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RESEARCH PAPER

Pilot-scale process development for low-cost production of a thermostable biodiesel refining enzyme in *Escherichia coli*

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Abstract
Biodiesels produced from vegetable oils have a major quality problem due to the presence of steryl glucosides (SGs), which form precipitates that clog filters and cause engine failures. Recently, we described an enzymatic process for removing SGs from biodiesel. However, industrial adoption of this technology was hindered by the cost of the steryl glucosidase (SGase) enzyme used. Here we report the development and validation at the pilot scale of a cost-efficient process for manufacturing the SGase. First, we tested various low-cost carbon sources for the *Escherichia coli* producing strain, ultimately developing a fed-batch fermentation process that utilizes crude glycerol as a feedstock. Next, we designed an efficient process for isolating the SGase. That process uses a novel thermolysis approach in the presence of a non-ionic detergent, centrifugation to separate the solids, and ultrafiltration to concentrate and formulate the final product. Our cost analysis indicates that on a large scale, the dose of enzyme required to eliminate SGs from each ton of biodiesel will have a manufacturing cost below $1. The new process for manufacturing the SGase, which will lead to biodiesels of a higher quality, should contribute to facilitate the global adoption of this renewable fuel. Our technology could also be used to manufacture other thermostable proteins in *E. coli*.

Keywords Bioprocess development · Microbial thermolysis · Biofuels

Introduction

Biodiesels are renewable fuels mostly produced from vegetable oils, including those from soybean, palm, sunflower, rapeseed, jatropha, and others. Currently, biodiesel mandates are set in more than 60 countries, many of which belong to high-consuming regions like the European Union and North America [1]. Global biodiesel production is projected to continue its rapid increase and reach more than 40 billion liters by 2020 [2]. Unfortunately, the acceptance of biodiesel as an alternative fuel is affected by the presence of contaminants that form sediments and cause the failure of engines. Steryl glucosides (SGs), present in different biodiesels at concentrations ranging from 10 to 300 ppm [3, 4], have been identified as the major component of such sediments [5–7]. Thus, the selective removal of SGs could produce biodiesels of a superior quality, increasing the likelihood that these renewable fuels will be adopted by consumers. Currently, the only available method capable of completely removing SGs from biodiesel is distillation, an energy-intensive and expensive process [8, 9].

Recently, we described an efficient method for removing SGs from biodiesel. The method is based on the use of enzymes with steryl glycosidase (SGase) activity. The most efficient SGase tested so far is a thermostable β-glucosidase from *Thermococcus litoralis*. Because this hyperthermophilic archaeon is difficult to cultivate, a synthetic codon-optimized version of the SGase gene was designed and successfully expressed in *E. coli* [10]. The producing strain was then optimized through extensive engineering, which included the use of different promoters and the co-expression of molecular chaperones to significantly increase the

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production of the SGase. Additionally, a high cell density, fed-batch fermentation process has been developed [11]. However, much remains to be done to achieve a cost-effective process for manufacturing SGase.

Currently, industrial enzymes produced by fermentation are considered commodities. The carbon source used in the fermentation processes can account for up to 50% of the total manufacturing cost [12, 13]. In addition, downstream processes involving a few simple operations can greatly reduce capital expenditures and operating costs [14–17]. In this work, we analyze the impact of different carbon sources and the post-induction feeding strategy on the productivity of the SGase in *E. coli*, aiming to develop a commercial fermentation process for producing this enzyme on a large scale. Additionally, a simple thermolysis method is described for facilitating the recovery of the enzyme. The results described here could be used to design processes for producing other thermostable enzymes in *E. coli* in a cost-effective manner.

Materials and methods

General

Enzymes were obtained from New England Biolabs (USA) and used as recommended. *E. coli* Top10 (Invitrogen) was used for plasmid propagation during cloning steps. *E. coli* BL21(DE3)AI served as the expression host. *E. coli* strains were made chemically competent with a kit from Zymo Research (USA). The concentrations of kanamycin and chloramphenicol used were 50 and 20 mg/L, respectively.

Insertion of a cassette for metabolizing sucrose

The *cscAKB* operon was amplified by PCR using oligonucleotides that match the 5′ and 3′ ends of the operon. *Sca*I restriction sites were added at the end of both oligonucleotides, and the resulting DNA fragments were inserted into identical sites of the plasmid pACYC184. The resulting plasmid was digested with *Bcl*I and *Sac*II, and the fragment containing the operon and the *Cm* resistance cassette was inserted into the *lacZ* locus of *E. coli* BL21(DE3)AI, as described by Datzenko et al. [20].

Growth conditions for expression studies in 96-well microplates

From stocks stored at − 80 °C, strains were grown overnight on Luria–Bertani (LB) agar plates at 37 °C. Individual colonies were used to inoculate 5 mL of HM medium in 50 mL glass tubes, the medium being supplemented with the appropriate carbon source. The cultures were incubated overnight at 37 °C with shaking at 200 rpm. Microbial growth was determined by measuring optical density at 600 nm (OD_{600}) in a General Electric NovaspecIII spectrophotometer. HM medium consists of 20.8 g/L KH_2PO_4, 3 g/L $(NH_4)_2PO_4$, 3.25 g/L K(OH), and 4 g/L NaH_2PO_4.

Microplates (Thermo Fisher Scientific) were prepared by adding to each well, 190 µL of HM medium supplemented with 1% of the source of carbon being tested and the necessary antibiotics. Each well was inoculated with 10 µL of the centrifuged culture. The microplates were incubated at 37 °C for 12 h with continuous shaking in a microplate reader (Synergy HT). OD_{600} were recorded every 30 min.

Growth conditions for expressing the SGase

Recombinant *E. coli* strains were grown overnight at 37 °C in 2 mL of LB medium supplemented with the appropriate antibiotic.

To test the expression of the SGase, 5 mL of HM medium was supplemented with different carbon sources (glycerol, crude glycerol, glucose, sucrose, or molasses) at 1% (w/v) and was inoculated with an overnight culture to an initial $OD_{600} = 0.1$. Following incubation at 200 rpm and 37 °C, protein expression was induced at $OD_{600} = 1$ with 0.4 g/L of L-arabinose. Growth was allowed to continue at 37 °C for 6 additional hours.

Cell disruption

Cells and supernatants of the cultures were separated by centrifugation. The cell pellets were resuspended in 20 mM citrate (pH 6.0) and 20 mM NaCl, normalized to a final $OD_{600} = 4$ per mL. Cells were disrupted on ice in a GEX 600 Ultrasonic Processor.

Activity assays

Expression of the SGase under different growth conditions was determined by measuring β-Glucosidase activity, according to a method previously developed in our laboratory [18]. One unit (U) was defined as the amount of enzyme required for the hydrolysis of 1 µmol of pNPG per min, under the described assay conditions.

High cell density fermentation

Seed cultures of *E. coli* BL21(DE3)AI and *E. coli* NK5, harboring the plasmids pKCN-BAD-SGase and pGro7 [11], were prepared in 1-L Erlenmeyer flasks containing 0.1 L of HM medium. The medium was supplemented with glycerol, crude glycerol, glucose, sucrose, or molasses as the carbon source. The cultures were grown at 37 °C in an incubator with shaking at 200 rpm.

Fed-batch fermentations were conducted in a 3-L bioreactor (New Brunswick BioFlo 115, USA) containing 1 L of the same medium. The temperature and stirring were maintained at 37 °C and 1200 rpm, respectively. The pH was maintained at 7.0 by addition of 28% NH_4OH. The concentration of dissolved oxygen was maintained at 30% of saturation. The feeding process was initiated when the carbon source initially present in the medium was exhausted. A feeding solution containing 600 g/L of glycerol, crude glycerol, glucose, sucrose, or molasses, plus 20 g/L $MgSO_4$·7H2O, was added by following an exponential feeding rate until protein expression was induced.

The feeding rate (F, mL/h) was determined by Eq. (1) [19], to maintain the specific growth rate at 0.25 1/h:

$$F = X_0 V_0 \mathrm{e}(\mathrm{u.t}) / S_0 Y_{X/S}$$

X_0 is the biomass concentration (g/L) when the feeding is started. V_0 is the initial volume (L). u is the desired specific growth rate (1/h). S_0 is the carbon source concentration in the feeding solution (g/L). $Y_{X/S}$ is the substrate yield.

When the OD_{600} reached 100, expression of the SGase was induced by adding L-arabinose at a final concentration of 0.4 g/L. Afterwards, the feeding rate was maintained at 8 g/h, except in the post-induction optimization experiments.

Pilot-scale fermentations were conducted at 1300 L. The culture medium, relative glycerol feeding rate, pH, and temperature were those used for the laboratory-scale fermentations. The inoculum was prepared in a 50 L bioreactor, to obtain an inoculum of the same relative size as that in the laboratory-scale experiments. After the fermentation, cells were disrupted on ice at 1000 bars through a high-pressure homogenizer (GEA NiroSoavi, Panda Plus 2000), except in the thermolysis experiments.

Thermolysis assays

High cell density cultures were incubated with none, 0.2, or 2% of the indicated detergent, and the mixtures were heated at 70 °C for 30 min. Then, the mixtures were centrifuged at 10,000 rpm for 20 min in a bench centrifuge (Eppendorf 5804R). Purified samples were analyzed by SDS–PAGE. Concentrations of the SGase were estimated by β-glucosidase activity.

Results and discussion

Analysis of different carbon sources for the production of the SGase

Bioprocesses based on *E. coli* mostly utilize for their carbon and energy source glucose obtained from the hydrolysis of cornstarch. This glucose has an average cost of $800 per metric ton. To explore lower-cost alternatives (below $200 per ton), we decided to test sucrose, glycerol, and industrial products containing these compounds—such as molasses and biodiesel-derived crude glycerol—as feedstocks for the production of SGase. While derivatives of *E. coli* BL21 can grow efficiently by utilizing glucose or glycerol, these strains cannot use sucrose as a carbon source, because they lack the genes for transporting and metabolizing this disaccharide. *E. coli* W strains grow with sucrose as the sole carbon and energy source using the *cscB, cscA*, and *cscK* genes, which mediate the transport and hydrolysis of sucrose and the phosphorylation of fructose, respectively. Therefore, we first engineered the BL21-based producing strain by inserting a sucrose utilization cassette containing these three genes into the *lacZ* locus of the chromosome. For this step, the operon containing the genes and the native promoter was amplified by PCR using oligonucleotides with extensions homologous to the target sequence in the chromosome. The operon was inserted using the Lambda red system described by Datsenko et al. [20]. The engineered strain was named NK5 and was tested along with the native strain for its ability to grow on sucrose, glucose, or glycerol supplementing minimal medium. For each strain, three independent clones were assessed.

Table 1 Growth of BL21(DE3)AI/pKCN-BAD-SGase pGro7 and NK5/pKCN-BAD-SGase pGro7 strains with different carbon sources

Carbon source	BL21(DE3)AI/pKCN-BAD-SGase pGro7	NK5/pKCN-BAD-SGase pGro7
Final OD_{600nm}		
Glucose	2.65 ± 0.19	2.75 ± 0.10
Glycerol	2.65 ± 0.13	2.46 ± 0.18
Crude glycerol	2.68 ± 0.25	2.49 ± 0.17
Sucrose	0.48 ± 0.19	2.67 ± 0.09
Molasses	1.35 ± 0.14	3.08 ± 0.25
μ (h^{-1})		
Glucose	0.63 ± 0.09	0.62 ± 0.05
Glycerol	0.55 ± 0.07	0.54 ± 0.09
Crude glycerol	0.59 ± 0.07	0.57 ± 0.08
Sucrosa	–	0.52 ± 0.07
Molasses	–	0.55 ± 0.06

Growth parameters are shown for the strains BL21(DE3)AI and NK5, when both harbor the plasmids pGro7 and pKCN-BAD-SGase. The strains were cultivated in 96-well plates with a minimal medium. The experimental error is the standard deviation for $n = 3$

Table 1 shows that, as expected, the native BL21-derived and the NK5 strains grew well on glucose and glycerol. Only the NK5 strain, which harbors the cassette for metabolizing sucrose, could grow on sucrose. The strains also grew on crude glycerol at a rate comparable to that obtained

for refined glycerol. Likewise, the NK5 strain exhibited a growth profile in molasses similar to the one shown for sucrose. The minor growth observed for the BL21 strain in molasses is probably due to a small amount of glucose being naturally present in this feedstock.

Next, shake flask cultures were grown in minimal medium supplemented with either 10 g/L of glucose, 11.5 g/L of refined or crude glycerol (containing 87% of glycerol), 10 g/L of sucrose, or 16.7 g/L of molasses (with a sucrose concentration of 60%), to analyze the impact of each carbon source on the level of expression of the SGase. Table 2 shows the results. In all of the cases, a similar final OD_{600} was reached, confirming that all of the tested carbon sources efficiently support the growth of the producing strains. The specific production of the SGase was comparable for all of the feedstocks, being about 10% lower for refined glycerol and sucrose.

Fermentation process development

To test the efficacy of the different carbon sources in the high cell density cultures typically used for the industrial production of enzymes, we developed fed-batch processes using glucose, refined and crude glycerol, sucrose, or molasses. In all of the cases, a 3-L lab scale bioreactor was used. The recombinant strains were grown at 37 °C in 1 L of HM medium supplemented with 20 g/L of the corresponding carbon source. The pH was maintained at 6.9 by the addition of 28% NH_4OH. The concentration of dissolved oxygen was kept above 30% of saturation. After the initial amount of a carbon source was exhausted, cultures were fed with a solution of 60% of the corresponding substrate. A feeding strategy using a balance mass equation was used to maintain the specific growth rate at 0.25 [19, 21]. When cultures reached an OD_{600} of 100, 0.4 g/L of L-arabinose was added to induce the production of the SGase. The feeding rate was kept constant at a rate of 8 g/h of substrate per L.

Figure 1 summarizes the results. In all of the cases, the maximum OD_{600} and SGase activity were obtained after 25 h of cultivation. The highest biomass concentration (160 OD units) and SGase production (280 units/mL) were obtained for refined and crude glycerol. This result is consistent with multiple reports indicating that glycerol, contrary to glucose and sucrose, is an attractive feedstock for use in *E. coli* fed-batch fermentations, because acetate is not produced. The accumulation of this byproduct has been widely reported to be the major factor affecting both cell growth and the production of recombinant proteins in *E. coli* in this type of fermentation [19, 22–24].

Bruschi and co-workers obtained identical yields when producing DAMP4, a surfactant peptide from either glucose or sucrose in an engineered strain, when the same set of genes for sucrose metabolism used here were expressed from a multicopy plasmid [25]. In our experiments, sucrose yielded a lower final biomass and less SGase activity than glucose did. However, in the fed-batch fermentations using sucrose or molasses as a carbon source, we detected the accumulation of sucrose (data not shown). This accumulation might account for the poorer performance. A possible explanation is a gene dose effect, in which the integration of a single copy of the genes involved in sucrose metabolism into the chromosome might cause insufficient protein expression, compared to expression from multicopy plasmids.

Our results and the economic advantage of using a waste product induced us to choose crude glycerol as the preferred carbon source for producing the SGase. All further experiments were conducted with this feedstock.

Effect of post-induction strategies for feeding crude glycerol on the production of the SGase

The post-induction strategy for feeding nutrients greatly impacts cell growth and protein production [26, 27]. Therefore, we further optimized our process by comparing four

Table 2 Expression of the SGase in shake flask cultures

Carbon source	Glucose	Glycerol	Crude Glycerol	Sucrose	Molasses
OD_{600nm}					
BL21(DE3)AI/pKCN-BAD-SGase pGro7	6.07 ± 0.23	5.14 ± 0.11	5.23 ± 0.24	–	–
NK5/pKCN-BAD-SGase pGro7	5.95 ± 0.11	5.29 ± 0.22	5.91 ± 0.31	5.11 ± 0.10	5.26 ± 0.08
SGase Activity (U/DO)*					
BL21(DE3)AI/pKCN-BAD-SGase pGro7	3.35 ± 0.38	2.98 ± 0.29	3.41 ± 0.32	–	–
NK5/pKCN-BAD-SGase pGro7	3.22 ± 0.31	3.07 ± 0.21	3.12 ± 0.15	2.96 ± 0.21	3.41 ± 0.40

Strains BL21(DE3)AI and NK5 harboring pGro7 and pKCN-BAD-SGase, were cultivated in shake flasks in HM medium with different carbon sources. The activity of the SGase was analyzed. The experimental error is the standard deviation for $n = 3$

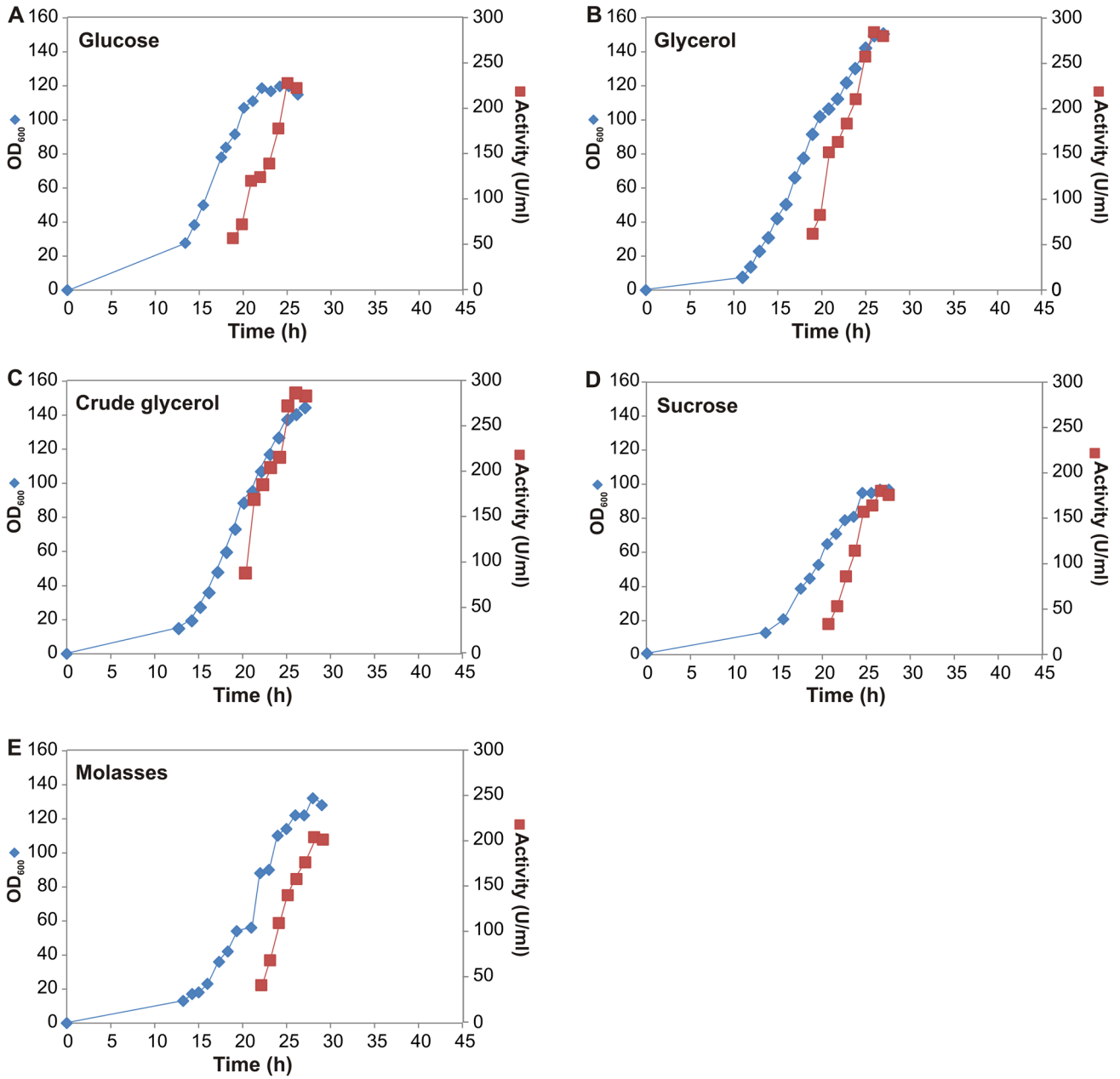

Fig. 1 Fed-batch fermentations using different carbon sources. Growth curves of fed-batch cultures in minimal medium with **a** glucose, **b** glycerol, **c** crude glycerol, **d** sucrose, and **e** molasses as the sole carbon source for the BL21(DE3)AI (**a**–**c**) and NK5 (**d**–**e**) strains. NK5 harbors the plasmids pKCN-BAD-SGase and pGro7. Fermentations were conducted in duplicate. The figure shows the average of the duplicate runs. In all of the cases, the data varied by less than 10%

different feeding strategies: (1) keeping the post-induction feeding rate of crude glycerol constant at 8 or (2) 12 g/L.h, (3) a strategy in which the feeding started at 8 g/L.h and was linearly increased at a rate of 0.56 g/L.h, and (4) a feeding profile in which the initial rate of 10 g/L.h was increased based on the culture OD_{600} (0.1 g/L.h per OD unit). In all of the cases, samples were analyzed hourly. The cultures were harvested when the SGase activity started to decline.

Table 3 shows the results. The three alternative post-induction feeding strategies yielded higher final activities of the SGase than did the original condition of feeding glycerol at a constant rate of 8 g/L h. Moreover, the duration of the fermentations decreased. A maximum productivity of 16.2 U/mL.h was achieved with a constant feeding rate of 12 g/L h, 50% higher than the productivity of the original protocol.

Table 3 Effect on the productivity of the SGase of different post-induction strategies for feeding crude glycerol

	SGase (U/mL)	Final OD_{600}	Fermentation process time (h)	Productivity (U/ml h)
Constant fed 8 g/l/h	280	137	26	10.8
Constant fed 12 g/l/h	405	140	25	16.2
Linear fed increase	390	146	25	15.6
Fed proportional to OD_{600}	355	133	25	14.2

Four strategies were tested: constant feed rates at 8 or 12 g/L h; a linearly increasing feed rate at 0.7 g/L.h; and an OD_{600}-based increasing feed rate at 0.1 g/L h per OD_{600} unit. Fermentations were conducted in duplicate. In all of the cases, the data varied by less than 10%

Different feeding strategies have been employed by various authors for the post-induction phase of recombinant cultures. The strategies include the following: a constant feeding rate [28–30]; a feeding rate that changes linearly [27]; a feeding rate that changes exponentially, beginning during the pre-induction phase [31, 32]; and a feeding rate with feedback control that resembles a DO-stat or a pH-stat [33]. In all of these cases, the productivity varied widely, such that a "universal" post-induction feeding profile could not be established. Based on the data available in the literature, it seems that optimization is required for each new bioprocess in *E. coli*. In our case, the productivity of the SGase increased with the amount of glycerol fed. However, further increments in the feeding rate were not tested, because a higher feeding rate would create an oxygen demand that would be difficult to satisfy in large-scale fermentations.

Scale-up of the fermentation process

To validate our laboratory-scale process at the pilot scale, we conducted fermentations in a 1300 L fermenter using a constant impeller tip speed (7 m/s) as a scale-up criteria [34–36]. Because of its simplicity, this is one of the most used scale-up methods. The pilot-scale tank had a height/diameter ratio of 2.8 and was equipped with three equally spaced Rushton turbines that have six blades and four diametrically opposed baffles (Supplementary Fig. 1). An inoculum bioreactor was used as a step between shake flask cultures and the pilot-scale bioreactor, so that the inoculum for the latter would have the same high concentration of cells and relative volume. The components of the medium, relative glycerol feeding rate, and pH and temperature of the process were the same as those used for the laboratory-scale fermentations.

Figure 2 and Table 4 show the growth and the SGase activity for the pilot-scale fermentation. The final cell density, substrate yield, and SGase productivity were similar to those obtained in the laboratory-scale fermentations. Glycerol did not accumulate, indicating that the substrate was efficiently consumed throughout the fermentation. Values of dissolved oxygen tension reached using 1 VVM of air were also similar for the laboratory and pilot-scale fermentations, indicating that the working conditions were successful in achieving similar oxygen transfer rates, one of the major limitations when scaling-up fermentation processes [37].

Scaling up *E. coli* fermentation processes from the laboratory to the pilot plant often lowers yields due to various factors, including insufficient oxygen transfer [38], plasmid loss [39], and acetate formation from high local concentrations of glucose caused by insufficient mixing [40–42].

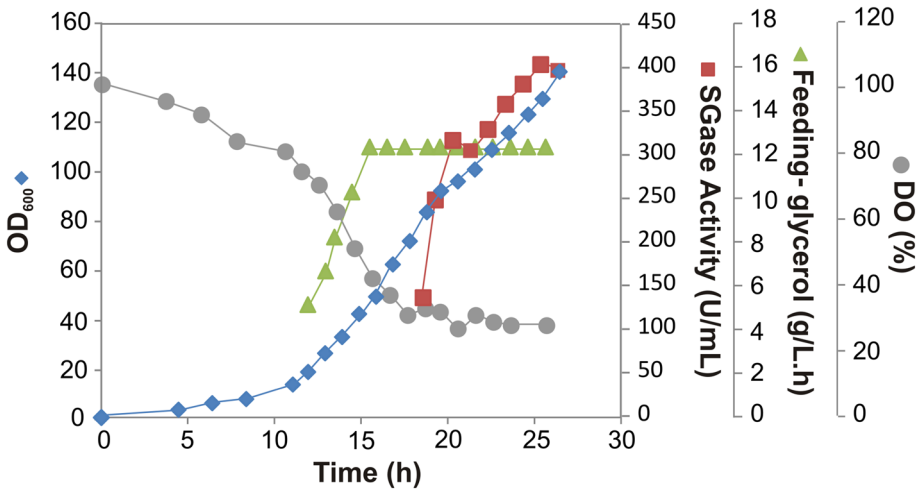

Fig. 2 Fed-batch fermentation of BL21(DE3)AI strain harboring the pKCN-BAD-SGase pGro7 plasmid at a 1300-L scale. Time courses of $OD_{600\,nm}$ (blue diamond), SGase activity (red square), flow rate of glycerol (green triangle), and dissolved oxygen (gray circle) are shown. The fermentations were conducted in duplicate. The figure shows the average of the duplicate runs. In all of the cases, the data varied by less than 10%

Table 4 Comparison of fed-batch fermentations at the laboratory and pilot plant scales

Scale	1300 l	3 l
Final OD_{600}	140	150
Residual glycerol (g/l)	0	0
Y/S (dry cell weight/glycerol consumed)	0.41	0.40
SGase activity (U/ml)	410	400
Fermentation process time (h)	25	25
Productivity (U/ml/h)	16.4	16

Fermentations of *E. coli* BL21(DE3)AI strain harboring the pKCN-BAD-SGase pGro7 plasmid were conducted in duplicate. The table shows the average of the duplicate runs. In all of the cases, the data varied by less than 10%

In our pilot-scale experiments, the SGase activity was slightly higher than what we expected, when we considered the data from our small-scale fermentations. The yield coefficient $Y_{X/S}$ was close to the theoretical value for glycerol (0.45) and similar to the yields obtained at the laboratory-scale, indicating that the scale-up criteria adopted for the bioprocess were successful. Taken together, these results suggest that further scale up to 25 m^3 bioreactors for the commercial production of the SGase is feasible.

Isolation of the SGase by thermolysis

E. coli has a major disadvantage as a host for producing proteins, in that this bacterium does not efficiently release the product to the medium. Thus, methods for releasing recombinant proteins from cells following fermentation are required. High-pressure homogenizers and bead mills have typically been used at the industrial scale [43, 44]. The disruption of cells by mechanical methods, however, presents several problems: (1) all soluble cellular proteins are released, which renders subsequent purification difficult and more expensive; (2) mechanical disruption results in the micronization of cell debris and increases viscosity by releasing DNA, which reduces the efficiency of particulate removal during subsequent centrifugation or membrane filtration; and (3) the required machines have a high capital cost.

Since the SGase is a thermostable protein, we decided to evaluate a thermolysis approach to isolate it. First, we tested the effect of heat on the release of the product, by raising the temperature of the fermentation broth directly at the end of the fermentation at the 1300 L scale. The activity and the purity of the SGase in the supernatant were analyzed. Unfortunately, these initial tests showed no activity recovered from the supernatant. In all of the cases, SDS PAGE analysis revealed that the SGase remained associated with the insoluble fraction after the heat treatment (Fig. 3a).

The SGase is highly homologous to BGPh from *Pyrococcus horikoshii*, which is associated with the membrane in the native organism [45]. This information suggested that the SGase might associate with the *E. coli* membrane after cells are disrupted by heat and that adding a detergent could help to release the enzyme.

The subsequent experiment confirmed that addition of 0.2% Triton X-100 to the fermentation broth releases the enzyme from the insoluble fraction (Fig. 3b). However, the high cost of Tritron X-100 prevents its use for producing the SGase at an industrial scale. Therefore, we sought to identify a cost-effective substitute for Triton X-100. We repeated the thermolysis procedure, again by incubating the broth at 70 °C for 30 min, but in the presence of various ionic and

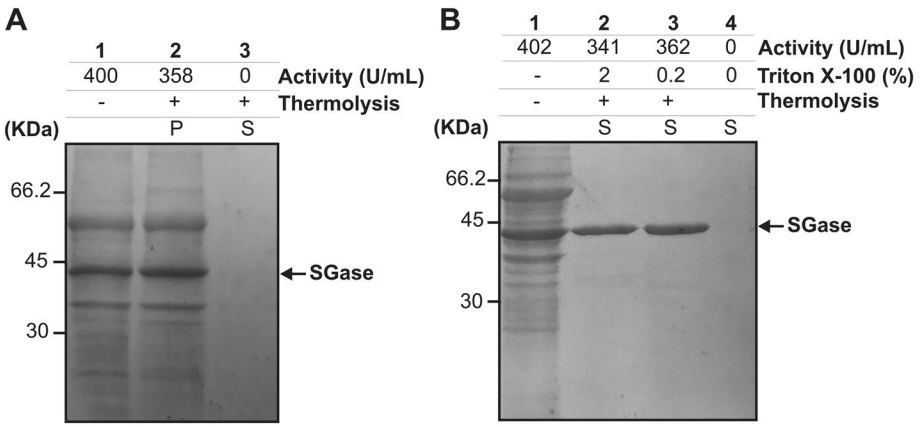

Fig. 3 Isolation of the SGase by thermolysis. **a** SDS PAGE of cell extracts obtained from a fermentation, by high-pressure homogenization without centrifugation (line 1), or by thermolysis without detergent followed by centrifugation (lanes 2–3). **b** SDS-PAGE of cell extracts obtained a fermentation, by high-pressure homogenization without centrifugation (lane 1), or by thermolysis in the presence of 2, 0.2%, or no Triton X-100 (lanes 2, 3 and 4, respectively) followed by centrifugation. The figure shows the average values of three experiments that have standard deviation less than 10%. *P* pellet, *S* supernatant

non-ionic detergents. Figure 4 shows that all of the non-ionic detergents tested resulted in a high recovery of the SGase. 0.2% lauryl alcohol ethoxylate (7 mol) led to a recovery of the SGase in the soluble fraction that was greater than 90%. This detergent was chosen for use at the industrial scale because of its low toxicity and cost. The addition of ionic detergents abolished nearly all activity, presumably by denaturing the SGase. After thermolysis, continuous centrifugation easily removed the solids. 10K hollow-fiber cartridges concentrated the resulting supernatant five-fold, producing a final SGase preparation that was stable at room temperature (data nor shown).

Several reports indicate that incubation of *E. coli* above 60 °C releases cytoplasmic proteins [16, 17]. Watson et al. [46] and Ren et al. [15] reported the release of cytoplasmic proteins from *E. coli* within ten minutes of exposure to temperatures above 70 °C. Recently, an effective thermolysis procedure was described to isolate peptides fused to a thermostable protein [47]. In addition to reduced capital expenditures and simplified recovery of the target protein, thermolysis has further advantages for use at the industrial scale, including killing the host and deactivating proteases.

The procedure described here to recover the SGase is, to the best of our knowledge, the first one combining thermolysis with the use of a non-ionic detergent. The protocol might serve to purify thermostable proteins associated with membranes or that are prone to aggregate, because hydrophobic interactions are favored at the high temperatures used to disrupt cells.

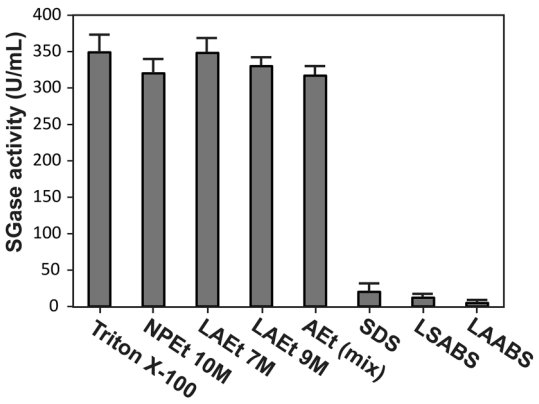

Fig. 4 Recovery of the SGase by thermolysis in the presence of different detergents. SGase activity was measured after thermolysis in the presence of 0.2% of each detergent. NPEt 10M: nonylphenol ethoxylate 10 mol, LAEt 7M: lauryl alcohol ethoxylate 7 mol, *AEt* commercial mixture of alcohols ethoxylate, *SDS* sodium dodecyl sulfate, *LSABS* linear sodium alkylbenzene sulphonate, *LAABS* linear ammonium alkylbenzene sulfonate

Cost analysis

Previously, we demonstrated that 260,000 units of the SGase (which is equivalent to 7 g of the enzyme) is sufficient to completely remove the average amount of SGs (100 ppm) present in one metric ton of soybean-derived biodiesel [10, 11]. The process illustrated in in Fig. 5 consist of a high cell density, fed-batch fermentation, a thermolysis step in the presence of a non-ionic detergent added to the fermenter followed by solids removal by continuous centrifugation and ultrafiltration to concentrate and formulate the final product.

At 1300 L scale, and considering an efficiency of recovery of 70%, approximately one liter of culture could provide the amount of enzyme required to treat one ton of biodiesel. Based on this calculation, and assuming a similar yield, we estimated the cost of producing the SGase at the 25,000 L scale, using crude glycerol as a feedstock and thermolysis followed by centrifugation and ultrafiltration to recover the enzyme. Figure 6 and Supplementary File 1 show the results. The final cost per dose is estimated to be $0.71.

Raw materials include all of the ingredients of the culture medium, where crude glycerol represents 50% of the total cost in our case. Utilities comprise cooling and process

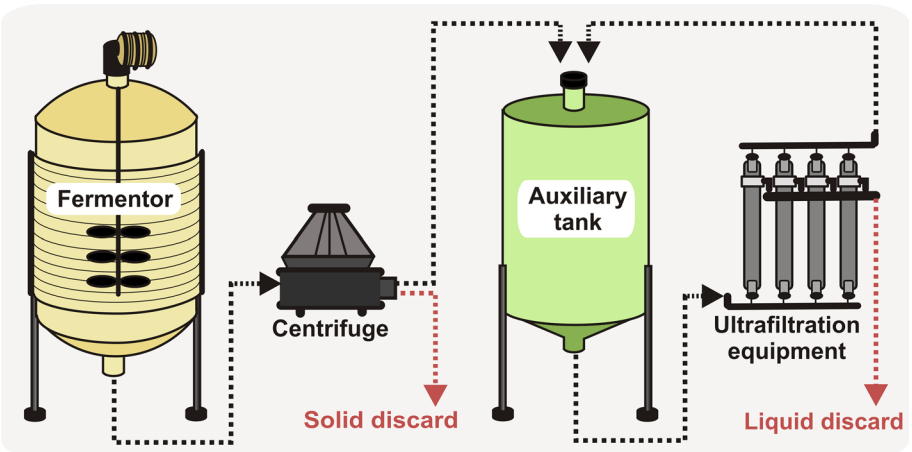

Fig. 5 Flow diagram for the proposed process to produce and purify the SGase. The process includes high cell-densitiy fermentation, thermolysis, continuous centrifugation and ultrafiltration

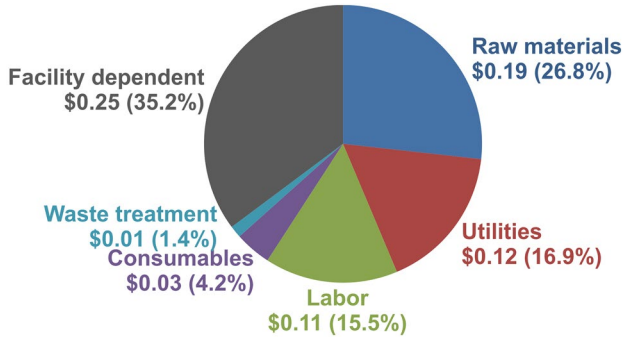

Fig. 6 Cost estimate for the production of the SGase. Values were estimated for the process described in Fig. 5 at the 25,000 L scale, using crude glycerol as a carbon source

water, steam, and electricity. Facility-dependent costs include depreciation and insurance. All of our costs were estimated for a facility located in Argentina, one of the largest producers of biodiesel in the world.

Detailed cost analyses for producing industrial enzymes are scarce in the public domain. Recently, Potvin and coworkers presented a cost analysis for the large-scale production of phytase in *Pichia pastoris*. They estimated a production cost equivalent to 5 cents per gram [48], lower than the cost presented here, which is approximately 10 cents per gram. However, they used a semi-continuous fermentation at the 180,000 L scale, which due to longer production times and reduced culture downtimes results in higher volumetric productivities. Klein-Marcuschamer et al. analyzed how the cost of cellulases impacted ethanol production, finding that the breakdown of the production cost for the enzymes is similar to the one that we report here [49].

In our case, the production of the SGase at the 25,000 L scale could provide a highly affordable solution for eliminating SGs that contaminate biodiesel. A facility with this capacity should provide sufficient enzyme to treat more than 8 million tons of biodiesel, a value which represents 40% of worldwide production in 2016 [50].

Conclusions

We have developed a cost-effective bioprocess for producing a SGase in *E. coli*. The SGase is an enzyme capable of removing biodiesels derived from vegetable oils SGs, the major contaminant present. The process involves a high cell density, fed-batch fermentation that uses crude glycerol as the carbon source,a waste product from biodiesel production. The fermentation is followed by thermolysis in the presence of a non-ionic detergent to release the enzyme from cells. Centrifugation separates the solids from the supernatant. Ultrafiltration concentrates and formulates the final product. We estimate that, using this process on a large scale, the dose of enzyme required to eliminate SGs from biodiesel will have a manufacturing cost below $1. This novel technology should be attractive to biodiesel producers, by facilitating the global adoption of the renewable fuel. In addition, the low-cost process described here could be a general method for producing other thermostable proteins in *E. coli*.

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