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β-Glucosidase immobilisation on synthetic superparamagnetic magnetite nanoparticles and their application in saccharification of wheat straw and *Eucalyptus globulus* pulps

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β-Glucosidase from *Trichoderma reesei* was immobilised on synthetic superparamagnetic magnetite (Fe_3O_4) nanoparticles with a mean diameter of 10 nm and were used to supplement cellulase in the enzymatic hydrolysis of three substrates: wheat straw pretreated by steam explosion, *Eucalyptus globulus* pretreated by hydrothermolysis and *E. globulus* pretreated by hydrothermolysis followed by alkaline extraction. The hydrolysis yields for each pretreated material, using immobilised *β*-glucosidase (I-*β*G) and free cellulase, were 76.1%, 83.6% and 75.6%, respectively, and resulted in an improved hydrolysis yields compared with only cellulase. These yields were at most 10% lower than yields reached with free enzymes. The (I-*β*G) was magnetically recovered and successfully reused twice. The differences in the hydrolysis yields were not significant ($p > 0.05$) in the case of steam-exploded wheat straw and *E. globulus* pretreated by hydrothermolysis followed by alkaline extraction. The immobilisation of enzymes provides an opportunity to reduce the costs of enzymes in the bioethanol production process.

Keywords: magnetite nanoparticles; superparamagnetism; *β*-glucosidase immobilisation; saccharification

1. Introduction

Studies of the production of bioethanol from lignocellulosic biomass have dramatically increased over the years due to its potential use as an alternative fuel [1] with various benefits including the reduction of greenhouse gas emissions by approximately 30–85% in comparison with gasoline, promotion of energy independence and increased rural economic development [2].

Lignocellulose is the most abundant renewable biomass produced by photosynthesis and consists primarily in plant cell wall materials, a complex natural composite with three main biopolymers: cellulose, hemicellulose and lignin [3,4]. The bioconversion of this substrate to bioethanol involves three major steps: a pretreatment process to reduce substrate recalcitrance; enzyme catalysed hydrolysis of cellulose and hemicellulose components to fermentable sugars and fermentation to produce ethanol [3].

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Enzymatic hydrolysis is a process catalysed by a group of enzyme-denominated cellulases with different enzymatic activities that synergically act over cellulose. Cellulases are produced extracellulary by a large number of microorganisms including fungi, actinomycetes and bacteria [5]. Cellulase from *Trichoderma reesei* is the most common choice for enzymatic hydrolysis in bioethanol production due to its high level of extracellular cellulase [5,6].

There are three major types of cellulase enzymes. Endo-β-1,4-glucanase (EG or endo-1,4-β-D-glucan-4-glucanhydrolase, EC 3.2.14) produces nicks in the cellulose polymer exposing reducing and non-reducing ends. Exoglucanases, including cellodextrinases (1,4-β-D-glucan-4-glucanhydrolase, EC 3.2.1.74) and cellobiohydrolase (CBH or 1,4-β-D-glucancellobiohydrolase, EC 3.2.1.91), act upon reducing and non-reducing ends to liberate cello-oligosaccharides, cellobiose and glucose. Finally, β-glucosidase (BG-EC 3.2.1.21) cleaves the cellobiose to release glucose, thereby completing the hydrolysis process [5].

The technology to generate fermentable sugars by enzymatic hydrolysis is a key step in the production of bioethanol from pretreated lignocellulose material, although the enzyme cost has been significantly reduced in the last decade still is considered an economic barrier to full-scale process commercialisation [7] because it contributes to cellulosic ethanol production cost in approximately 20–40% [5]. This high cost is due to the poor activity of cellulases, the large amounts used and long incubation times [2].

Since covalent bonds provide the strongest linkages between enzyme and carrier [8], covalent enzymatic immobilisation presents an opportunity to retain and recycle an expensive ingredient of the bioethanol process reducing the costs of catalyst production. Magnetite (Fe_3O_4) nanoparticles, a magnetic iron oxide, have been widely applied to immobilise enzymes and proteins such as lipase [9], glucose oxidase [10], bovine serum albumin (BSA) [11] and homing peptides [12]. Cellulase and β-glucosidase has also been successfully immobilised using Fe_3O_4 as support [13–16]. Immobilised β-glucosidase (I-βG) from *Aspergillus niger* on Fe_3O_4 particles functionalised with polyethyleneimine was used in enzymatic hydrolysis of lignocellulosic material with a good hydrolysis yield [14]. It has also been immobilised on nanoparticles functionalised with 3-aminopropyltriethoxysilane (3-APTS) [15] and on polyacrylamide-Fe_3O_4 beads [16].

The main limitation of commercial cellulase is the relatively low amounts of β-glucosidase enzyme. Therefore, the cellulose complex must be supplemented with β-glucosidase enzyme to reach higher concentrations of glucose and to avoid the inhibition of endo- and exoglucanases by cellobiose. In this work, the covalent immobilisation of β-glucosidase enzyme on synthetic superparamagnetic Fe_3O_4 nanoparticles and their application in the saccharification process were studied. The magnetic property of the bioconjugates enzyme/Fe_3O_4 nanoparticles should improve the recovery of the enzyme magnetic support system in biotechnology applications, such as the saccharification process of pretreated lignocellulosic materials used in bioethanol production.

2. Materials and methods

2.1. *Materials*

All reagents were pro-analysis and used as received without further purification. $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, ammonia 25% wt, 3-APTS, glutaraldehyde 25% wt, cellobiose, D-glucose, D-xylose and D-arabinose were purchased from Merck. *p*-Nitrophenyl β-D-glucopyranoside and *p*-nitrophenol were purchased from Sigma. Nanopure water (NPW) was used in all preparations, unless otherwise stated. Cellulase complex (NS50013) and β-glucosidase (NS50010) enzymes were kindly provided by Novozymes (USA).

2.2. *Synthesis of Fe_3O_4 nanoparticles*

Superparamagnetic Fe_3O_4 nanoparticles with a mean diameter of 10 nm were synthesised by co-precipitation route from Fe^{2+}/Fe^{3+} in aqueous solutions (molar ratio 1:2) by adding ammonia under constant stirring at 10,000 rpm [17]. Shape, morphology and size distribution of the Fe_3O_4 nanoparticles were determined using transmission electron microscopy (TEM). The images were taken using a JEOL JEM 1200EXII microscope at an accelerating voltage of 120 kV. The samples were re-dispersed in a NPW:ethanol (1:1) matrix, and then deposited on a 200-mesh carbon-nitrocellulose-coated TEM copper grid. To determine the size distribution and the mean average size, 100 particles were measured for each sample.

2.3. *Covalent immobilisation of β-glucosidase*

Covalent bonding between enzymes and carriers is based on a chemical reaction between the active amino acid residues on the enzyme surface and the active functional groups that are attached to the carrier's surface [18]. The first step to immobilise the enzyme is to prepare amino-functionalised Fe_3O_4 nanoparticles. Thus, 1.5 g of dry Fe_3O_4 was re-dispersed in 200 mL of ethanol for 30 min by sonication. Then, 2.0 mL of 3-APTS were added and this solution was kept at room temperature overnight under magnetic stirring. Silanized nanoparticles were magnetically harvested and washed repeatedly with ethanol and dried under vacuum at room temperature. Fourier transform infrared spectroscopy (FT-IR, Perkin Elmer) was used to confirm chemical bond formation between active Fe_3O_4 and 3-APTS. The magnetic properties of Fe_3O_4 uncoated and coated by 3-APTS were analysed at 300 K using a MPMS superconducting quantum interference device magnetometer (SQUID, Quantum Design, XL model, applied field ± 20 kOe).

Subsequently, 1.0 g of dry amino-functionalised Fe_3O_4 was re-dispersed by sonication in 100 mL of 5% glutaraldehyde 50 mM and phosphate buffer (pH = 7.4) and later stirred overnight at room temperature. The nanoparticles were then magnetically harvested and washed with buffer solution. Afterwards the nanoparticles were combined with a 50 mL solution of 2% v/v β-glucosidase (Novozymes β-glucosidase) in buffer solution. This mixture was gently stirred overnight at room temperature. (I-βG) on Fe_3O_4 nanoparticles were magnetically separated and washed with phosphate buffer 50 mM at pH 7.4, re-suspended in this buffer solution and stored at 4°C until used. The supernatant and the washing solutions were used to determine the unbound proteins concentration on the functionalised Fe_3O_4 nanoparticles. Immobilised proteins concentration was calculated from the difference between protein concentration in the initial solution and the unbound proteins concentration in the supernatant and washing solutions. These concentrations were determined using the Bio-Rad Protein assay kit based on the Bradford's method through visible spectrometry (Shimadzu UV-1650 PC, 595 nm, $b = 1.0$ cm). BSA was used as protein standard for the calibration curve (linear range 0.2–1.0 mg mL^{-1}, $R = 0.994$). β-Glucosidase specific activity was determined by monitoring the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside and expressed as international units per mg of protein (IU mg^{-1}) [18,19]. Cellulase activity was determined using International Union of Pure and Applied Chemistry procedure [20].

2.4. *Carbohydrates analysis*

A Merck Hitachi LaChrom HPLC equipped with a refractive index detector, a Aminex HPX 87H column (Bio-Rad, Hercules, CA, USA) at 45°C and H_2SO_4 5.0 mM as mobile phase at a flow rate of 0.6 mL min^{-1} was used to quantify cellobiose and monosaccharide concentrations.

Calibration curves of cellobiose (linear range 0.5–7.0 g L^{-1}, $R = 0.999$), D-glucose (linear range 0.5–6.0 g L^{-1}, $R = 0.999$), D-xylose (linear range 0.6–7.0 g L^{-1}, $R = 0.999$) and D-arabinose (linear range 0.1–0.8 g L^{-1}, $R = 0.999$) were made. All experiments were performed in triplicate.

2.5. *(I-βG) time stability*

Percentage of hydrolysis of cellobiose (2.5 g L^{-1}) in sodium citrate buffer 50 mM at pH 4.8 was used to determine the stability of immobilised enzyme for 45 days.

2.6. *Substrate preparation*

Wheat straw was chipped, and 110 g of dry weight were pretreated by steam explosion (WS-SE) at 205°C and 400 psi for 5 min. Pretreated wheat straw was recovered in a cyclone.

Eucalyptus globulus chips were pretreated by hydrothermolysis (EG-H) at 180°C for 15 min in 1 gal reactor (Parr Instruments, Moline, IL, USA). Part of the pulp was subjected to delignification through alkaline extraction (EG-HA) to prevent unproductive binding of the enzymes to lignin. The hydrothermal pulp obtained was mixed with 8% NaOH solution at 10% consistency, 68°C for 1 h in 1 gal reactor. Chemical characterisation of raw and pretreated materials was performed following TAPPI Standard Method [21] and Puls et al. [22].

2.7. *Enzymatic hydrolysis*

Enzymatic hydrolysis was performed using a commercial preparation of *T. reesei* cellulase complex (61.0 FPU mL^{-1}). 20 FPU of cellulase per gram of dry pretreated material was used, and was supplemented with I-βG on 1.0 g of silanized Fe_3O_4. Control experiments were carried out using 20 FPU of cellulase per g of dry pretreated material supplemented with free β-glucosidase (F-βG) and without supplementation of β-glucosidase.

Enzymatic hydrolysis of the pretreated material at 5.0% (w/w) of consistency in sodium citrate buffer 50 mM at pH 4.8 was performed in a shaker at 160 rpm and 50°C for 72 h. Samples were taken at 6, 12, 24, 48 and 72 h. The content of cellobiose and the glucose released by the enzymatic hydrolysis were analyzed by HPLC. The hydrolysis yield was expressed as the percentage of glucose released in relation to the potential glucose in the pretreated material.

3. Results and discussion

3.1. *Fe_3O_4 silanization*

The physical–chemical characterisation of synthesised superparamagnetic Fe_3O_4 nanoparticles with a mean diameter of 10 nm and saturation magnetisation (M_S) of 72.0 emu g^{-1} is well documented in Valenzuela et al. [17]. TEM image shows that the synthesised Fe_3O_4 nanoparticles have spherical shaper with a size distribution between 4 and 15 nm with mean diameter on 10 nm (Figure 1).

Figure 2 displays the FT-IR spectrum of (a) Fe_3O_4 and (b) Fe_3O_4 chemically bonded to 3-APTS. The Fe_3O_4 spectrum shows the characteristic absorption bands of Fe–O bond at 583.05 and 633.75 cm^{-1}. When silanized, these absorption bands slightly shifts to higher wave numbers 584.07 and 634.80 cm^{-1}, respectively, a phenomenon attributed to the formation of Fe–O–Si bonds. Fe–O–OH groups on the surface of Fe_3O_4 nanoparticles are replaced by Fe–O–$Si(O–)_2$–R. Since –$Si(O–)_2$ is more electronegative than H, the strength of the Fe–O bond is enhanced, producing the shift of the absorption bands [23]. The absorption band at 1129.38 cm^{-1}, assigned to the stretching vibration of the C–N bond; the band at 1029.42 cm^{-1}, due to the stretching vibration of the Si–O bond; the band at 3397.08 cm^{-1}, due to the stretching vibrations of –NH_2 and the bands at 1568.04 and 692.92 cm^{-1}

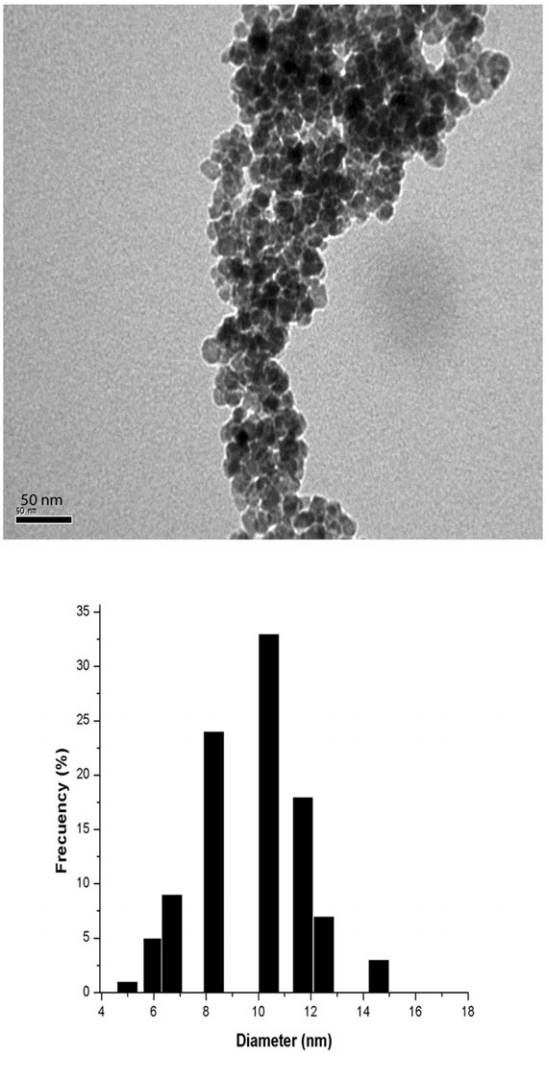

Figure 1. TEM image and diameter distributions of magnetite nanoparticles.

corresponding to bending vibrations of the $-NH_2$ group, confirmed that 3-APTS was chemically bonded to Fe_3O_4.

Figure 3 shows magnetic properties for Fe_3O_4 coated and uncoated with 3-APTS at 300 K. In both the cases, the magnetic curves showed no hysteresis loop and they are reversible. Additionally, neither coercivity nor remanence was observed. The saturation magnetization (M_s) for uncoated Fe_3O_4 and coated were 72.0 and 55.9 emu g^{-1}, respectively. These results agree with Yamaura et al. [24] proving that 3-APTS coating contributes as non-magnetic mass to the total sample volume.

3.2. *ß-Glucosidase immobilisation*

Mean concentration of I-βG determined by the Bradford method was 0.10 ± 0.02 mg mL^{-1}. Specific enzymatic activity of I-βG and F-βG was 110.7 ± 0.2 and 112.3 ± 0.3 UI mg^{-1}, respectively.

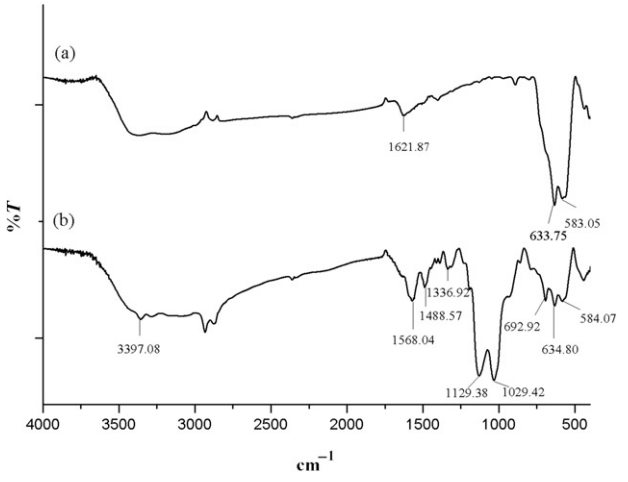

Figure 2. FT-IR spectra of magnetite and magnetite coated with 3-APTS.

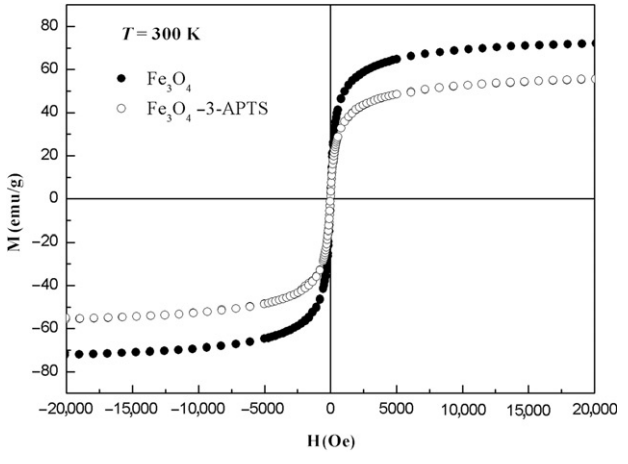

Figure 3. Hysteresis loops magnetite and magnetite coated with 3-APTS.

I-βG hydrolysed 100% of cellobiose 2.5 g mL^{-1} in 60 min and can be magnetically harvested and reutilised over 45 days without losing its ability to hydrolyse cellobiose. These results indicate that the enzyme enzymatic activity was maintained and no denaturation of the immobilised enzyme is observed when stored at 4°C and used for different time periods at 50°C.

3.3. *Enzymatic hydrolysis of pretreated material*

Table 1 shows the chemical composition of raw wheat straw, WS-SE, raw *E. globulus*, EG-H and EG-HA.

The dry weight in basis of glucane in WS-SE was 31.9%, corresponding to 87.6% of solid recovery of glucane from the initial raw material. The xylane component was practically completely solubilised and low amount of lignin was removed, which are characteristics feature

Table 1. Chemical composition of raw wheat straw, WS-SE, raw *E. globulus*, EG-H and EG-HA.

	Wheat straw		*E. globulus*		
Compound	Raw material % dwb	WS-SE % dwb[a]	Raw material % dwb	EG-H % dwb[a]	EG-HA % dwb[a]
Glucane	36.4	31.9	45.6	45.1	44.2
Arabinane	1.9	nd	0.1	nd	nd
Xylane	19.8	1.2	17.5	6.4	2.3
Lignin	13.6	11.0	26.2	23.8	10.5

Notes: nd = not detectable.
[a]Dry weight in basis of raw material.

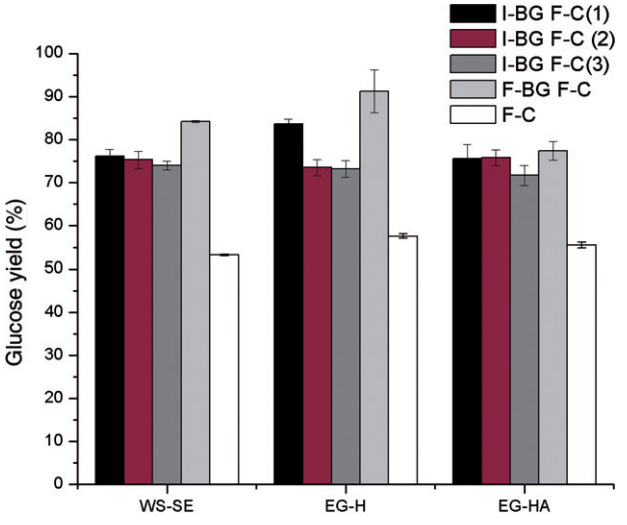

Figure 4. Glucose yields from enzymatic hydrolysis of wheat straw and *E. globulus* pulps at 72 h with immobilised β-glucosidase and free cellulase used three times. I-βG F-C (1), (2), (3): immobilised β-glucosidase and free cellulase (numbers of use); F-βG F-C: free β-glucosidase and free cellulase; F-C: free cellulase.

of this pretreatment [2]. The recovery of glucane content from *E. globulus* in EG-H and EG-HA were 98.9% and 96.9%, respectively. Hydrothermolysis pretreatment removed only 9.2% of lignin whereas alkaline extraction subsequent to hydrothermolysis removed 59.2% of lignin.

The results of enzymatic hydrolysis of the pretreated materials by cellulase supplemented with I-βG and F-βG and without supplementation are displayed in Figure 4. The highest yields of glucose were reached at 72 h and it can be seen that the mean hydrolysis yields on pulp basis obtained for cellulase supplemented with I-βG were 76.1%, 83.6% and 75.6% for WS-SE, EG-H and EG-HA, respectively. The I-βG were magnetically recovered and reused twice and the differences in the hydrolysis yields were not significant ($p > 0.05$) for WS-SE and EG-HA, whereas the I-βG recycled from EG-H hydrolysis suffer a significant decrease of 10% in the yield of hydrolysis that can be attributed primarily to the loss of Fe_3O_4 during the multiple washes after the first recovery mainly due to the complex matrix composition of *E. globulus*

material resulting from the hydrothermolysis pretreatment that has higher content of lignin (Table 1). In the third recycle, the yield of hydrolysis decreases notoriously near 20% for WS-SE and for EG-H indicating loss in the enzymatic activity or loss of enzyme from the support during the washes. Since no improvement in the enzymatic hydrolysis was found when the hydrothermal pulp was subjected to alkaline extraction with NaOH due to the redistribution over the fibre of the 10.5% of the remaining lignin by the alkali treatment, thus decreasing the accessibility of the enzymes to the cellulose surface [25], a third reutilisation was not performed.

The sedimentation of I-βG during orbital shaking may explain lower hydrolysis yields for all cases when compared with the hydrolysis of cellulase supplemented with F-βG. The mean specific enzymatic activity after the third hydrolysis was $107.5 \pm 1.5\,\mathrm{IU\,mg^{-1}}$, similar to the value before hydrolysis.

The yield of glucose during enzymatic hydrolysis using cellulase without β-glucosidase supplementation was lower than using supplementation with F-βG or I-βG, increasing the concentrations of cellobiose and glucose (data not shown) that produces end-product inhibition of the endo- and exoglucanases components of the cellulase complex.

4. Conclusion

(I-βG) on superparamagnetic amino functionalised Fe_3O_4 nanoparticles was successfully used to supplement cellulase for the saccharification of wheat straw pretreated by steam explosion and *E. globulus* pretreated by hydrothermolysis and hydrotermolysis followed by alkaline extraction. The results showed that after 72 h the enzymatic hydrolysis by cellulase supplemented with I-βG are lower than the enzymatic hydrolysis with cellulase supplemented with F-βG. This difference can be attributed to the sedimentation of the enzyme/Fe_3O_4 support. Immobilised enzymes were reutilised twice maintaining the hydrolysis yield without losing their specific enzymatic activity after which the yield of hydrolysis decreases.

Further studies with immobilised cellulolytic enzymes must be performed with higher concentrations of Fe_3O_4 or with nanoparticles with broad size to immobilised higher concentration of enzymes to increase the consistency of pretreated solids to obtain more fermentable sugars. Also simultaneous saccharification of fermentation with immobilised enzymes must be studied to consider the interaction between the pretreated solid material chosen, the enzyme/Fe_3O_4 complex and fermentation microorganisms, even though Fe_3O_4 has been used to immobilise *Saccharomyces cerevisiae* due to its low toxicity level. The reusability of immobilised enzymes on Fe_3O_4 nanoparticles without losing activity in addition to new technologies used to produce cheaper enzymes with higher enzymatic activities provide a good opportunity to reduce the overall cost of the bioethanol production process.

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