C (15 min).

The sterol structure was determined based on obtained *m/z* values: 43, 55, 400 for campesterol, 55, 83, 412 for stigmasterol, and 43, 55, 414 for β-sitosterol. Avenosterol was not detected by GCMS, but the content of the corresponding glucoside in the starting material was negligible.

3. Results and discussion

3.1. High cell-density fermentation with pure glycerol

Previous reports have shown that the recombinant production of GlyHy in *E. coli* is challenging and results in the production of IBs and a low β-glucosidase activity [22]. All previously reported GlyHys exhibiting affinity towards steryl glucosides, including the one studied in this work, have a thermophilic origin [12,21]and contain one cysteine residue.

Enzyme production in native thermophilic microorganisms revealed that the optimal growth temperature, which is usually 50–80 °C [16], is necessary to obtain the proper enzyme quarter structure and conformation which were proven to be crucial for enzyme activity [23]. Moreover, even a low induction temperature does not always eliminate IB formation during the expression of thermostable enzymes in *E. coli* [19]. Koma et al. [24] demonstrated that a high induction temperature (46 °C) was the key factor for the expression of soluble thermophilic enzymes in *E. coli* [24]. These reports demonstrate a possible correlation between the high temperature and proper folding of thermostable proteins.

Based on the above arguments, the range of different induction temperatures was screened for GlyHy expression in the high cell-density (HCD) fermentation process. As shown in Fig. 1, the lag phase of cell growth at 37 °C took approximately 4 h, after which cells entered the exponential phase. The expression of GlyHy was induced in the mid-exponential phase, and the induction temperature was changed in all processes. The lowest specific enzyme activity was reached in expression at 20 °C, and it increased with increasing induction temperature, which is in agreement with previous reports of thermophilic gene expression [22,24]). The same trend was confirmed by crude extract SDS-PAGE electrophoresis (Figure S1; the highest level of insoluble GlyHy was reached by expression at 20 and 30 °C. Even when expressed at 40 °C, the production of IBs was detected, but the specific enzyme activity of the crude enzyme extract was 7-fold higher compared to that at 20 °C. The highest biomass concentration was reached when the temperature was reduced to 30 °C. Based on the specific enzyme activity and the difference in biomass concentration, which was observed approximately 2 h after induction, it can be assumed that at 37 °C (Fig. 1C), the enzyme expression was increased and biomass production was less intensive. A similar trend was observed at 40 °C. Although fermentation at 37 and 40 °C proceeded similarly, and the final biomass concentration was higher at 37 °C, the highest specific enzyme activity (0.15 ± 0.01 U/mg_{DCW}) and volumetric productivity (195 U/l/h) were reached at 40 °C (Table 1). The further increase of induction temperatures to 46 and 53 °C, resulted in *E. coli* growth inhibition, and no GlyHy was produced (data not shown).

For comparison, in Tabandeh et al. [25], expression of human growth hormone in *E. coli* was in high cell density fermentation heat-induced by 2 h temperature shift from 37 to 42 °C and then reduced to 37 °C for the next 4 h. Under these conditions, the final biomass concentration fed-batch mode reached 100 g/l after 40 h. The authors hypothized, that the improvement of expression was achieved due to the degradation of proteases at a higher temperature[25]. Compare to this, in our work whole 6 h induction period was carried out at 40 °C, and the fermentation was performed in the batch mode, which in 16 h process resulted in the lower biomass of 22 g/l. The improvement of enzyme expression was also apparent.

Koma et al. [24] explored the expression of thermophilic aminotransferases at a temperature range of 15–46 °C in *E. coli*. Enzymes were expressed in soluble form only at a temperature above 37 °C and the highest expression level was reached at 46 °C. Authors assumed that improvement in solubility was reached mainly due to the lower protein synthesis rate compared to enzyme folding rate[24]. However, no HCD fermentations under these conditions were investigated. In the field of high cell density fermentation of *E. coli* and inductions at higher than

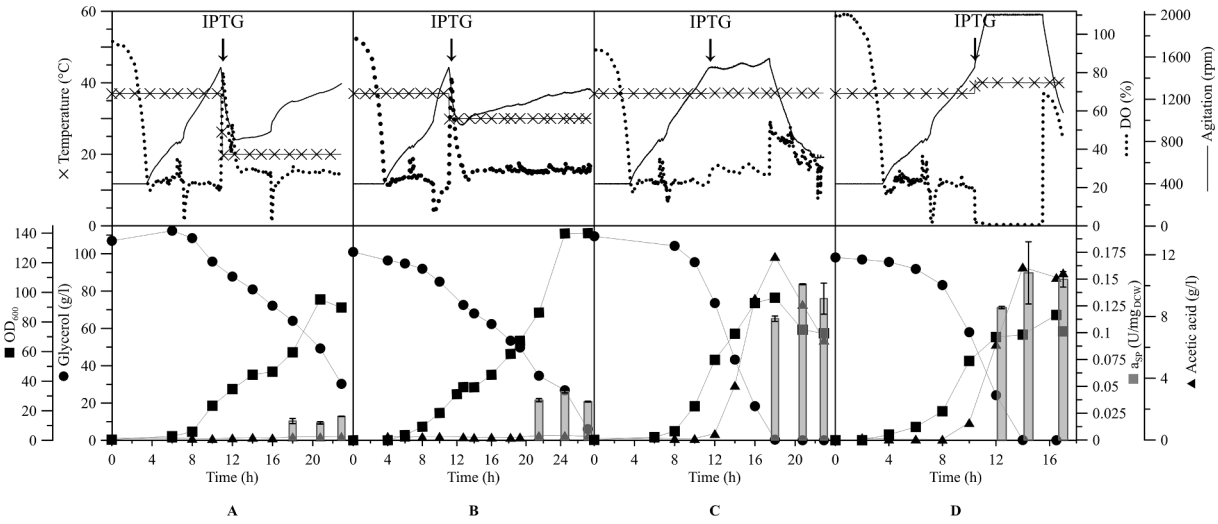

Fig. 1. HCD fermentation of *E. coli* expressing GlyHy at 20 °C (A), 30 °C (B), 37 °C (C), and 40 °C (D) performed in Mini Bioreactor System. All fermentations were initiated at 37 °C until the induction when the temperature was adjusted to 20, 30, and 40 °C (A, B, D) or remained at 37 °C (C). The arrow labeled with IPTG indicates induction of GlyHy expression.

Table 1
Summary of HCD fermentations at different induction temperatures.

Induction Temperature (°C)	Final Biomass Concentration (g/l)	Total Dry Cell Weight (g)	Enzyme Activity (U/mg_{DCW})	Total Produced Enzyme Activity (U)	Volumetric Productivity (U/l/h)
20	27	4.1	0.022	89	26
30	42	6.3	0.036	227	55
37	22	3.3	0.132	430	126
40	22	3.3	0.150	498	195
46	10	1.5	ND	ND	ND
53	9	1.4	ND	ND	ND

ND – not detected due to insufficient expression.

optimal temperature other systematic studies are missing. We assume that this method of improved recombinant enzyme production might be applied also to other thermostable enzymes.

3.2. High cell-density fermentation with crude glycerol

To demonstrate the sustainability and scalability of the process, the optimized HCD fermentation protocol was further applied to partially purified and neutralized crude glycerol to 1.3-l BioFlo 115 fermenters. Both glycerols contain various impurities that may impact cell growth and enzyme expression. Their composition differs, and the precise effect on the fermentation process could not be predicted. To prevent the immediate inhibition due to the combination of crude glycerol and a higher induction temperature and to obtain comparable results, the induction phase was initiated at a higher biomass concentration, and the length of induction was chosen as a constant parameter. Fermentation with pure glycerol was performed as a control (Fig. 2). Process transfer to higher-volume fermenters resulted in a faster substrate utilization (90 g/l of pure glycerol), which was therefore not sufficient to perform 6 h of induction in the single batch mode, as optimized above. The substrate was depleted after 14 h, which includes only 2.8 h of induction. The culture was therefore fed with glycerol (up to 30 g/l), and the fermentation was extended until the expression duration reached 6 h. Besides the change of the feeding strategy and the partial re-utilization of acetic acid, the obtained biomass concentration and specific enzyme activity corresponded with those reached on the smaller scale (Fig. 1D).

When partially purified crude glycerol was used (Fig. 3), the lag phase was extended to 5.5 h. However, when the culture entered the exponential growth phase, it took only 6 h to reach induction cell density. Expression was initiated at 11.5 h, which corresponds to pure glycerol fermentation (Fig. 2). As expected, the crude glycerol utilization was slower, and no additional substrate feed was needed to achieve 6 h of induction. In the case of neutralized glycerol, the lag phase was markedly extended compared to pure or partially purified crude glycerol. Even after entering the exponential growth phase, it took 9 h to reach induction cell density (Fig. 4). As expected, the glycerol utilization rate was probably decreased by the higher level of impurities. Due to the extension of the initial fermentation phase, the glycerol was depleted in 19.3 h, which corresponded to a 3-h-long induction. The culture was therefore fed with 30 g/l of crude glycerol.

Crude glycerol affected the early fermentation stage by the decrease of the initial growth of the culture; however, no difference in the obtained fermentation profiles and GlyHy expression was observed. Even more, the specific enzyme activity reached with crude glycerols was comparable to that of the pure one (Table 2). Comparing two crude glycerol samples, partially purified crude glycerol was more favorable for specific enzyme activity, although a higher volumetric productivity was achieved with neutralized glycerol despite the longer lag phase (Table 2.). To summarize, both tested crude glycerol samples were suitable for the production of recombinant GlyHy and are worth of further process upscale.

One of the major problems of crude glycerol valorization by microorganisms is the presence of impurities that inhibit cell growth and enzyme expressions such as fatty acids, inorganic salts, soups, and alcohols [26]. Fortunately, a wide scope of microorganisms was found to tolerate these impurities and to use crude glycerol as a sole carbon source. Despite its carbon source potential, there is still a lack of information at recombinant enzyme manufacturing from crude glycerol. Nguyen et al. (2013), Lee et al. [27], and Sudheer et al. [28] explored the recombinant enzyme expression in *E. coli* growing on crude glycerol but

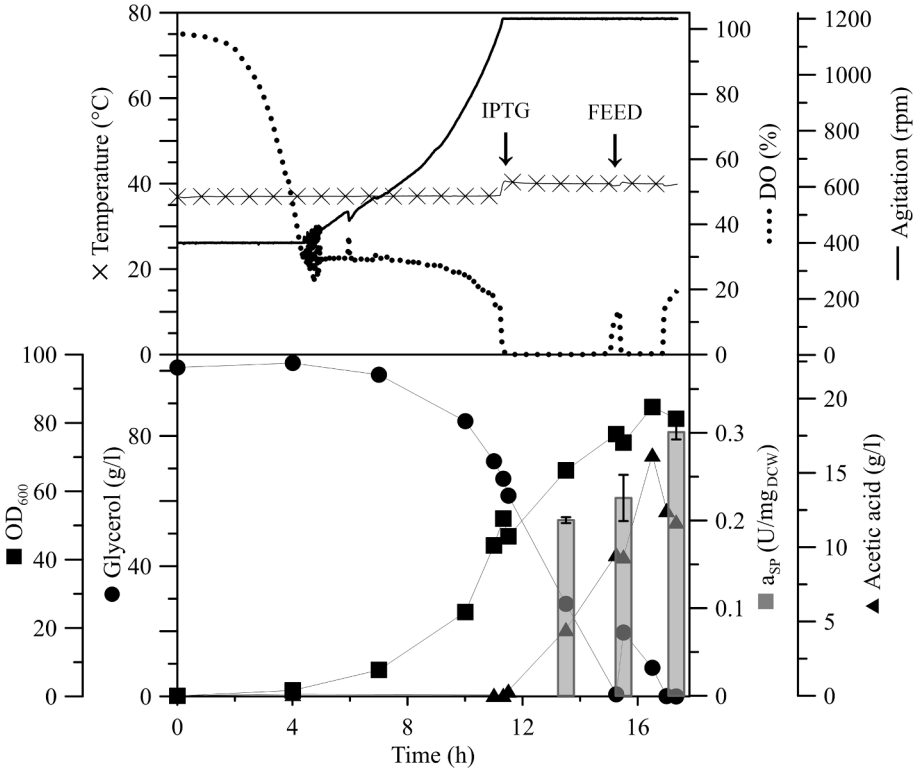

Fig. 2. HCD fermentation of *E. coli* expressing GlyHy at 40 °C with pure glycerol. The arrow labeled with IPTG indicates induction of GlyHy expression. The arrow labeled with FEED indicates the addition of a feeding medium.

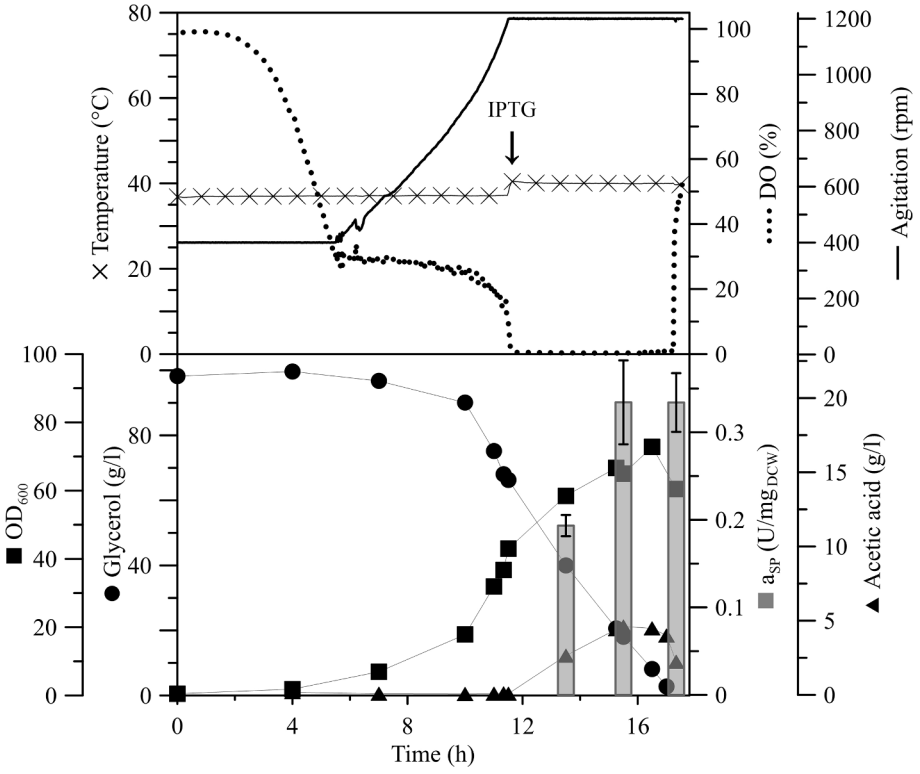

Fig. 3. HCD fermentation of *E. coli* expressing GlyHy at 40 °C with partially purified crude glycerol. The arrow labeled with IPTG indicates induction of GlyHy expression.

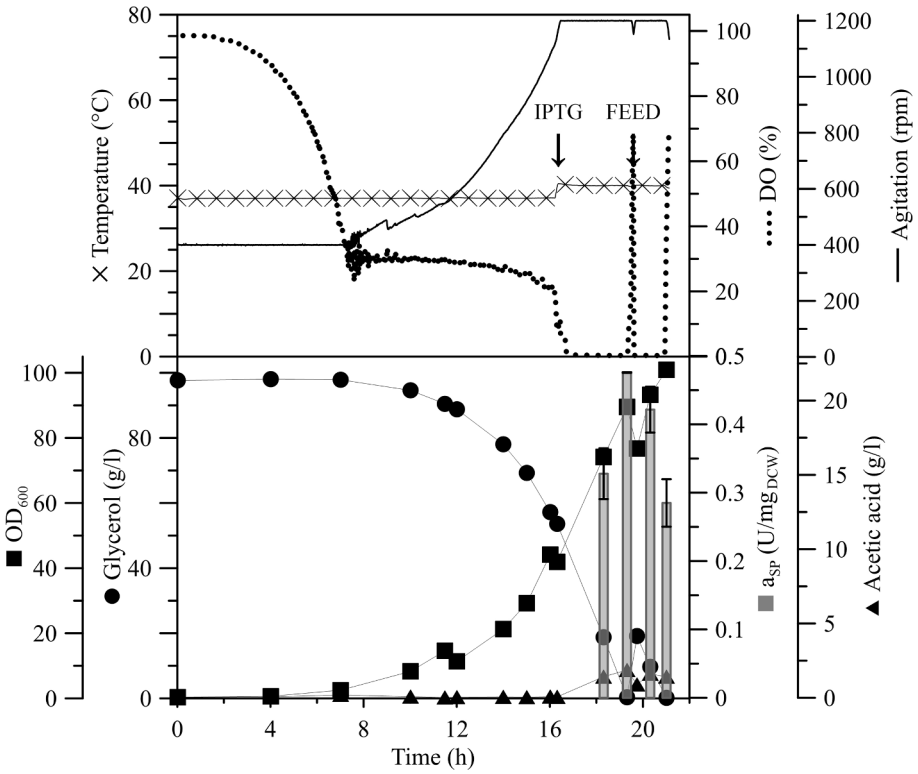

Fig. 4. HCD fermentation of *E. coli* expressing GlyHy at 40 °C with neutralized crude glycerol. The arrow labeled with IPTG indicates induction of GlyHy expression. The arrow labeled with FEED indicates the addition of a feeding medium.

Table 2
Summary of HCD fermentations with pure and crude glycerol.

Glycerol Type	Final Biomass Concentration (g/l)	Total Dry Cell Weight (g)	Enzyme Activity (U/mg_{DCW})	Total Produced Enzyme Activity (U)	Volumetric Productivity (U/l/h)
pure	25	18.4	0.301	5528	424
partially purified	18	12.7	0.334	4251	346
neutralized	30	22.9	0.285	6514	411

the main product was not the enzyme itself but secondary metabolites [26–28]. As the fermentation parameters differed from GlyHy fermentation parameters this again cannot be compared comprehensively but all studies agreed that *E. coli* is a suitable protein producer from crude glycerol and its tolerance towards impurities can be improved by strain mutations. In the present results; however, there were no differences in tolerance towards partially purified and neutralized glycerol.

3.3. Purification and characterization of recombinant glucoside hydrolase

Isolation of His-tagged recombinant proteins produced in *E. coli* by immobilized metal affinity chromatography (IMAC) is one of the most frequent purification methods, especially due to its simplicity and high selectivity [18,29]. Despite the intensive optimization of this affinity isolation process, GlyHy expressed at 20 and 30 °C could not be purified effectively. The binding of the enzyme to Ni Sepharose resin was weak, it was eluted at a low imidazole concentration (116 mM) together with different non-specifically bound proteins repeatedly, and only 1.24 ± 0.05 U/mg of β-glucosidase activity was reached. These results suggest that besides IBs formation, even soluble GlyHy did not gain the proper conformation. The His tag was assumingly hidden in the improper three-dimensional structure and was not sufficiently exposed on the enzyme surface to bind to Ni Sepharose resin [30].

There are, however, other ways that could improve the solubility of recombinant enzymes produced in *E. coli* [29,31]. Based on previous studies of thermophilic enzyme expression in mesophilic hosts [19,23,32] and based on our unsuccessful attempts of the IB solubilization, it was assumed that the key factor of proper GlyHy folding is the increase of the induction temperature to 40 °C during its production.

This step principally increased not only the expression of GlyHy but also significantly improved its downstream processing. Once expressed under 40 °C, GlyHy was successfully purified using the same IMAC protocol. Under these production conditions, the enzyme could bind to Ni Sepharose resin with higher affinity and was eluted at 404 mM of imidazole without any other contaminating proteins in the final processed sample (Figure S2). As high as 32 ± 2 $\mu g/ml_{CE}$ of purified GlyHy, with 16 U/mg of β-glucosidase activity, was reached, which represents a 2.7-fold and 13-fold increase compared to GlyHy expressed at lower temperatures, respectively. The kinetic parameters of the prepared enzyme were determined as $V_{MAX} = 24.95$ μmol/min and $K_M = 0.44$ mM at pH 7 and 80 °C, which were optimal conditions for hydrolysis (Fig. 5). Obtained results correspond with the kinetic parameters of similar GlyHys from thermophilic bacteria expressed in *E. coli* [33].

The structure of thermophilic enzymes varies from those derived from mesophilic organisms in many ways [34–36]. Interestingly, these differences are mostly promoted by non-covalent interactions and small structural modifications [36]. These findings imply the importance of the quaternary structure of thermostable proteins that, on the other

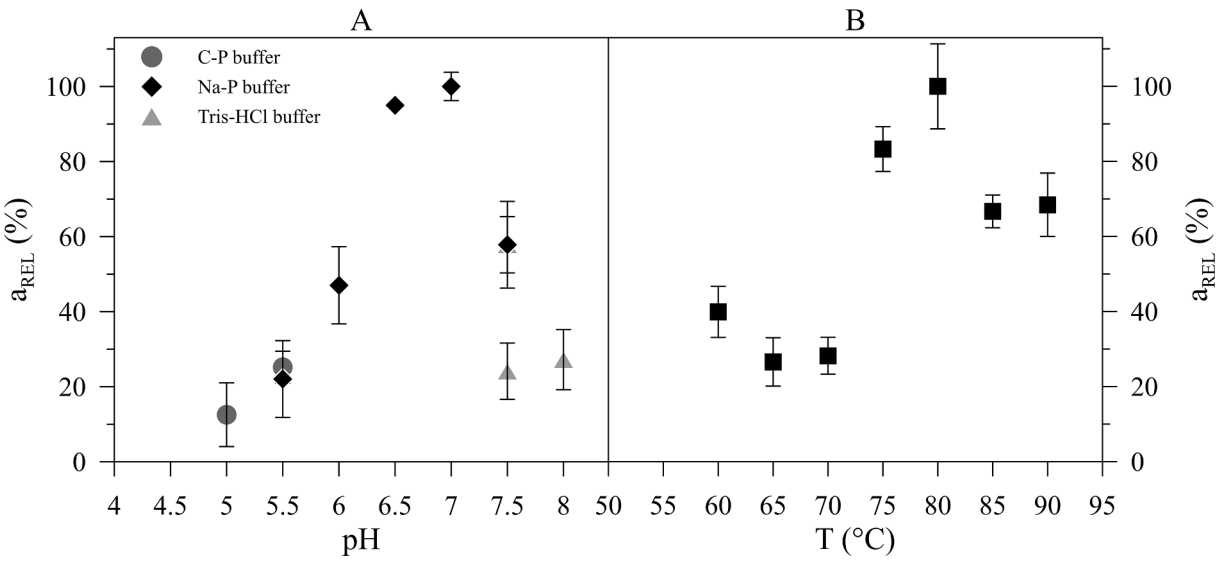

Fig. 5. pH profile of GlyHy obtained in different 50 mM buffers at 80 °C (A). Temperature profile of GlyHy obtained at different temperatures in 50 mM Na-P buffer pH 7(B).

hand, depends on the production temperature.

The presented results are in a good agreement with these reports and clearly demonstrate the correlation between production temperature and the proper folding of thermostable enzymes since only a small increase in induction temperature in mesophilic expression host improved the solubility and specific enzyme activity of recombinant thermostable GlyHy, as well as the exposure of His tag on its surface.

3.4. Enzymatic hydrolysis of steryl glucosides

Steryl glucosides are the major biodiesel contaminants that form insoluble precipitates and affect the quality of biodiesel. These compounds contribute to the total contents of insoluble contaminants and need to be removed in order to meet the requirements of biodiesel standards [37]. In industry, these contaminants are removed by distillation which is expensive and time-consuming. One of the possible ways of their elimination is enzymatic elimination by glycoside hydrolase. The content of steryl glucosides in biodiesel varies from 10 to 500 ppm [12]). However, it usually does not exceed the concentration of 300 ppm [13,22]. Therefore, the GlyHy prepared in this work was tested for the hydrolysis of 300 ppm of steryl glucosides mixture and reached 100 % conversion after 18 h at optimized conditions. Despite many reports of enzymatic hydrolysis of steryl glucosides, the products (free sterols) are either detected indirectly by glucose detection or directly. In both cases, however, there is a lack of data explicitly proving that the expected products are free sterols. For this reason, the products of enzymatic GlyHy hydrolysis prepared in this work were analyzed by GCMS. As expected, the obtained sterols were campesterol, stigmasterol, and β-sitosterol (2.8.3, Figure S3. Steryl glucosides, used in this work, are the most frequent biodiesel contaminants; however, their contents depend on the feedstock used for its production and might even differ from one batch to another.

The necessity to prevent the climate change crisis has become a global challenge. Therefore, newly developed small- or large-scale technologies are expected to come up with ecologically sustainable ideas. The presence of steryl glucosides in biodiesel, together with other challenges of biofuel manufacturing, impede their implementation. In this work, we propose the technology of direct waste stream use for the production of the biocatalyst that completely removes contaminants of biodiesel. This process contributes to carbon recycling and simultaneously improves biodiesel quality and its application potential. Besides the hydrolysis of steryl glucosides, thermophilic hydrolases are widely applicable in the biofuel industry [38,39]; these enzymes show tremendous stability during the hydrolysis of starch and lignocellulosic materials [40]. Several thermophilic glucoside hydrolases exhibit affinity toward biologically active plant glycosides [41,42]. Deglycosylated free aglycones of these compounds exhibit even higher biological activity and have therefore become of pharmaceutical interest. This emphasizes the importance of thermostable glucoside hydrolases beyond the biofuel industry. Apart from the direct use of crude glycerol to produce recombinant enzymes applicable in biorefineries, this technology could be further used for the manufacturing of high-value compounds of pharmaceutical interest.

4. Conclusions

In this work, crude glycerol from biorefineries was applied for the recombinant GlyHy production by *E. coli*. Optimized HCD fermentation with crude glycerol reached the same amount of biomass and specific enzyme activity compared to the pure one. The production of soluble GlyHy was achieved by an induction temperature increase to 40 °C, which eliminates IB formation and improves enzyme folding and His tag purification. The produced GlyHy was applied for the hydrolysis of 300 ppm of steryl glucosides mixture and reached 100 % substrate conversion within 18 h. The enzyme has a high application potential in biorefineries and the pharma industry.

CRediT authorship contribution statement

Dominika Gyuranová: Methodology, Investigation, Writing – original draft, Visualization, Data curation. **Vladimír Krasňan:** Methodology, Investigation, Data curation. **Ivan Špánik:** Validation, Resources. **Martin Rebroš:** Conceptualization, Methodology, Investigation, Writing – review & editing, Funding acquisition, Supervision, Project administration, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fuel.2022.125550.

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