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Research article

Ethanol production from cassava stem using *Saccharomyces cerevisiae* TISTR 5339 through simultaneous saccharification and fermentation

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Abstract

The stems of cassava (*Manihot esculenta* Crantz) are agro-waste that can provide an alternative source for the production of glucose as a bioenergy resource. The potential was studied of cassava stem as a raw material in the production of ethanol, through a simultaneous saccharification and fermentation process using *Saccharomyces cerevisiae* TISTR 5339. Steam explosion was applied as the pretreatment process to break the structure of cellulose, hemicellulose and lignin. The results indicated that after the pretreatment process at 210°C for 5 min, a higher yield of α-cellulose (91.06%) was obtained at a significance level of 95%. Experimental designs were used to optimize the appropriate nutrients for ethanol production. The maximum achieved value of ethanol concentration at 8.77% (weight per volume; w/v) corresponded well with the predicted value of 8.76% (w/v), under the optimized conditions at a solid loading of 20% and yeast extract of 2.05%.

Introduction

In the past decade, energy supply has been a primary concern as the global supply of petroleum is depleting (Owusu and Asumadu-Sarkodie, 2016). First generation biofuels that are made from starch, sugar and vegetable oil from crops, have raised some concerns regarding their economic benefits and the inability to produce adequate biofuels without threatening food supply (Boondaeng et al, 2015). As such, the search for new sources of biofuels has captivated global interest with lignocellulosic biomass— the most abundant feedstocks for a second generation biofuel —being of particular interest due to its low cost, as it can serve as an alternative to various raw materials such as agro-wastes, forestry wastes and pulp wastes (Saha, 2004; Talebnia et al., 2010; Chen, 2011; Singh and Bishnoi, 2012). Most importantly, the abundance of renewable carbohydrates from agricultural residues each year can be utilized in the production of ethanol. Through advanced biotechnology, those substrates can be used to produce ethanol and alleviate the issue of waste disposal (Tang et al., 2006).

The complex structure of lignocellulosic biomass, consisting of cellulose, hemicellulose, and lignin, is very difficult to break down by enzymatic hydrolysis and therefore, a pretreatment process is necessary to separate cellulose from a matrix of polymers-lignin and hemicellulose, and to provide access for enzymatic hydrolysis to occur. Steam explosion is one of the most effective and common methods for opening up the fibers and enhancing the enzymatic digestibility of lignocellulosic biomass (Pielhop et al., 2016). Advantages of steam explosion pretreatment compared to other pretreatment methods are that it is environmental-friendly, requires lower capital investment and uses less hazardous process chemicals (Garrote et al., 1999). The principle of this pretreatment method is biomass heating with hot steam (160−290°C) and high pressure (20−50 bar) followed by a sudden decompression, which results in a separation of the fibers. (Neves et al., 2007; Balat et al., 2008).

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The treatment can increase the effectiveness of cellulose digestibility and hemicellulose solubilization (Nibedita et al., 2012). Alkali delignification has been investigated to remove lignin residue and to make cellulose and hemicellulose available for enzymatic hydrolysis (Kobkam et al, 2018).

Cassava (*Manihot esculenta* Crantz) is a starchy root vegetable that is widely grown in Africa, South America and Asia including Thailand (Uchechukwu-Agua et al., 2015; Martín et al., 2017). With a high carbohydrate content in its root, cassava can be processed into various kinds of human food, animal feed and alcoholic beverages (Falade and Akingbala, 2010; Martín et al., 2017). Cassava is regarded as a major economic plant in Thailand, which is the world's largest producer and exporter of cassava (Klinpratoom et al., 2015). Many reports estimated that a large quantity of cassava stems—around 116 million t—are produced globally each year (Martín et al., 2017). Nonetheless, a mere 10–20% of cassava stems are used in propagation for the next season and the rest are abandoned as agro-wastes (Wei et al., 2014; Zhu et al., 2015). In Thailand, cassava has been planted on approximately 1.28 million ha in 48 provinces. Of the 20–25 million t of cassava roots harvested, 75% are exported (Papong et al., 2010). Good cassava stems are selected by the farmer for use in establishing the crop in the following season. Approximately 4 million t of the large amount of cassava stems left in the field are eliminated by burning or plowing in. Therefore, a large quantity of lignocellulosic cassava stem residues remains unused (Klinpratoom et al., 2015). Indeed, cassava stem can be used as a feedstock in the fermentation process, because of its high ratio of cellulose and low cost (Zhu et al., 2015, Martín et al., 2017). *Saccharomyces cerevisiae*, one of the most well-known species of yeast, is generally used in the production of ethanol in combination with starch and feedstock obtained from various types of sugars (Drapcho et al., 2008; Hidalgo et al., 2013). Accordingly, this study considered the utilization of cassava stems in a pretreatment process using steam explosion, with the hydrolysate produced used as a feedstock for fermentation. In addition, the response surface methodology (RSM), based on a central composite design (CCD), was used in the experimental design to optimize the conditions of the pretreated cassava stems. The ethanol yield was analyzed and investigated using a developed regression model and the Design-Expert software, respectively.

This study was the first of its kind that used a pretreatment process with steam explosion to prepare cassava stems as a raw material for second generation biofuel to produce ethanol through the simultaneous saccharification and fermentation (SSF) process. The possibility of producing ethanol from a cassava stem was investigated based on its abundance and low cost as a source of renewable biomass.

Materials and Methods

Raw materials and yeast strain

Cassava stems were collected in Nakhon Sawan province, located in northern Thailand used as a raw material. They were cut into small pieces (between 1 cm and 3 cm in length), air-dried at the room temperature and stored until use.

Saccharomyces cerevisiae TISTR 5339 (Thailand Institute of Scientific and Technological Research, Thailand) was used in this study. It was grown on a yeast extract peptone dextrose (YPD) plate, containing: 20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone and 15 g/L agar, for 24–48 hr. After that, it was transferred to 250 mL Erlenmeyer flasks containing 100 mL of YPD broth for seed culture preparation. After incubation for 48 hr with continuous shaking at 150 revolutions per minute (rpm), cell cultures were centrifuged, washed and used as a seed culture for further experiments.

Content determination of wood component

Dried cassava stem wood was milled to a particle size of 40 mesh, using a Wiley mill (Kinematica AG Co. Ltd.; Tokyo, Japan). The chemical compositions of the dried cassava stem wood were determined according to the standard industry methods, namely: T203 om-88 (Technical Association of Pulp and Paper Industry, 1992) for α-cellulose and T222 om-88 (Technical Association of Pulp and Paper Industry, 1988) for Klason (acid-insoluble) lignin. The residual yield and its chemical composition were studied during the delignification process using acidified sodium chlorite to determine the holocellulose content (Browning, 1967).

Pretreatment of raw material

The pretreatment process with steam explosion was performed at 195°C and 210°C for 5 min. Approximately 200 g of dried cassava stem wood were steam-exploded in a 2.5 L stainless steel batch digester (Nitto Koatsu Co. Ltd.; Tokyo, Japan), at 20 MPa and at varying temperatures. The solid residues, which were separated using filtration, were immersed in hot water at 80°C for 60 min and rinsed until having a neutral pH. After that, they were delignified by soaking in a 15% (weight per volume; w/v) NaOH solution and were incubated at 90°C for 30 min. After filtration, the obtained solid residues were rinsed with tap water until having a neutral pH. The delignified solids were air-dried and stored until use.

Enzymatic hydrolysis

Saccharification was enzymatically performed on the cellulose obtained from a cassava stem (5% w/v), using Cellic CTec2 (185 FPU/mL; Novozyme A/S; Basgsværd, Denmark) in a citrate buffer (50 mM; pH 4.8) at 30°C and 150 rpm for 24 hr. Enzyme loading was varied (20 FPU/g substrate, 25 FPU/g substrate and 30 FPU/g substrate). The Nelson-Somogyi method was used for the glucose analysis (Somogyi, 1952)

Simultaneous saccharification and fermentation

The delignified cassava stem wood samples were used in the production of ethanol based on the SSF process. The samples were slurried in 250 mL Erlenmeyer flasks with a working volume of 100 mL, in citrate buffer (100 mM; pH 4.8) using enzymatic

saccharification with Cellic CTec2 (25 FPU/g substrate). The seed culture of *Saccharomyces cerevisiae* TISTR 5339 (optical density of a sample measured at a wavelength of 600 nm) and yeast nutrients, comprising: 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4.7H_2O$, 1 g/L yeast extract and 13.8 g/L $NaH_2PO_4.H_2O$, were added to each sample. Each mixture was agitated at 150 rpm and 30°C for 96 hr, and was determined every 24 hr for ethanol concentration using gas chromatography (Chromosorb-103, GC4000; GL Sciences; Tokyo, Japan). Gas chromatography was performed with an HP5 capillary (30 m × 0.32 mm × 0.25 µm; JW Scientific; CA, USA) and an FID detector under the following conditions: split ratio of 50:1; split flow of 25.1 mL/min; air flow of 400 mL/min; H_2 flow of 40 mL/min; initial oven temperature of 40°C/min for 5 min and 15–250°C/min for 15 min; and injection volume of 1 µL).

Experimental design

The effects of each parameter on ethanol production were evaluated using a response surface methodology (RSM). The investigated factors were substrate loading (12.93−27.07%) and yeast extract (1.29−2.71), with each at five different levels based on the factorial design at two levels, as shown in Table 1. The CCD consisted of 2^2 factorial points with four star points ($\alpha = \pm 1.41$). The three replicates at the center point were also designed. Eleven experiments were performed to optimize the parameters. The effects of each independent variable were evaluated using a polynomial quadratic equation (Equation 1):

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{11}X_1^2 + + a_{11}X_2^2 + + a_{12}X_1X_2 \ldots\ldots\ldots\ldots \quad (1)$$

where Y is the predicted response (ethanol concentration, measured in percent), a_0 is a constant term, a_1 and a_2 are linear terms, a_{11} and a_{22} are quadratic terms, a_{12} is an interaction term and X_1 and X_2 are the test variables studied.

The response surface of each variable was analyzed using the Design Expert software (Version 7.0; Stat Ease; Minneapolis, MN, USA). Subsequently, the predicted values obtained from the software analysis were validated.

Results and Discussion

Chemical composition of cassava stem wood

The chemical composition of cassava stem wood consisted of holocellulose (69%), cellulose (38%), hemicellulose (31%) and lignin (22%) as shown in Table 2. Aside from the cassava stem, other lignocellulose residues were also reported (Table 3). The cellulose content from the cassava stem was compared to other reported cellulose content such as 44% for *Acacia mangium* and 42% for *Acacia* hybrid (Boondaeng et al., 2015), 26–43% for bamboo (Sánchez, 2009), 42–45% for corn cob (Kuhad and Singh, 1993; Prasad et al., 2007; Liu et al., 2010), 20–25% for hardwood (McKendry, 2002), 43% for the empty fruit bunch of oil palm (Garcia-Nunez et al., 2016), 28–36% for rice straw (Chen et al., 2008; Saini et al., 2015), 27–30% for softwood (McKendry, 2002), 42–48% for sugarcane bagasse (Kuhad and Singh, 1993; Rocha et al., 2015; Saini et al., 2015) and 5–34% for switch grass (Butkute et al., 2013; Saini et al., 2015). These figures indicated that cassava stem has a high potential to be used as a feedstock in bioconversion processes for ethanol fermentation, especially as it can be obtained in large quantities from local growers.

Table 1 Experimental design used in response surface methodology of two independent variables, substrate loading (X_1), and yeast extract concentration (X_2) with three center points and the observed and predicted ethanol concentration

Treatment	Level		Actual level		Ethanol concentration (%)	
number	X_1	X_2	X_1	X_2	Observed	Predicted
1	1	1	25	2.50	2.73	3.01
2	1	-1	25	1.50	5.00	5.25
3	-1	1	15	2.50	5.03	5.69
4	-1	-1	15	1.50	2.87	3.49
5	1.41	0	27.07	2	3.27	2.88
6	0	1.41	20	2.71	3.26	2.85
7	-1.41	0	12.93	2	5.72	5.58
8	0	-1.41	20	1.29	6.93	6.23
9	0	0	20	2	8.87	8.77
10	0	0	20	2	8.92	8.77
11	0	0	20	2	8.51	8.77

Table 2 Chemical composition of cassava stem before and after steam explosion pretreatment (% dry weight)

Component	Raw material	After pretreatment	
		195°C, 5 min	210°C, 5 min
Holocellulose	68.73	93.64	96.65
Cellulose	37.66*	87.59*	91.06*
Hemicellulose	31.07	6.05	5.59
Lignin	22.07	18.48	16.25

* significance level = 95% using Duncan's multiple range test.

Table 3 Chemical composition of some lignocellulosic residues

Source	Cellulose	Hemicellulose	Lignin	References
Cassava stem	38	31	22	Current study
Acacia mangium	44	30	24	Boondaeng et al., 2015
Acacia hybrid	42	32	23	Boondaeng et al., 2015
Bamboo	26–43	15–26	21–31	Sánchez, 2009
Corn cob	42–45	35–39	14–15	Kuhad and Singh, 1993; Prasad et al., 2007; Liu et al., 2010
Hardwood	20–25	45–50	20–25	McKendry, 2002
Oil palm (empty fruit bunch)	43	21	15	Garcia-Nunez et al., 2016
Rice straw	28–36	23–28	21–14	Chen et al., 2008; Saini et al., 2015
Softwood	27–30	35–40	25–30	McKendry, 2002
Sugarcane bagasse	42–48	19–25	20–42	Kuhad and Singh, 1993; Rocha et al., 2015; Saini et al., 2015
Switch grass	5–34	30–50	10–40	Butkute et al., 2013; Saini et al., 2015

Pretreatment and enzymatic hydrolysis

Comparison of the steam explosion pretreatments at 195°C and 210°C for 5 min showed that 210°C for 5 min was the most effective at reducing the recalcitrance of the lignocellulose by partially removing hemicelluloses, partially degrading lignin and improving accessibility to the cellulosic fibers. The contents of steam-exploded cassava stem were 88% and 91% at 195°C and 210°C, respectively (Table 2). Thus, using 210°C for 5 min can readily generate a digestible cellulose substrate, leading to easy enzymatic hydrolysis. Hence, this condition was selected for further study on enzymatic hydrolysis and SSF, based on a statistical design of the experiment.

Enzymatic hydrolysis of cassava stem after the pretreatment was conducted using Cellic CTec2 cellulase enzyme at 20 FPU/g substrate, 25 FPU/g substrate and 30 FPU/g substrate. The highest concentration of the reducing sugar (43 g/L at 72 hr) was obtained using 25 FPU/g substrate (Fig. 1). The concentration of the reducing sugar decreased with either more or less than this amount of enzyme. This result corresponded with other reports. Triwahyuni et al. (2015) studied the effects of substrate loading on the SSF process in the production of bioethanol from empty fruit bunches of oil palm. Pretreatment using alkali solutions resulted in a cellulose content of 75%. At 25% substrate loading, 18 FPU Cellic CTec2 /g substrate and 20% Cellic HTec2, and 1% yeast *Saccharomyces cereviceae* produced the maximum ethanol concentration of 8.34% (w/v), whereas, 30 FPU Cellic CTec2/g substrate produced a lower ethanol concentration (4.55% w/v). Wilkinson et al. (2016) studied the effect of enzyme loading (Cellic CTec2, 10–160 FPU/g biomass) on saccharification. They reported an increased glucose concentration from using an excess of Cellic CTec2 (160 FPU/g biomass) and increasing the solid loading from 5% to 25%, but the theoretical glucose yield percentage decreased. A low dose of Cellic CTec2 (10 FPU/g biomass) also increased the glucose liberated with an increase in solids loading from 5% w/v to 25%. However, using an excess enzyme dose and a low enzyme dose resulted in slightly different on theoretical percentage yields as the solids loading was increased from 5% to 25%. The results implied that using a low enzyme dose was optimal at a higher solid loading in terms of enhancing the efficiency of the biomass process using large quantities. However, the amounts of individual enzymes during lignocellulosic biomass saccharification need to be optimized for a specific individual biomass.

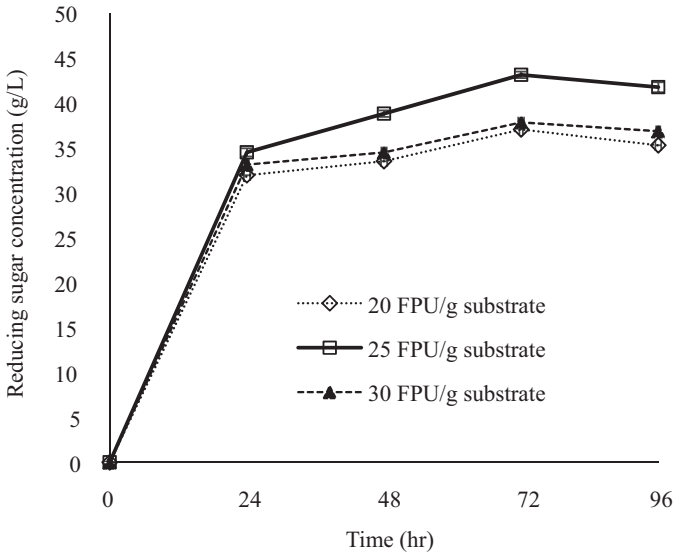

Fig. 1 Effects of enzyme loading (Cellic Ctec2) at different concentrations in citrate buffer (50 mM, pH 4.8) at 30°C on reducing sugar concentration

Simultaneous saccharification and fermentation

RSM based on CCD was used to identify the optimal response region of ethanol production and to optimize the variables, solid loading (X_1) and yeast extract concentration (X_2). The experimental design, as shown in Table 1, was used to evaluate the two-variable quadratic polynomial regression model to predict the concentration of ethanol. The predicted production of ethanol was provided using Equation 2:

$$Y = 8.77 + 0.013X_1 + 0.23X_2 - 1.11X_1X_2 - 2.97X_1^2 - 1.44X_2^2 \quad (2)$$

where Y is the percentage ethanol concentration, X_1 is the percentage solid loading and X_2 is the percentage of yeast extract (%).

The analysis of variance, as illustrated in Table 4, indicated that the model was statistically significant. According to the Fisher's F-test, the probability value was considerably low (p-model > F = 0.001).

In addition, the determination coefficient (R^2) was 0.9669, which illustrated a strong correlation between the observed and predicted values. Thus, it can be inferred that the regression model provided an appropriate explanation of the relationship between the independent and dependent variables. Moreover, the relationships between solid loading and yeast concentration (X_1X_2), and between the quadratic term of solid loading (X_1^2) and yeast extract concentration (X_2^2) were significant (Table 4). The 3D response surface was plotted using Eq. (2) to explain the effects of the difference in levels of two process variables on the concentration of ethanol. The effects of solid loading and yeast extract concentration on the ethanol concentration during the fermentation of cassava stem at 30°C, are illustrated in Fig. 2 as a 3D response surface plot. The plot indicated that the concentration of ethanol was low at 15% solid loading. The ethanol concentration increased as the solid loading increased but began to decline after the solid loading reached 22.5%. Additionally, the concentration of ethanol increased with the concentration of yeast extract up to 2.25%, after which the concentration of ethanol began to decrease, as illustrated in Fig. 2. These results were in accordance with those reported by other researchers. Luo et al. (2014) found that the concentration of solid juice had a significant effect on ethanol production. As the concentration of solid juice increased, the concentration of ethanol increased correspondingly. However, when the solid juice concentration reached 13%, the ethanol concentration began to decrease. Moreover, they found that the temperature of fermentation and the yeast load had no significant effect on the yield and final concentration of ethanol. Singh and Bishnoi (2012) conducted an experiment in which they optimized the production of ethanol from a microwave-alkali pre-treatment of rice straw, using *Saccharomyces cerevisiae*. Their results indicated that the ethanol concentration increased up to 3% (w/v) inoculum and decreased beyond that level. Stewart et al. (1988) and Bafrncová et al. (1999) reported that yeast extract had an effect on the growth and viability of yeast during very high gravity ethanol fermentation using 300 g/L of glucose as the carbon source in a synthetic medium. In addition, they found that the yeast was better able to withstand the osmotic pressure and high temperature when the concentration of yeast extract was increased.

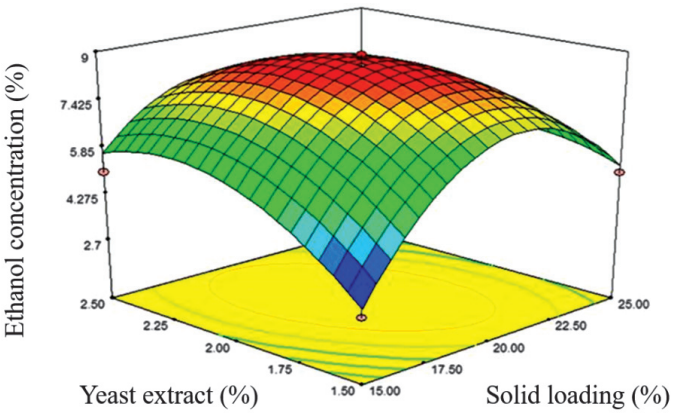

Fig. 2 Response plot of combined effects of solid loading (X_1) and yeast extract concentration (X_2) on ethanol fermentation (%) by *Sacchromyces cerevisiae* TISTR 5339 from pretreated cassava stem wood at 30°C

The experimental model was validated by repeating the four additional runs three times, under different combinations of solid loading and yeast extract concentration. The maximum response of the verification experiments produced an observed value of 8.77% (w/v), which was almost identical to the predicted value at 8.76% (w/v). The observed value was obtained at a solid loading of 20% and a yeast extract concentration of 2.05%. Furthermore, the yield and the productivity obtained were 0.46 g/g sugar and 1.22 g/L/hr, respectively. The production of ethanol from other lignocellulosic residues has been reported. Ko et al. (2016) produced ethanol through a separate hydrolysis and fermentation (SHF) process from dilute acid-pretreated hydrolysates of rice straw and hardwood (oak). After 72 hr, they obtained an ethanol yield of 0.43–0.46 g/g sugar and productivity of 0.25–0.29 g/L/hr. Martínez-Patiño et al. (2015) produced ethanol from the pretreatment of pruned olive trees with dilute phosphoric acid using *Escherichia coli*. The yield of ethanol was 0.46 g/g sugar and 0.13 g/g material from the pretreated liquid fraction and solids after enzymatic hydrolysis, respectively. Another experiment conducted by Govumoni et al. (2013) reported an ethanol yield of 0.44 g/g sugar and ethanol productivity of 0.68 g/L/hr after 36 hr using wheat straw hydrolysate and *Saccharomyces cerevisiae* with an SHF process. On the other hand, Dussán et al. (2016) investigated ethanol fermentation using an SHF process with sugarcane bagasse hydrolysate and two strains of xylose-fermenting yeast. The ethanol yield and productivity from *Scheffersomyces shehatae* were 0.42 g/g sugar and 0.25 g/L/hr, respectively. The yield and productivity of ethanol from cassava stem were relatively higher compared to those from other lignocellulosic

Table 4 Analysis of variance for model regression representing ethanol concentration of cassava stem

Source	Sum of squares	Degrees of freedom	Mean square	F value	*p* value
Model	57.29	5	11.46	29.23	0.0010*
X_1	0.013	1	0.22	1.400E−0.03	0.9547
X_2	0.23	1	0.22	1.13	0.3371
X_1^2	-2.97	1	0.31	12.53	< 0.0001*
X_2^2	-1.44	1	0.26	30.02	0.0028*
X_1X_2	-1.11	1	0.26	-0.119	0.0166*
Residue	1.96	5			
Lack of fit	1.86	3	0.62	12.70	0.0739
Total	59.25	10			

*significance level = 95%; coefficient of determination (R^2) = 0.9669; Adjusted-R^2 = 0.9338.

residues. The results obtained from the current study confirmed the possibility of using cassava stem as a feedstock in ethanol fermentation. In conclusion, the response surface methodology based on a central composite design was successfully implemented to improve the production of ethanol from steam-exploded cassava stems using *Saccharomyces cerevisiae* TISTR 5339. The optimal conditions at 20% solid loading and 2.05% yeast extract concentration produced ethanol concentration, yield, and productivity of 8.77% (w/v), 0.46 g/g sugar and 1.22 g/L/hr, respectively. The ethanol yield derived from this process was relatively higher than those obtained from other lignocellulosic feedstocks. Accordingly, cassava stem may potentially serve as a low-cost and abundant resource for ethanol production.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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