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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS - ORIGINAL PAPER

Strain engineering and process optimization for enhancing the production of a thermostable steryl glucosidase in *Escherichia coli*

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Abstract Biodiesels produced from transesterification of vegetable oils have a major problem in quality due to the presence of precipitates, which are mostly composed of steryl glucosides (SGs). We have recently described an enzymatic method for the efficient removal of SGs from biodiesel, based on the activity of a thermostable β-glycosidase from *Thermococcus litoralis*. In the present work, we describe the development of an *Escherichia coli*-based expression system and a high cell density fermentation process. Strain and process engineering include the assessment of different promoters to drive the expression of a codon-optimized gene, the co-expression of molecular chaperones and the development of a high cell density fermentation process. A 200-fold increase in the production titers was achieved, which directly impacts on the costs of the industrial process for treating biodiesel.

Keywords Steryl glucosidase · Biodiesel · *Thermococcus litoralis* · Thermostable β-glucosidase · Process development

Abbreviations

IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria–Bertani
PCR	Polymerase chain reaction
pNPG	*p*-Nitrophenyl β-D-glucopyranoside
SG	Steryl glucoside
SGase	Steryl glucosidase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Biodiesels are renewable fuels consisting of fatty acid methyl esters synthesized via transesterification of oils and fats with short chain alcohols. They offer several technical advantages over petrodiesel, including superior lubricity, higher cetane number, lower toxicity, higher biodegradability, higher flash point, negligible sulfur and aromatics content, and lower overall exhaust emissions [18]. However, being mostly produced from vegetable oils, including those from soybean, palm, sunflower, rapeseed and jatropha, some unwanted side products that form insoluble contaminants are also generated. These sediments may plug filters in engine fuel delivery systems and form deposits on engine injectors [6], which affects the acceptance of biodiesel as an alternative fuel [17, 30]. Steryl glucosides (SGs), present in different biodiesels at concentrations ranging from 10 to 300 ppm [25, 29], have been identified as the major component of such sediments [8, 17, 22, 30]. Particles composed of clumped SG molecules also promote

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aggregation or precipitation of other compounds in the biodiesel, such as saturated monoacyl glycerides (SMGs), which further reduces the fluidity of biodiesel and increases the likelihood of clogging [6, 19]. The presence of SGs thus becomes the greatest limitation for the implementation of diesel blendings with higher amounts of biodiesel obtained from soybean and palm oil, in some cases limited to be no more than 5% [19].

The selective removal of SGs may produce biodiesels of superior quality, helping these renewable fuels to be adopted by consumers and therefore contributing to the development of the biodiesel industry. SGs can be completely removed from biodiesel by distillation, but the process is energy-intensive and expensive and therefore reduces the cost efficiency and gain in net energy of biodiesel production [1, 23, 29]. Other methods, including cold soak filtration, centrifugation and filtering through diatomaceous earth, magnesium silicate and bleaching earth have shown to have limited utility in providing biodiesels with high quality [19, 20, 29].

We have recently described an efficient method for the removal of SGs from biodiesel based on the use of an enzyme with steryl glycosidase activity (SGase) [21]. The enzyme hydrolyzes SGs, generating sterols, which are completely soluble in biodiesel, and glucose which is eliminated subsequently during the water-washing steps that follow transesterification. The SGase employed in this enzymatic method is a thermostable β-glucosidase from *Thermococcus litoralis*, which was expressed in *Escherichia coli*. The production method has not yet been optimized, and because the enzyme is the main consumable for this process to treat biodiesel, it constitutes a critical step in developing a cost-effective procedure. In the present work, we describe the development of a cost-effective process for the production of SGase in an engineered *E. coli* strain, based on modifications of the expression system, the co-expression of molecular chaperones and the optimization of growth conditions.

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, and *E. coli* BL21(DE3)AI served as expression host. *E. coli* strains were made chemically competent with a kit from Zymo Research (USA). The concentrations of kanamycin and chloramphenicol used in the study were 50 and 20 mg/L, respectively.

Vector construction

All the expression vectors constructed were based on the backbone of the pET24a plasmid (Novagen), maintaining the promoter regions integrated at the same location in all cases. PCR primers used to amplify the promoter regions are listed in Table 1. PCR products and the pET24a vector backbone were digested with BglII and NdeI and ligated to generate all the vectors of the pKCN series. The synthetic gene of SGase (WP_004069094.1) from *T. litoralis* was inserted in each vector between the NdeI and HindIII restriction sites. The resulting plasmids are listed in Table 2.

Table 1 Oligonucleotides used in this study

Name	Sequence (5′–3′)	References
Cp8fwBglII	AGGAGATCTCATTCTTTAGTTTA	[10]
Cp8revNdeI	AGGCATATGAACAGTACTCACCT	
LacfwBglII	AGGAGATCTTAACCAGGCTTTAC	[28]
LacrevNdeI	AGGCATATGGGATCCTTCCACAC	
TacfwBglII	AGGAGATCTGACTTCATATACCC	[2]
TacrevNdeI	AGGCATATGGGATCCATTATACG	
BADfwBglII	AGGAGATCTCTGACGCTTTTTAT	Thermo Fisher Scientific
BADrevNdeI	AGGCATATGCAGTAGAGAGTTGC	
T5fwBglII	AGGAGATCTTAAAATTCAAAA	[7]
T5revNdeI	AGGCATATGGGATCGAATCT	
T710%fwBglII	AGGAGATCTGAATTCGAAATTAATACGG	[9]
T710%revNdeI	AGGCATATGGTACCAAGCTTTCT	
T725%fwBglII	AGGAGATCTGAATTCGAAATTAATACGT	
T725%revNdeI	AGGCATATGGTACCAAGCTTTCT	

Table 2 Plasmids used in the present work

Plasmids	Relevant properties	Drug resistance	References
pET24a-SGase	P_{T7lac}::SGase[a]	Kan^R	Novagen
pKCN-cp8-SGase	P_{CP8}::SGase[a]	Kan^R	This work
pKCN-lacUV5-SGase	P_{lac}::SGase[a]	Kan^R	This work
pKCN-tac-SGase	P_{tac}::SGase[a]	Kan^R	This work
pKCN-BAD-SGase	P_{BAD}::SGase[a]	Kan^R	This work
pKCN-T5-SGase	P_{T5}::SGase[a]	Kan^R	This work
pKCN-$T7_{25\%}$-SGase	$P_{T725\%}$::SGase[a]	Kan^R	This work
pKCN-$T7_{10\%}$-SGase	$P_{T710\%}$::SGase[a]	Kan^R	This work
pTf16	P_{araB}::*tig*[b]	Cm^R	Takara
pG-Tf2	$P_{Pzt\text{-}1}$::*groEL/ES/tig*[b]	Cm^R	Takara
pGro7	P_{araB}::*groEL/ES*[b]	Cm^R	Takara
pG-KJE8	P_{araB}::dnaK/J. $P_{Pzt\text{-}1}$::*groEL/ES*[b]	Cm^R	Takara
pKJE7	P_{araB}::dnaK/J[b]	Cm^R	Takara

Kan^R kanamycin resistance, *Cm^R* chloramphenicol resistance

[a] Plasmids contained the replication origin of pBR322 and SGase gene is under control of the indicated promoters

[b] Plasmids contained the replication origin of pACYC184 and genes encoding chaperones under control of the indicated promoters

Growth conditions for comparative expression studies

Recombinant *E. coli* strains were grown overnight at 37 °C in 2 mL of Luria–Bertani (LB) medium supplemented with the appropriated antibiotic. For expression experiments, 5 mL of semi-defined HM medium [15] was inoculated with the 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 0.12 g/L isopropyl β-D-glucopyranoside (IPTG), or 0.5 g/L of L-arabinose for the *araBAD* promoter. Growth was continued for 6 additional hours at 20, 25, 30, 37, 42 or 46 °C.

Cell disruption and protein analysis

Cells and supernatants of the cultures were separated by centrifugation. The cells pellets were resuspended in a buffer of 20 mM citrate pH 6.0, and 20 mM NaCl, normalized to a final $OD_{600} = 4$ per mL. These cells were disrupted on ice in a GEX 600 Ultrasonic Processor. The cell extracts obtained were separated by SDS-PAGE on 12% gels, and stained with Coomassie Brilliant blue.

Activity assays

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

High cell density fermentation

A seed culture of *E. coli* BL21AI harboring pKCN-BAD-SGase and pGro7 was prepared in a 1-L Erlenmeyer flask containing 0.1 L of LB medium cultivated at 37 °C in an incubator shaking at 200 rpm. Fed-batch fermentation was carried out as previously described [24], in a 2-L fermenter (New Brunswick Bio Flo 115 USA) containing 1 L of semi-defined HM medium. The temperature, stirring and the pH were maintained at 37 °C, 1200 rpm and 7 (by addition of 25% NH_4OH), respectively. The concentration of dissolved oxygen was maintained at 30% of saturated air by enriching the air stream with pure oxygen when necessary. The feeding solution contained 800 g/L of glycerol and 20 g/L of $MgSO_4 \cdot 7H_2O$. The specific growth rate was maintained at 0.25 L/h.

Expression of SGase gene was induced at $OD_{600} = 100$ by adding L-arabinose at a final concentration of 0.5 g/L. Afterwards, the feeding rate was maintained at 15 mL/h. After the fermentation, the cells were disrupted on ice at 1000 bars through a high-pressure homogenizer (GEA Niro Soavi, Panda Plus 2000). The cell debris was heated at 80 °C for 10 min and then centrifuged at 10,000 rpm for 30 min in a bench centrifuge (Eppendorf 5804R). SGase concentration was estimated both by β-glucosidase activity and by densitometric quantification in SDS-PAGE using a scanner and a standard curve (1, 0.75, 0.5 and 0.25 µg/well) of purified His_6-SGase (purity >90% by HPLC) included on each gel [21]. ImageJ software was used to perform the quantitation of the scanned images.

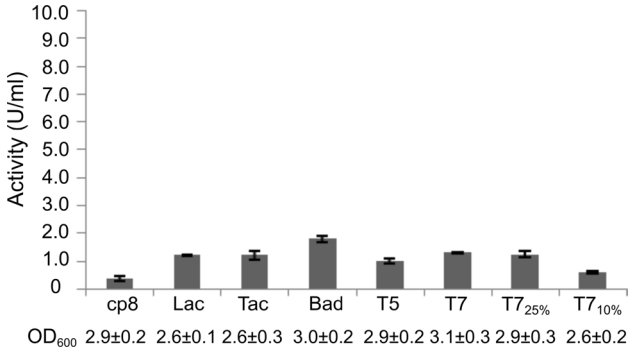

Fig. 1 Profiles of the expression of SGase in *E. coli* BL21AI under different promoters, as determined by β-glucosidase activity. Cell densities (OD_{600}) reached after 6 h of induction are indicated for each culture condition. The results are expressed as mean and deviation standard of at least three independent experiments

Results and discussion

Optimization of SGase expression

In previous work, we were able to produce an SGase from *Thermococcus litoralis* through heterologous expression in *E. coli* [21]. The expression system comprised the SGase gene cloned under the T7 promoter in a pET28a vector, using the *E. coli* BL21(DE3) strain as the host. That production system allows reaching, after 6 h of induction at 30 °C, a final β-glucosidase activity of 1.3 U/mL (see below, Fig. 1), which is not sufficient to establish a cost-effective manufacturing process for SGase at industrial scale. Attempts to produce SGase in alternative hosts such as *Pichia pastoris* and *Corynebacterium glutamicum*, by means of secretory systems for protein expression, were unsuccessful (data not shown). We therefore focused our efforts on improving the productivity of *E. coli* to increase the yields of SGase by means of different strategies.

Selection of the optimal promoter for SGase expression

In general, the development of efficient recombinant gene expression protocols occurs on case-by-case, trial-and-error approaches [3]. This is the situation for the selection of an optimal promoter, where many alternatives are available with different induction systems (i.e., constitutive, positively regulated, and negatively regulated) that also exhibit variable strengths. To select the most effective promoter for the expression of SGase, we constructed a set of vectors where the expression was driven by eight different promoters, the inducible *Lac*, *Tac*, *araBAD*, *T5*, *T7* (and its attenuated versions *T7*$_{25\%}$ and T7$_{10\%}$), and the constitutive *CP8* (Table 2). To reduce potential effects on expression unrelated to the features of the promoter systems themselves, all vectors used were designed in such a way that the backbones were identical and the expression cassettes were in all cases integrated at the same location. The strain *E. coli* BL21AI was used as host, because it carries a chromosomal copy of the T7 polymerase under the control of the *araBAD* promoter (which is required to drive the expression from *T7* promoters) and also because it is unable to catabolize L-arabinose, the inducer of the *araBAD* promoter system. This strain was transformed with each plasmid, and the resulting strains were tested for SGase production in batch cultures incubated at 30 °C, where the expression of the enzyme was triggered by adding the corresponding inducer. In all cases, the final OD_{600} reached after 6 h of incubation was similar (Fig. 1). The production of SGase was measured by analyzing its β-glucosidase activity in extracts obtained from cultures normalized to a final $OD_{600} = 4$, and the results obtained show that the highest activity was reached using the *araBAD* promoter (Fig. 1). The activity obtained with this strain represents a 40% increase compared to that of the starting production strain based on a *T7* promoter expression. In addition to the higher yield, the use of *araBAD* promoter has a great advantage over the remaining inducible promoters for producing SGase on an industrial scale, because the cost of L-arabinose is about 10 times lower than that of IPTG.

Co-expression of SGase with molecular chaperones

The analysis of soluble and insoluble protein fractions obtained from *E. coli* BL21AI harboring pKCN-BAD-SGase reveals that, despite exhibiting enhanced expression, most of the SGase synthesized exists in insoluble aggregates (Fig. 2a). The co-expression of recombinant proteins with molecular chaperones has been previously used to efficiently reduce the formation of aggregates [4, 5]. However, the complexity of predicting the optimal chaperone system for a particular protein requires trial-and-error experiments [26]. For this reason, we evaluated the effect of co-expressing SGase with different combinations of chaperones expressed from five different plasmids, pG-KJE8, pGro7, pKJE7, pG-Tf2 or pTf16. The chaperones encoded on each plasmid are detailed in Table 2. All these plasmids contain origins of replication and selection markers compatible with the pKCN-BAD-SGase expression vector.

Each chaperone plasmid was co-transformed with pKCN-BAD-SGase in *E. coli* BL21AI, and SGase expression was evaluated in batch cultures. β-glucosidase activity analysis revealed that SGase production increased 2.5-fold when the expression of SGase was carried out in the presence of the chaperones GroES/EL provided by the plasmid pGro7 (Fig. 2b). The remarkable increase in the activity can be explained by an enhanced solubility of SGase due to the overexpression of GroES/EL, as shown by SDS-PAGE analysis of soluble and insoluble protein fractions of crude

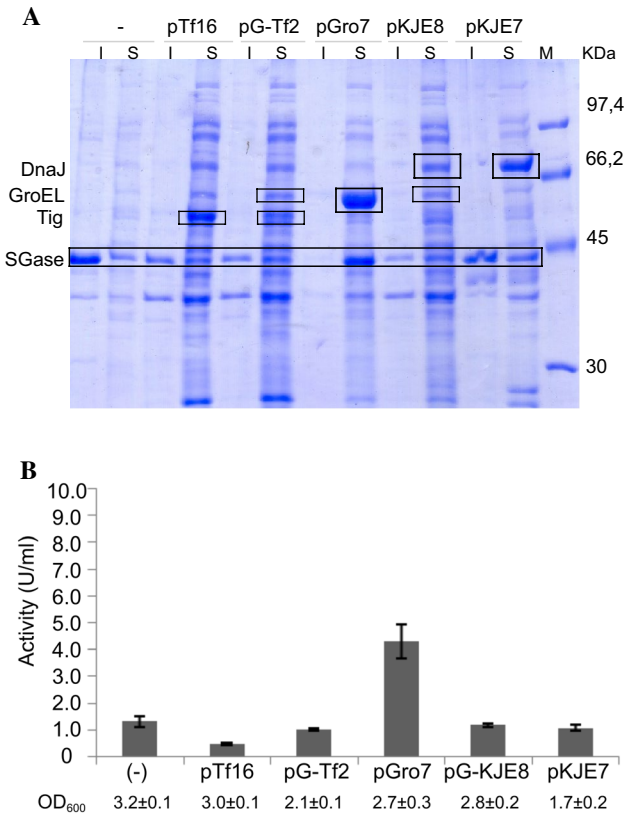

Fig. 2 Expression of SGase in *E. coli* BL21AI assisted by different chaperones. **a** SDS-PAGE analysis of strains harboring plasmid pKCN-BAD-SGase and each of five plasmids containing different combinations of chaperones. *M* protein markers, *S* soluble fraction of whole cell protein; and *I* insoluble fraction of whole cell protein. **b** Profiles of SGase expression as determined by β-glucosidase activity. Cell densities (OD_{600}) reached after 6 h of induction are indicated for each culture condition. The results are expressed as mean and deviation standard of at least three independent experiments

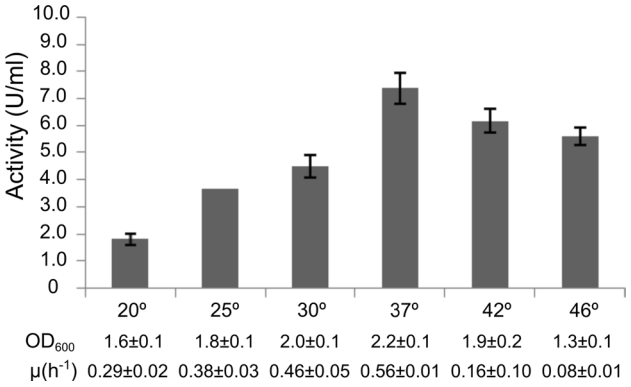

Fig. 3 Profiles of the expression of SGase in *E. coli* BL21AI incubated under different temperatures of induction, as determined by β-glucosidase activity. Cell densities (OD_{600}) reached after 6 h of induction, and specific growth rate [$\mu(h^{-1})$] are indicated for each culture condition. The results are expressed as mean and deviation standard of at least three independent experiments

cell extracts (Fig. 2a). This analysis also reveals that the co-expression of some chaperones, such as those present on pG-Tf2, pKJE7 and pG-KJE8, can negatively influence the yield of total protein. Similar observations have been previously reported and these side effects of co-expressing chaperones were attributed to the inhibition of cell growth and the proteolysis of recombinant protein [11, 14].

The use of a single inducer to drive the expression of proteins from pGro7 and pKCN-BAD-SGase is very advantageous in terms of costs and operations. However, the great effect of GroES/EL on SGase production raised the question whether pGro7 could exert a greater effect on SGase expression when driven by other promoters. To test this, the strains used in the previous section in the comparative experiment of SGase expression under different promoters were transformed with pGro7, and SGase production assayed as previously described. The results obtained were comparatively similar to those presented in Fig. 1 (Supplementary Fig. 1), confirming the *araBAD* as the preferred promoter for SGase expression.

Optimization of process conditions

Lowering growth temperatures is a well-known strategy to reduce the in vivo aggregation of recombinant proteins, due to a slower rate of protein synthesis and the reduction of hydrophobic interactions, which strongly depend on temperature and in turn determine the extent of aggregation [12, 27]. The experiments described above were performed at a temperature of induction arbitrarily set at 30 °C, to provide a growth rate compatible with an industrial process. To determine the effect of the temperature of incubation on the expression of SGase with the GroES/EL chaperone, *E. coli* BL21AI harboring plasmids pKCN-BAD-SGase and pGro7 was induced to express SGase in batch cultures incubated at 37, 30, 25 and 20 °C. However, not only did lower temperatures not increase SGase production, but the highest levels of β-glucosidase activity were observed at a temperature of induction of 37 °C (Fig. 3). An increase in the yield of folded recombinant protein at higher temperatures was previously reported in *E. coli* for four thermophilic enzymes, where their expression in the soluble fraction was possible only at temperatures as high as 46 °C [13]. When SGase expression was tested at 42 and 46 °C after induction, β-glucosidase activity did not increase compared to that obtained at 37 °C and, as expected, the growth rate of the cells was lower, therefore reducing the volumetric productivity (Fig. 3).

Fed-batch production of SGase

To test the robustness of the engineered *E. coli* strain created in high cell density cultures, a fed-batch fermentation

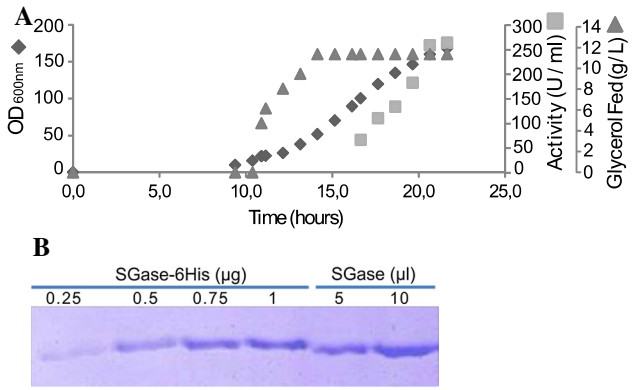

Fig. 4 Development of a fed-batch process for SGase production. **a** Time courses of SGase activity (*grey squares*), biomass (*black diamonds*), and the flow of glycerol (*grey triangles*) during a fed-batch fermentation in the 1-L fermenter with a semi-defined medium. **b** SDS-PAGE of cell-free extracts obtained from this fermentation process. *Lanes 1–4* were loaded with increasing amounts of a standard of purified His_6-SGase. *Lanes 5–6* were loaded with 5 and 10 µL of fermentation samples diluted 1/60. Densitometric quantification of the scanned images employing ImageJ software indicated a concentration of 7 g/L of SGase in the extract

process was developed in a 1-L lab-scale bioreactor. The recombinant strain *E. coli* BL21AI harboring plasmids pKCN-BAD-SGase and pGro7 was grown at 37 °C in 1 L of semi-defined medium using glycerol as a carbon source. After consumption of the initial amount of glycerol, a balance mass equation was used to determine the feeding rate in order to maintain the specific growth rate at 0.25 h^{-1} [16]. When cultures reached an OD_{600} of 100, 0.5 g/L of L-arabinose was added to induce the expression of GroES/EL and SGase. The temperature was kept at 37 °C, and the feeding rate was maintained at a constant value of 15 mL of nutrient feeding solution per liter per hour.

Figure 4a shows that the basal expression of SGase provided 66 U/mL at the point where the inducer was added to the culture, and that the production of SGase continuously increased until a maximum activity of 260 U/mL was reached 6 h after the induction. The final activity achieved represents a 200-fold overall improvement in production titers from the starting process. The productivity of SGase, growth profile and glycerol consumption remained unaltered in more than ten different fermentation experiments, confirming the stability of this expression system and its suitability to be used in an industrial process. Protein quantification of the extracts obtained in these fermentation processes indicates a final concentration of SGase of 7 g/L (Fig. 4b).

The amount of fermentation broth required for the enzymatic removal of SG from 1 ton of a typical industrial crude soybean biodiesel containing 75 ppm SGs is about 1 L [21]. It has been estimated that the cost of *E. coli* industrial fermentation is far below $1 per liter [31]. Considering that the cost of removing SG by distillation is estimated to be between $20 and $35 per ton [21], the manufacturing process for SGase presented here makes the use of this enzyme a cost-effective alternative for the implementation of this environmentally friendly process at industrial scale.

Conclusions

Here we describe the optimization steps to increase the microbial production of an SGase enzyme, the main consumable of a novel process for the removal of SGs from biodiesel. A high cell density fermentation process was developed for the engineered strain, with a fed-batch strategy using low-cost salt medium, carbon source and inducer. The improvements in the production process resulted in a 200-fold higher activity of SGase. The results presented here will facilitate the implementation of an enzymatic biodiesel refining process and may therefore contribute to the adoption of this renewable fuel.

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Compliance with ethical standards

Conflict of interest The authors declare that they have competing interests as inventors of a patent. The authors declare that they have no non-financial competing interests.

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