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**Efficient Saccharification of Agave Biomass Using *Aspergillus niger* Produced  
Low-cost Enzyme Cocktail with Hyperactive Pectinase Activity**

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**Abstract**

To develop a cost-effective, time-saving and efficient saccharification system for converting biomass into mono-/oligo-saccharides for production of bioethanol or other biochemicals, a relatively low recalcitrant and widely available biomass *Agave americana* was selected as feedstock. During the investigation of efficient enzyme cocktail, pectinase, which usually is neglect for biomass saccharification, was confirmed that it dramatically improves the saccharification of agave biomass. A production-friendly fungal strain of *Aspergillus niger* Gyx086 was employed for low-cost enzyme cocktails production using wheat straw as substance. The enzyme cocktail which was with hyperactive pectinase activity of  $6.29 \pm 0.42$  U/ml could efficiently saccharify un-pretreated agave biomasses. As a result, under a mild condition at  $35^{\circ}\text{C}$  in less than 72 h, most of the polysaccharides were completely converted into reducing sugar. The low-cost, process-simplified, and efficient biotechnology should stimulate the development of agave as feedstock for green energy and bio-based products production.

**Keywords**

Agave, Saccharification, *Aspergillus niger*, Pectinase, Low-cost enzyme cocktail

## 1. Introduction

To relieve the escalating energy crisis and environment pollutions, a bio-based economy which is based on the biofuel and biochemical production from biomass has been abstracting extensive attention (Zhao et al., 2012a). Such as the second generation bioethanol, it is a viable alternative method for producing renewable fuel from lignocellulosic agriculture residues without competition with food and feed production (the first generation bioethanol deriving from sucrose or starch) and also it can lower 45–65% of greenhouse gases emissions (Daylan & Ciliz, 2016; Sindhu et al., 2016). Three key steps, namely pretreatment, enzymatic saccharification and fermentation, are involved in the production of bioethanol or other biochemical production from lignocellulosic feedstocks. However, releasing the fermentable sugars from lignocellulose has become a bottle-neck for industrializing lignocellulose biorefining due to its compact and rigid structure in cell wall known as biomass recalcitrance (Zhao et al., 2012a).

Biomass recalcitrance of enzymatic hydrolysis mainly is derived from the complex matrix structure of cellulose, hemicellulose, lignin, and pectin in plant cell walls (Himmel et al., 2007; Xu et al., 2012). Usually, hemicellulose is combined with lignin, cellulose, and pectin via both non-covalent and covalent bonds and enhances the resistance to enzymatic degradation of the cell wall (Abasolo et al., 2009; Chen, 2014). De-lignin, de-hemicellulose and/or de-crystallinity are usually used as tactics for reducing the biomass recalcitrance by mechanical, physic-chemical, chemical or biological pretreatment method (Guo et al., 2017; Hendriks & Zeeman, 2009; Yoon et

al., 1995). Although the digestion rate can significantly be improved by physical and chemical methods, lots of drawbacks, such as high energy consumption, high production cost, special equipment requirements, generating inhibitors and environmentally unfriendly cause these methods to become economically unviable (Lee et al., 1995; Mosier et al., 2005; Saha et al., 2017; Zhao et al., 2012b). Biological pretreatment has been proposed as an economical, eco-friendly technology to improve biomass digestibility base on it can be performed at a milder condition in a simple equipment and it is less harmful to the environment (Chen et al., 2010; Rabemanolontsoa & Saka, 2016). Brown, white and soft rot fungi were widely investigated for delignification of the lignocellulosic biomass (Sindhu et al., 2016) but the process is too slow, usually more than two weeks, and it is not recommended for industrial purposes (Chaturvedi & Verma, 2013).

Accordingly, a low recalcitrance and widely available biomass are preferable because it avoids the costly and time-consuming pretreatment and the side-effect from pretreatment which will affect the downstream process. Thus, it will become distinctly economical and implementable. In this study, we presented an economical, simple and efficient saccharification of lignocellulose feedstock via selecting a low recalcitrance agave which is further saccharified by enzyme cocktails from *Aspergillus niger* (*A. niger*). The polysaccharide of agave biomass could be completely hydrolyzed into reducing sugar in a mild condition at 35 °C. The efficient and low-cost biotechnology provides a bright prospect for green energy and bio-based industry.

## 2. Materials and methods

## 2.1 Agave biomass and strains

*Agave americana* biomass (kindly provided by Jeffrey Phelps of Redding, California, USA) was dried at 70 °C in a drying oven (Thermo Fisher Scientific, Canada) for 24 h, grounded in a Wiley mill, and then sieved through U.S. standard sieve series of 20, 40, 80, and 100 mesh (Chicago, Precision Scientific Co.). The biomass was stored at room temperature. The Gyx086 was isolated from ancient ginkgo tree soil and selected from 107 of fungal isolates in our previous work (Wang et al., 2013).

## 2.2 Biomass component analysis

Total extraction analysis was executed four times extraction of 0.10 g samples at 65 °C for 30 min each as described by Guo et al. (2017) and Shrestha et al. (2015) with minor modifications. Briefly, twice in 1.5 ml hot water, once in 1.5 ml of absolute ethanol and once in 1.5 ml acetone. Then the feed-extraction sample was air-dry for 2 days following dried for 8 h at 105 °C. Structural carbohydrates and lignin were determinated using two-step acid degradation as outlined by NREL (Sluiter et al., 2010) with some minor modifications. In brief, 0.10 g sample was incubated in 0.5 ml of 72 % sulfuric acid at an indoor temperature for 1 h with vortexing every 15 min, following adjust the volume to 15 ml and autoclave at 121 °C for 1 h. The residue was separated from the supernatant by centrifuging at 12000 ×g for 5 min and used for analyzing Kalson lignin by a weight method. Acid-soluble lignin and reducing sugars were measured at the absorbance of 205 nm (Dence, 1992) and at 540 nm by 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959), respectively. Acid-soluble lignin (ASL) in the acid hydrolysate was concluded by the formula: ASL (g/L) = A<sub>205</sub>/ 110 × dilution factor, then further

shown as a percentage in the composition. The monosugars in the supernatant was quantified by using an ion chromatography, Dionex, ICS 5000, Thermofisher Scientific, equipped with CarboPacTM SA10 column and an electrochemical detector (ED) (Dionex-300, Dionex Corporation, Canada). 1.00 mM of KOH was used as the eluent with a flow rate of 1.2 ml/min and the column temperature was set at 30 °C (Fatehi et al., 2016).

### 2.3 Enzymatic hydrolysis

Four commercial enzymes were used for hydrolyzing agave biomass: E1: Glucosidase 49291-1G from *Aspergillus niger* (Sigma, USA); E2: Cellulase “Onozuka R-10” derived from *Trichoderma viride* (PhytoTechnology Laboratories, USA); E3: Pectinase “Guojiaomei” (Hengsheng Bio-Tech. Co, China); E4: Cellulase “Celluclast 1.5L” from *Trichoderma reesei* ATCC 26921 (Sigma, USA). 10 mL of the single or mixed enzyme cocktail was prepared by 50 mM citrate buffer (pH 5.0) with 0.05 % of sodium azide as antimicrobial. Using a self-prepared enzyme cocktail, the crude enzymes were adjusted to pH 5.0 before mixing with isopyknic citric buffer (0.1 M, pH 5.0). Agave biomass was loaded at a solid concentration of 1.0 % (w/v) in 15 ml plugged tubes. The saccharification experiment was conducted at 50 °C and 150 rpm in a shaking incubator for 72 h. 200 µl of hydrolysate were taken every 12 h for reducing sugar and/or monosugars analysis. The reducing sugar yield was calculated as reducing sugar yield (%) = released reducing sugar / total carbohydrates × 100.

### 2.4 Production of lignocellulosic enzymes by *A.niger*

By using a small hole puncher with a diameter of 0.5 cm to take 3 pieces of agar covered by spores of *A. niger* Gyx086 in petri dish plate and inoculated them into 50 ml of various liquid medium with 5.0 % of the different biomass in nature pH value. Fermenting for 8 days at 30 °C in the incubator with a rotate speed of 200 rpm, 1.0 ml of each sample was taken for enzymatic activity analysis every 24 h. In the single factor and optimized experiment, the same rotate speed was set under different other conditions. All of the experiments were performed in triplicates.

## 2.5 Analysis of enzymatic activities

Enzymatic activities of xylanase, CMCase and FPase were measured refer to the microplate method in our lab (Guo et al., 2017), with Beechwood xylan (Megazyme, Ireland), carboxymethyl cellulose (Acros organic, USA) and No. 1<sup>#</sup> filter paper (Whatman, England) as a substrate, respectively. Briefly, 10 µl of diluted crude enzyme liquid was mixed with 20 µl 1.0 % of various substance solution (pH 5.0) in each well of microplate, acting in the water bath at 50 °C for 10 min (FPase for 30 min), then instantly cooled down and added 60 µl of the DNS reagent and heated in boiling water for 5 min. 200 µl water was added into each well and take 200 µl mixed liquid for measuring the absorbance at 540 nm. The release amount of reducing sugar was calculated by the relevant standard curve. Polygalacturonase (PG) activity was analyzed refer to the above protocols using polygalacturonic acid (Sigma, USA) as substrate. The β-glucosidase activity was determinated by using 4-nitrophenyl β-D-glucopyranoside (pNPG) (Sigma, USA) as substrate according to described by Shrestha et al. (2015). Enzyme activity has been expressed in International Units (IU), as the amount of

enzyme which releases 1  $\mu\text{mol}$  of glucose, xylose, galacturonic acid or *p*-nitrophenol in 1 min.

### 2.6 XRD and FT-IR measurements

X-ray diffraction (XRD) and Fourier Transform Infrared (FT-IR) spectroscopy were used for investigating characteristics of air-dried agave's structure. XRD patterns were collected by using PANalytical X'pert Pro diffractometer (PANalytical, Holland) equipped with a conventional X-ray tube (CuK $\alpha$  40 kV, 20 mA, line focus) in transmission mode. The patterns of agave and cellulose crystal I were recorded in the range of 6–40° ( $2\theta$ ) at a step size of 0.0263° and 165 s/step. The Crystallinity index ( $CrI$ ) was concluded as:  $CrI (\%) = (I_{002} - I_{sm}) / I_{002} \times 100$ . FT-IR analysis was performed by a Bruker Tensor 37 FTIR Spectrophotometer (Bruker Optics, Inc., Billerica, MA). The spectra were recorded in transmission mode with 32 scans at a spectral resolution of 4  $\text{cm}^{-1}$  within the 4000 - 500  $\text{cm}^{-1}$  range.

### 2.7 Box-Behnken design (BBD) and statistical analysis

Response surface methodology (RSM) was used to optimize the production of PG. Three factors and three levels were selected for BBD based on the results of the single factor experiment; the ranges and levels of the independent variables are shown in Table 2. The BBD matrix was generated by SYSTAT 12 (Systat Software, Inc., USA). This software was also used for statistical analysis of the experiment data, describing the response surface, and drawing the contour maps. The goodness of fit of the second-order polynomial model equation, the determination coefficient  $R^2$  and the lack of fit were indicated by an F test at the 5 % level of significance. The most optimized

conditions were decided by the “Response Surface Method – Optimize – Ridge max analysis” program of SYSTAT 12. The statistical analysis was carried out in the one-way ANOVA program of the SAS system for Windows 8.02 (SAS Institute Inc., USA). Duncan’s multiple-range test was selected as the comparison method in the program and the significance level was set at 0.05.

### 3. Results and Discussion

#### 3.1 Characterization of *Agave americana*

To investigate agave biomass structure peculiarity and its potential use as a feedstock, characterization of agave biomass was first accomplished. The agave biomass has a water ratio  $10.35 \pm 0.01\%$  and a high extraction content  $27.47 \pm 1.24\%$  including water-soluble extraction  $26.18 \pm 0.74\%$ , ethanol absolute  $1.04 \pm 0.20\%$ , and acetone extraction  $0.25 \pm 0.11\%$ . Reducing Sugar content in the agave is  $1.64 \pm 0.01\%$  of the agave weight. Total lignin represents  $15.27 \pm 1.19\%$  of biomass, including 3.46 % of acid-soluble lignin and 11.8 % of Klason lignin, which is lower than 16 % in agave bagasse (Caspeta et al., 2014). Pectin content in the agave is  $6.02 \pm 0.15\%$  which higher than other biomass, such as wheat straw, ginkgo leaves, when it compares to our study (Data not showed). Total carbohydrate content is 37.76 % and it is assayed by DNS method via two-step acid hydrolysis (Selig et al., 2008). Six structural monosugars including arabinose, galactose, rhamnose, glucose, xylose and mannose were analyzed by ion chromatography. The total released monosaccharides from the agave via acid hydrolysis is  $29.11 \pm 1.07\%$  of the agave weight. Glucose occupies 71.8 % of the total amount of six monosugars and the hemicellulose monose mainly is xylose, galactose

and arabinose in the agave and the content is 12.5 %, 9.8 % and 3.5 %, respectively.

There is only  $0.22 \pm 0.01$  % of raminose and  $0.45 \pm 0.04$  % of manose in agave. Li et al. (2012) indicated that fructose occupies 25.2% of total sugar in agave juices of *Agave americana* leaves. However, fructose hasn't been detected by the ion chromatography assay. Oligosaccharides, such as cellobiose or xylobiose and other sugars, was also produced during hydrolysis of biomass (Rawat et al., 2014). This can be the explanation of why the determinated total monosugars was 29.11 % and it was lower than the value of 37.76 % which are determinated by the DNS method. In fact, the maximum theoretical value of sugar is higher than this value when all of polysaccharide and oligosaccharides are converted into monosugars. The spectra of X-ray was carried out to investigate the cellulose crystal in agave, which shown that there is the very little amount of crystal in the agave biomass, due to the 002 crystalline peak is tiny in the spectra of X-ray when it compares with nature cellulose crystal from wheat straw under the same analysis conditions. While the peaks of 002 and 101 crystal faces are the characteristic peaks of nature cellulose, the 002 crystalline peak was used for the calculation of the crystallinity index by the peak high (Lionetto et al., 2012). The *CrI* was 16.12 % in agave biomass which was far lower than 41.19 % in the natural cellulose of wheat straw. The result suggested that the cellulase has better accessibility to agave cellulose for hydrolysis due to the less crystalline region of cellulose. *Agave americana* exhibit crassulacean acid metabolism which allows *Agave americana* to grow well in arid and semi-arid regions of the world with limited water input (Cushman et al., 2015). Davis et al. (2017) indicated that healthy 3-year-old *A. americana* plots

could yield 4.0-9.3 Mg ha<sup>-1</sup> yr<sup>-1</sup> total biomass, thus the agave can be a widely available and potential alternative bioenergy crop.

### 3.2 Efficient enzyme cocktail for saccharification of agave biomass

To recognize what kinds of enzyme is efficient for agave saccharification, four commercial enzymes were used to degrade un-pretreated agave biomass (Table 1). The released reducing sugars were significantly different from different enzyme or their mixed enzyme cocktail. The amounts of released reducing sugars were significantly higher in hydrolysates which were hydrolyzed by E1+E2, E1+E3 and E3. The enzyme activity assay indicated that these enzyme cocktail have significantly higher cellulase, pectinase and/or xylanase activity (Table 1). Factor analysis between enzyme activities and the amount of released releasing sugar revealed that the order of the relation values is CMCase > BG > PG >Xylanase > FPA. However, the E2 with high xylanase activity only released very low amount of reducing sugar, especially glucose, thus probably due to its too low glucosidase. This enzyme is necessary for degrading the cellulose into monosugars (Himmel et al., 2007; Qiu et al., 2017). The E3, which is low FPA, glucosidase and xylanase activity but high pectinase activity, dramatically released a high amount of the reducing sugar. Thus, the result suggested that pectinase maybe play a critical role in the degradation of agave biomass. To further verify this, two enzyme cocktails from *A. niger* were used for saccharifying the agave biomass (Table 1), the 39E with high cellulase, xylanase activities and low PG activities released significantly lesser reducing sugar than the E28. As a result, pectinase makes a dramatic effect during the degradation of agave biomass. Unfortunately, this enzyme is usually

thought of a negligible effect for biomass deconstruction (Jørgensen et al., 2007; Sindhu et al., 2016; Zhao et al., 2012a). The agave biomass possesses 6 % of pectin by the extraction method of ammonium oxalate, which is higher than other biomass, such as wheat straw and maple leaves which are less than 2 % (Data not showed). The pectin fills in the plant primary cell walls and the middle lamellae (Caffall & Mohnen, 2009), as a result, it will reduce the accessibility of cellulose. While the pectinase deconstructed the plant cell of agave biomass and exposed cellulose to the cellulase which will improve the sugar yield. The FTIR spectra of agave biomasses are investigated and compared between un-hydrolyzed and hydrolyzed agave samples, which indicated that the structural difference presents on the broadband at 3000-3600 cm<sup>-1</sup> corresponds to O-H bonds, and on the peak at 2940-2846 cm<sup>-1</sup> is assigned to methine, methylene and methyl groups stretching vibrations (Gutiérrez-Hernández et al., 2016; Velazquez-Jimenez et al., 2013). The O-H bands are obviously reduced in the samples hydrolyzed by 28E which suggested that hemicellulose and cellulose have been massively hydrolyzed, while a lesser degradation was in the 39E. This was further proved by the peak reduction in 800-1200 cm<sup>-1</sup> and 1200-1800 cm<sup>-1</sup> which assigned to carbohydrates (Paradkar et al., 2003). Thus, draw a conclusion, pectinase is critical for saccharification of agave biomass.

### 3.3 Production of enzyme cocktail

Based on cellulase with high pectinase and/or xylanase is more efficient for saccharification of un-pretreated agave, *A. niger* Gyx086, which was selected from 107 fungi isolates in our previous work (Wang et al., 2013), was selected for producing

enzyme cocktail. The strain produced high FPase and xylanase activities when fermenting ginkgo leaves (Wang et al., 2013; Wang et al., 2018). In this work, pectinase activity has been proved (Table 1, E28). For producing low-cost enzyme cocktail, eight of available biomasses were used as a medium with 5 % of substance solid content without another additive. As shown in Figure 1-a, significantly higher PG activity was obtained from the wheat straw than from other biomass substances, accordingly, wheat straw was used as a medium substance in the following experiments. Moreover, 30-35 °C, pH 5.0 and 3 % of medium substance were more suitable for the production of higher enzyme activities (Figure 1-b to d). Dramatically, when under room temperature with pH 7.0, a higher PG activity appeared than pH 5 and pH 3 (Data not shown). Thus, a Box-Behnken design was executed with temperature 28 °C, 32 °C, 36 °C; pH 3, 5, 7 and time 48 h, 72 h, 96 h (Table 2). The data was analyzed statistically by response surface analysis. The PG activity could be expressed as a quadratic equation:

$$\begin{aligned} \text{PG activity} = & 5.614 - 0.685X_1 - 1.947X_2 + 0.099X_3 - 1.058X_1^2 - 1.594X_2^2 - 1.057X_3^2 \\ & - 0.138X_1X_2 - 0.820X_2X_3 - 1.96X_1X_3. \end{aligned}$$

The multiple R of the equation is 0.98, the regression p-value is 0.00 and the *p*-value of lack of fit is 0.27 (Table 3), which indicated that PG activities could be well explained by the factor X<sub>1</sub>-X<sub>3</sub> and the regression equation is reliable. Due to the regression *p*-value 0.00 is less than 0.01 (highly significant), the *p*-value also suggests that the equation could be reliable and without lack of fit (*p*-value >0.05) (Baş & Boyacı, 2007; Faravelli, 1989). All of the contour plots appeared as ellipses (Khuri & Mukhopadhyay, 2010) shown in Figure 2 suggested that the interaction exists among temperature, pH and

fermentation time. A maximum estimated response 7.24 with a 95% confidence interval from 5.62 to 8.87 was obtained within experimental parameters by the ridge analysis; while the determinated value was  $6.29 \pm 0.42$  by confirmatory quintuplicate tests.

### 3.4 Characterization of enzyme cocktail

Using the optimized enzyme cocktail for saccharification of agave biomass, the enzyme activities and characteristics were investigated. The results indicated that this is a partial acidic broth with pH value only 2.34, the acid derived from the secretion by *A. niger* during fermentation. The enzymatic activities unit of PG, xylanase, glucosidase and CMCase were  $6.29 \pm 0.42$  U,  $1.89 \pm 0.10$  U,  $0.12 \pm 0.02$  U and  $0.43 \pm 0.06$  U in per millilitre broth, respectively. This enzyme cocktail includes relatively high PG and xylanase activity but relatively low cellulase activities. Generally, 10-15 FPU of cellulase and 10-15 IU of  $\beta$ -glucosidase were loaded for per gram cellulose (Ballesteros et al., 2006; Volynets & Dahman, 2011), although excessive cellulase loading (up to 60 FPU and 64 pNPGU/g cellulose) is recommended by the USA National Renewable Energy Laboratory (NREL) to determinate the effect of pretreatment on substrate digestibility (Selig et al., 2008). According to these values, 1 % of solid agave in enzyme broth is suitable for saccharification. For understanding the enzymatic hydrolysis condition, these enzymatic characteristics were investigated as shown in

Figure 3. The most suitable reaction temperature of PG, xylanase and CMCase was 40 °C, 50 °C and 60 °C, respectively (Figure 3-a). The pH 5 was most suitable for PG,  $\beta$ -glucosidase and xylanase, but the pH 2 was more suitable to CMCase (Figure 3-b), thus pH 5.0 was decided as the pH condition of saccharification. Moreover, the

thermostabilities were enormously different among the enzymes as shown in Figure 3-c to d. The CMCase and  $\beta$ -glucosidase were very stable at 50 °C for 72 h without activity loss, but xylanase holds only half of the activity value at 50 °C after 24 h of water bath when compared with the initial value and the activity have been completely lost at 60h. However, PG holds only 8.4% of enzyme activity after 12 h at 50 °C. When less than 40 °C, the xylanase activity hasn't been reduced after 72 h, but the PG lost more half of activity after 12 h and the stable activity obtained only at 27 °C for 72 h which indicated the PG was not a thermos stable enzyme. The inconsistent conditions among these enzymes suggested that an overall consideration was needed in the case to obtain the optimized condition for saccharification.

### 3.5 Saccharification of agave biomass

The temperature condition affected enzymatic activity, thus it will further affect the sugar releasing. The cellulase shows a higher activity and good thermotolerance under 50 °C, but not the highest sugar releasing (Figure 4-a). This could be explained as due to PG and xylanase quickly lost their activities at the temperature, such as the cellulose embedded by pectin and hemicellulose couldn't be effectively de-structuralized. As a result, cellulose wasn't accessible to cellulase for further enzymatic hydrolysis. The temperature 35-40 °C was more suitable for higher sugar release, which indicated that a better coordination has occurred in this temperature because the maximal sugar releasing is depended on the coordination of various enzymes which can destructure the cell wall and hydrolysis cellulose (Betts et al., 1991; Chen, 2014; Himmel et al., 2007).

Figure 4-b to 6-d shown that the sugar release is affected by the ratio of enzyme cocktail

and buffer, biomass loading ratio and biomass sizes. Without diluting the enzyme cocktail broth, higher reducing sugar was released from agave biomass than the diluted broth due to the enzymatic activity unit was higher in the former.

When the agave biomass loading was 10 %,  $6.51 \pm 0.39$  g/L of reducing sugars were obtained via hydrolysis after 72 h of incubation,  $3.18 \pm 0.16$  g/L and  $4.87 \pm 0.36$  g/L of sugar liquor were obtained in 1 % and 5 % of solid loading, respectively (Figure 4-d). The result indicated that higher agave solid loading significantly increased the sugar concentration in the hydrolysate. Higher sugar content is usually preferable in practical production during saccharification because of higher content of ethanol or chemicals in per unit volume liquor can be obtained in downstream process (Koppram et al., 2014; Modenbach & Nokes, 2012). However, agave biomass conversion was decreased when increasing agave feedstock loading, probably due to high loading solid will increase the inhibitors, which results in lower performance of the enzymes (Jørgensen et al., 2007; Liu et al., 2014). The agave biomass with big size, 20-40 mesh, cause significantly decreasing on the releasing of reducing sugar when compared to the small size biomass ( $p < 0.05$ ), while the releasing value of reducing sugar was not significant between 80-100 mesh biomass and < 100 mesh biomass. Due to the particle surface is large enough for an effective enzymatic hydrolyzation, the excessive surface area will not increase the efficiency of the hydrolyzation (Zhu et al., 2009). Therefore, further reducing the particle size will not be beneficial to hydrolyzation. The results indicated that the agave biomass almost completely converted to reducing sugar with the

maximum yield 38.97 % when compared with 37.76 % of total carbohydrate (Table 1) which is hydrolysis for 72 h with material size less than 80 meshes.

#### 4. Conclusion

*A. americana* biomass is widely available and a low recalcitrance biomass as biorefinery feedstock. The feedstock can be effectively hydrolyzed by lignocellulolytic enzyme cocktail with high pectinase activity. *A. niger* Gyx086 using wheat straw as substance can produce such a low-cost enzyme cocktail. The optimized enzyme cocktail can completely convert agave polysaccharide into reducing sugars with 1.0 % of solid loading (< 80 mesh) in a mild condition of 35 °C and pH 5.0. The efficient and low-cost saccharification system is promising to promote agave bio-based industry and the work provides a value protocol for some kinds of biomass saccharification .

E-supplementary data of this work can be found in the online version of the paper.

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#### Notes

Declarations of interest: none.

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**Figure Captions**

**Figure 1** PG activities were affected by different biomass substance (a), temperature (b), pH value (c), and biomass content (d).

**Figure 2** The contour plots between temperature (X1) and pH (X2) and fermentation time (X3) appears the interactive effects to the PG activities.

**Figure 3** Enzymatic characteristics of lignocellulolytic enzymes from *A. niger* Gyx086.

(a) Various temperature under pH 5.0; (b) Various pH under 50 °C; (c) Thermal tolerance with pH 5.0 at 50 °C; (d) Thermal tolerance of PG with pH 5.0 at 27 °C, 35 °C and 40 °C

**Figure 4** Saccharification of agave biomass using an enzyme cocktail from *A. niger* Gyx086 in a 10 ml of hydrolysis volume. (a) Temperature affection using half of the enzyme broth with 1 % of 20 - 40 mesh agave biomass loading; (b) Enzymatic broth rate affection at 35 °C with 1 % of 20 - 40 mesh agave biomass loading; (c) Biomass solid rate affection using the whole enzyme at room temperature with feedstock size 20-40 mesh; (d) Biomass size affection using half of enzyme broth at 35 °C;

**Table 1** Enzymatic dosages for hydrolysis and sugars released from agave biomass using these enzyme cocktails

	E1+E2	E1+E3	E1+E4	E2	E3	28E	39E
FPA (U/g dw)	36.5±1.8 <sup>a</sup>	22.9±1.1 <sup>b</sup>	37.1±1.8 <sup>a</sup>	25.6±2.3 <sup>b</sup>	14.3±2.5 <sup>c</sup>	4.77±0.14	6.77±0.29
CMCase (U/g dw)	62.4±1.1 <sup>b</sup>	69.7 ±1.3 <sup>a</sup>	44.3±5.3 <sup>d</sup>	52.1±0.1 <sup>c</sup>	66.3±4.7 <sup>ab</sup>	14.31±0.29	13.82±0.38
Glucosidase (U/g dw)	31.3±0.76 <sup>a</sup>	38.5±4.7 <sup>a</sup>	31.8±4.9 <sup>a</sup>	2.1±0.3 <sup>c</sup>	5.2±0.11 <sup>b</sup>	2.51±0.18	2.96±0.37
Xylanase (U/g dw)	302.4±5.2 <sup>a</sup>	135.9±2.22 <sup>b</sup>	143.1±8.5 <sup>b</sup>	273.3±21.6 <sup>a</sup>	55.8±0.7 <sup>c</sup>	89.23±2.15	122.5±4.57
PG (U/g dw)	-	51.4±7.5 <sup>a</sup>	-	-	61.5±4.9 <sup>a</sup>	105.92±4.62	6.60±1.59
Released RS (mg/g)*	122.18±1.45 <sup>a</sup>	117.91±1.03 <sup>a</sup>	100.27±0.92 <sup>b</sup>	92.6±0.19 <sup>c</sup>	117.06±1.32 <sup>a</sup>	138.8±2.70	104.9±1.90
Total monosugars (mg/g) **	109.92±0.23 <sup>a</sup>	72.14±3.37 <sup>b</sup>	48.25±0.15 <sup>d</sup>	49.87±1.2 <sup>d</sup>	67.39±1.52 <sup>c</sup>	63.6±4.7	53.1±1.8
Arabinose	2.63±0.38	2.18±0.07	2.63±0.08	1.05±0.41	1.59±0.33	3.6±0.2	1.9±0.3
Galactose	38.93±0.45	8.16±0.30	3.90±0.15	10.05±0.19	5.37±0.15	5.1±0.6	5.2±0.5
Glucose	54.23±1.65	54.15±2.28	32.48±1.02	33.45±0.68	56.62±1.28	48.1±2.9	33.1±1.3
Xylose	5.78±0.08	2.03±0.13	4.51±0.09	3.92±0.05	1.34±0.08	4.5±0.3	7.4±1.1
Mannose	7.35±0.11	5.62±0.06	4.73±0.08	1.43±0.02	2.47±0.10	2.3±0.1	4.8±0.1

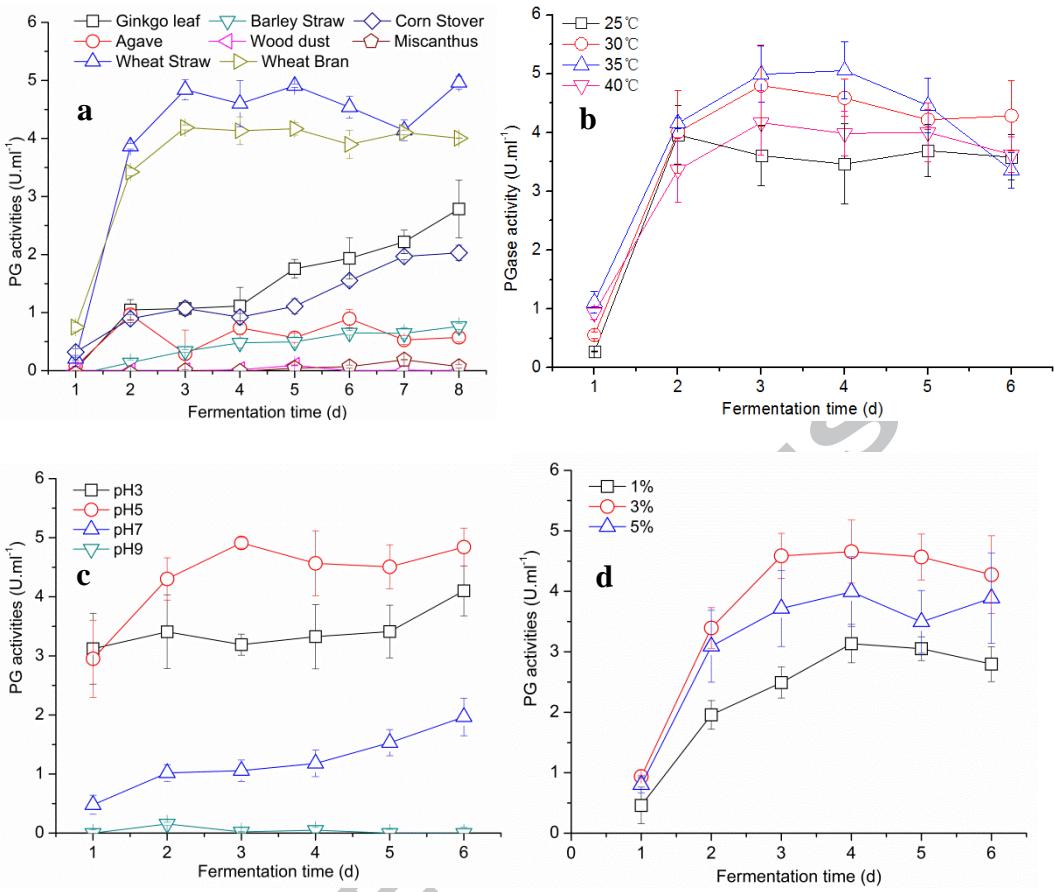
\* Released reducing sugar from agave in a 10 ml reaction volume with 0.1 g biomass for 72 h at 50 °C; \*\* Fructose hasn't been found using the ion chromatography; The same superscript alphabet on the same line indicated that there is a non-significant difference among these values.

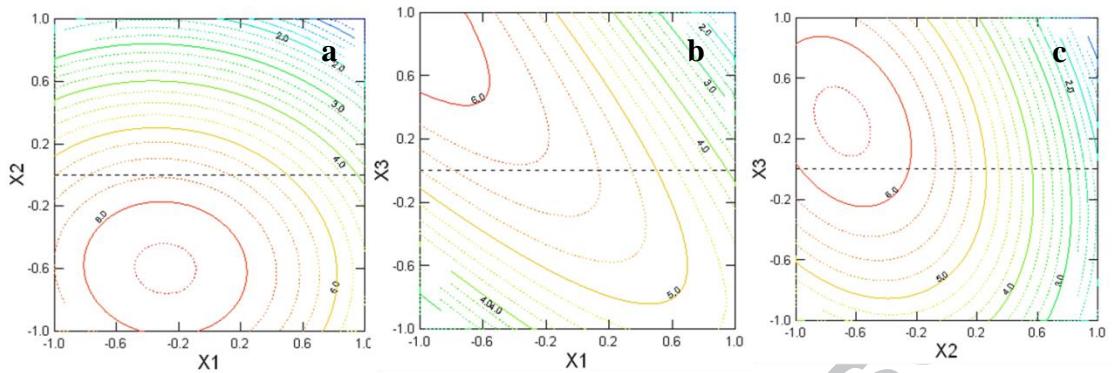
**Table 2** Box–Behnken design matrix for optimization of the PG activity

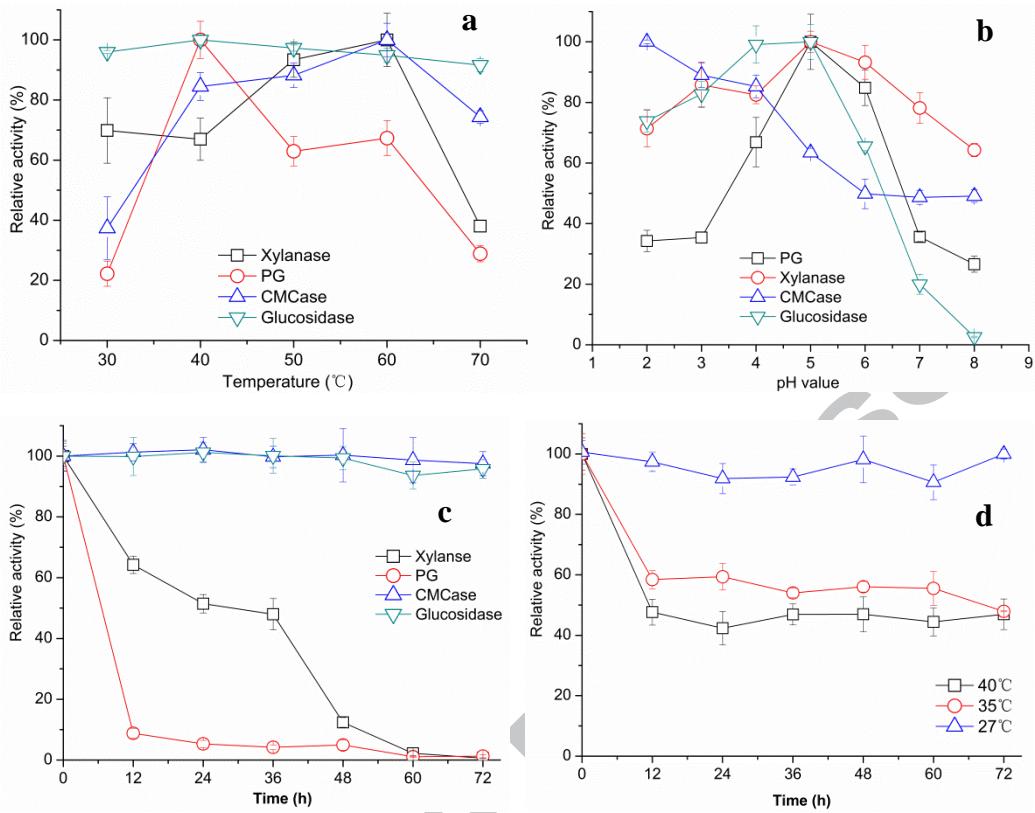
Run	X <sub>1</sub> Temperature(°C)	X <sub>2</sub> pH value	X <sub>3</sub> Time (h)	PG activities (U.ml <sup>-1</sup> )
1	-1 (28)	-1 (5)	0 (72)	5.65±0.68
2	1 (36)	-1	0	3.69±0.56
3	-1	1 (7)	0	2.51±0.35
4	1	1	0	0
5	-1	0 (6)	-1 (48)	1.64±0.20
6	1	0	-1	5.05±0.29
7	-1	0	1 (96)	5.87±0.13
8	1	0	1	1.44±0.05
9	0 (32)	-1	-1	4.29±0.37
10	0	1	-1	1.55±0.31
11	0	-1	1	6.01±0.33
12	0	1	1	0
13	0	0	0	5.10±0.28
14	0	0	0	5.69±0.34
15	0	0	0	6.05±0.12

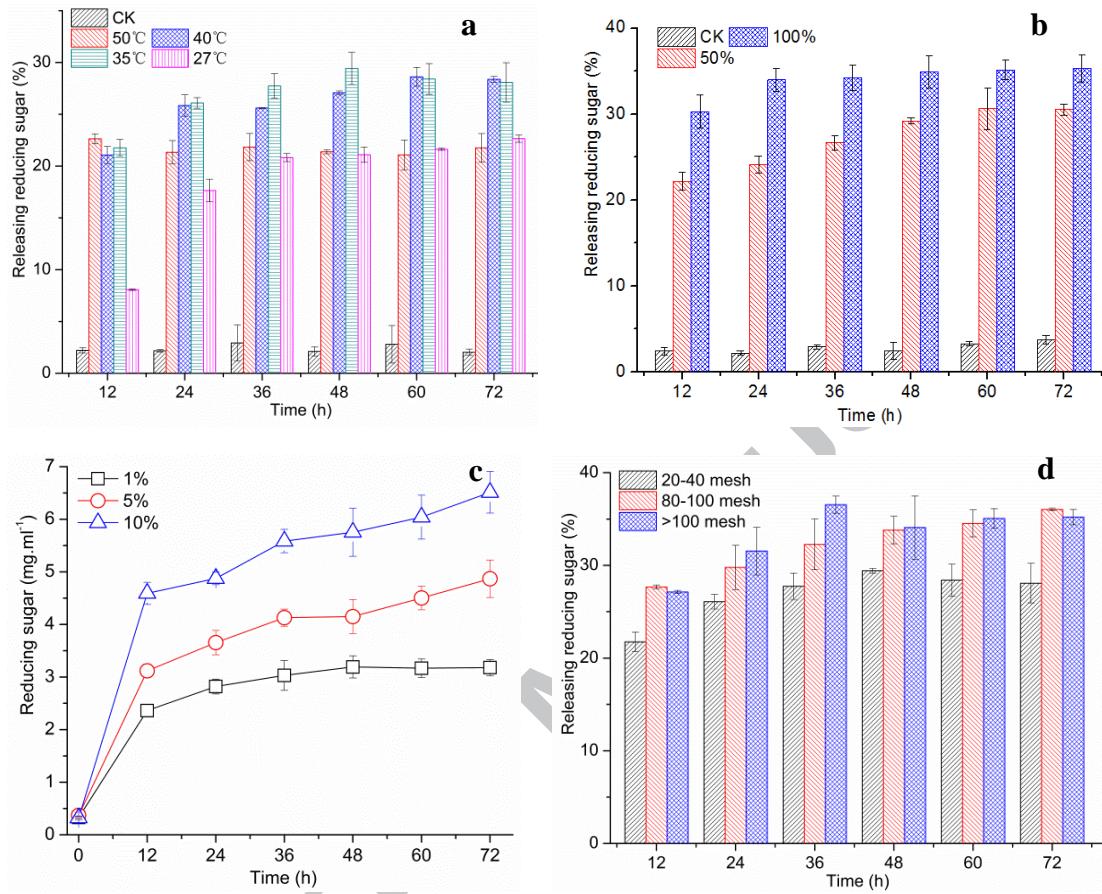
**Table 3** The variance analysis ANOVA and the lack of fit test for the response surface quadratic model

Source	df	SS	Mean Squares	F-ratio	p-value
Regression	9	67.73	7.53	15.58	0.00
Linear	3	34.15	11.38	23.57	0.00
Quadratic	3	15.45	5.15	10.66	0.01
Interaction	3	18.13	6.04	12.51	0.01
Residual Error	5	2.42	0.48		
Total Error	14	70.14			
Squared Multiple R	0.98				
Adjusted Squared Multiple R	0.97				
Lack of Fit	3	1.96	0.65	2.89	0.27
Pure Error	2	0.45	0.23		
Residual Error	5	2.42	0.48		

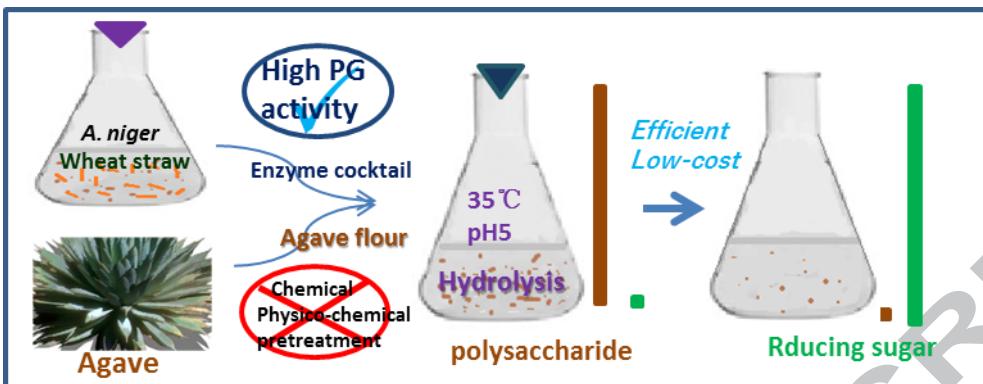
**Figure 1**

**Figure 2**

**Figure 3**

**Figure 4**

## Graphical abstract



***Highlight***

*A. americana* was proved to be a low recalcitrance biomass.  
Pectinase was confirmed which dramatically improved saccharification of agave.  
Cost-effective enzyme cocktail was produced using wheat straw as substance.  
Enzyme cocktail from *A. niger* Gyx086 can effectively saccharify agave polysaccharide.  
Un-pretreated agave biomass was near-completely hydrolyzed at 35°C in less than 72 h.