Study of enzymatic saccharification of *Agave* leaves biomass to yield fermentable sugars

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Abstract *Agave* is a good source of polysaccharides for the production of fermentable sugars as sustainable bioenergy feedstock solutions for semi-arid and arid lands. This plant grows in arid areas, which correspond to a large territory in northern Mexico. Having lignocellulose as the polysaccharide of interest, the information for the enzymatic saccharification of this kind of material is limited. *Agave* cell walls have a unique recalcitrant nature, but having a high cellulose content, makes this plant material an interesting research subject. In this work, acidic, alkaline and aqueous pretreatments were evaluated to generate a biomass rich in cellulose. The saccharification of pretreated *Agave* leaves-residue was evaluated under experimental designs to identify the most suitable conditions for enzymatic hydrolysis. Maximum value obtained was 31% glucose, which further increased to 41.4% at extended hydrolysis time of 96 h. The highest cellulose-saccharification reached was up to 61.81%, making *Agave atrovirens* an alternative for bioethanol production in its geographical area of cultivation.

Keywords Enzymatic hydrolysis · Maguey · Bioenergy · Glucose · Cellulase

Introduction

All types of lignocellulosic residues generated from agriculture and their processing industries, are useful biore-sources for various products (Corona-González et al. 2016) and processes (Montiel et al. 2016; Abdeslahian et al. 2011). Cellulose derived from these materials produced in vast amounts annually is a sustainable carbon source of renewable energy (Arenas-Cárdenas et al. 2016). The use of these resources holds great importance due to its abundance (Arreola-Vargas et al. 2015). As the gradual depletion of oil sources, alternatives for energy have been investigated, such as the production of bioethanol, biodiesel and many other processes using lignocellulosic residues (Caspeta et al. 2014). There are various reports available related to its processing-microbiology and enzymology at small and industrial levels, but more information is required for efficient and cost-effective use of cellulose from agricultural residues (Hidayat et al. 2012). *Agave* plants widely grow in Mexican semi-desert area under stressful conditions; having very low water requirement proliferates well in semi-arid regions. *Agave* plants possess a CAM metabolism (Crassulacean acid metabolism), which allows an efficient use of water and nutrients in semi-arid conditions (Black and Osmond 2003), accumulating more sugars than other plant species (Davis et al. 2011). *Agave* leaves are residues from alcoholic beverages production, and have been used for animal feed, as soil-conditioner, energy source for combustion in distillation, cloth-fabrics, wound-healing among many other uses (Nava-Cruz et al. 2014), but these traditional applications are of low economic importance. The attractive option to utilize *Agave* is its enzymatic hydrolysis to generate fermentable sugars, but it cannot be very easily executed due to its resistant structure (Saucedo-Luna et al. 2011; Hernández-Salas et al.

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2009). One of the key steps in the efficient use of agricultural residue is the pretreatment, making it more accessible to biotechnological processes (Da Silva et al. 2015); hence, the selection of a suitable pretreatment has a large impact on costs of the conversion-process (Barrera et al. 2016). For identification of the best saccharification conditions, experiments must be carried out to identify the effect of variables involved on hydrolysis and quantification of the response obtained by the manipulation of the process conditions (Van Dyk and Pletschke 2012). The aim of this work was to study the performance of tested pretreatment methods and determine the conditions of enzymatic hydrolysis of the *Agave* leave-fibers selected by the experimental design that would result in the highest glucose accumulation in the system.

Materials and methods

Plant material and physical pretreatment

Leaves of *Agave atrovirens* and *Agave salmiana* were obtained from the Saltillo Coahuila region, chopped to small cubes, then skin was peeled off to obtain inside contents (fibers). The fibers were dried at 70 °C, so they could easily be milled to a fine particle size (1–2 mm).

Acidic pretreatment

For this experiment, 200 mL of 1%, v/v HCl solution was added to 10 g of each plant material in Erlenmeyer flasks. The mixture was treated in an autoclave at 121 °C for 15, 30 and 45 min, respectively. After this operation, the material was washed to neutral pH with water and dried.

Alkaline pretreatment

For this process, 200 mL solution of 2%, w/v NaOH was added to 10 g of plant material in Erlenmeyer flasks. The mixture was treated in an autoclave and subjected to 121 °C for 15, 30 and 45 min, respectively. After this operation, the material was washed to neutral pH with water and dried.

Aqueous pretreatment

For this, 200 mL of water was added to 10 g of plant material in Erlenmeyer flasks. The mixture was placed in an autoclave and subjected to 121 °C for 15, 30 and 45 min, respectively. After this operation, the material was washed with water and dried.

All pretreatments were carried out in triplicate sets, washed samples were dried and stored for analysis and

saccharification. The cellulose content was determined in treated and untreated leaf-fibers by acid and neutral detergent fiber method (Van Soest et al. 1991). A commercial enzyme complex was used for saccharification (Powercell; Prozyn, Brazil) which is a mixture that must contain the required enzymes (endoglucanases, cellobiohydrolases and β -glucosidases) to degrade cellulose. After enzymatic hydrolysis, apparent cellulose degradation and glucose accumulation were measured by means of a RANDOX glucose enzymatic kit (England).

Exploratory design on enzymatic hydrolysis of cellulose

A Plackett–Burman design was applied to evaluate different factors affecting enzymatic hydrolysis of cellulose for subsequent glucose accumulation. The evaluated factors were: temperature, agitation, pH, enzyme loading, substrate, surfactant and *Agave* species. This design required the establishment of two levels for each factor (Table 1). After establishing the most influential factors, enzymatic hydrolysis of pretreated fibers was carried out for a period of 96 h.

Enzymatic hydrolysis and statistical optimization

In this design three evaluated factors were: enzyme loading, pH and substrate, using a Box–Behnken design, three levels were established for each factor (Table 2). This type of experimental design allows determining, as in this case, the optimal enzymatic hydrolysis conditions. The experiments were carried out in triplicate and results were analyzed with Statistica 7.0 software. After analysis, an extended hydrolysis was performed for 96 h to determine if hydrolysis yields could be improved.

Table 1 Plackett–Burman design variables used in experiments with the contribution percentage of each variable and its standard effect on the system

Variables	Low level (−1)	High level (+1)	Contribution (%)	Standard effect
Enzyme (U/L)	1000	2000	31.3	11.7
Agave Fiber type	<i>A. atrovirens</i>	<i>A. salmiana</i>	23.3	−8.72
Substrate (g)	0.4	0.6	15.9	−6.93
pH	4.6	5	14.4	−5.38
Temperature (°C)	46	50	12.4	4.61
Surfactant (%)	0.01	0.03	1.7	0.634
Stirring (rpm)	50	150	1.1	0.398

Table 2 Evaluated variables on the Box–Behnken design with cellulose enzymatic hydrolysis and glucose liberation results

Treatment	Enzyme	pH	Substrate	Glucose (mg/g)	Cellulose hydrolysis (%)
a	6000	4.6	0.3	264.38	39.39
b	2000	4.6	0.3	210.21	31.32
c	4000	4.6	0.4	310.90	46.32
d	4000	4.6	0.2	172.71	25.73
e	4000	4.4	0.3	266.69	39.73
f	2000	4.4	0.4	191.46	28.52
g	6000	4.4	0.4	306.04	45.60
h	2000	4.4	0.2	157.43	23.46
i	6000	4.4	0.2	178.26	26.56
j	2000	4.2	0.3	160.21	23.87
k	4000	4.2	0.2	176.18	26.25
l	4000	4.2	0.4	262.99	39.18
m	6000	4.2	0.3	240.07	35.77

Mathematical modeling of enzymatic hydrolysis

Two equations were used to predict values of (1) glucose accumulation and (2) cellulose hydrolysis, in a period of 96 h under the established conditions from the Plackett–Burman and Box–Behnken design processes.

$$\text{Glu} = \frac{(\text{Glu}_{\max})(t)}{(k + t)} \quad (1)$$

Where Glu = Amount of glucose obtained by enzymatic hydrolysis, $\text{Glu}_{\max}$ = Maximum glucose accumulation, k = Constant of glucose accumulation and t = Time of enzymatic hydrolysis.

$$\text{Cel} = \frac{(\text{Cel}_{\max})(t)}{(k + t)} \quad (2)$$

In the case of the cellulose hydrolysis equation Cel = Cellulose, $\text{Cel}_{\max}$ = Maximum cellulose degradation, k = Constant of cellulose hydrolysis and t = Time of cellulose enzymatic hydrolysis.

Results and discussion

Pretreatments

Physical procedures cutting and milling were very useful for the processing of the leaf-material. As the particle size decreased, this led to an increase in available surface area and also decreased the degree of polymerization of the polysaccharides present in the material, which contributed a favorable effect on the hydrolysis of lignocellulosic material (Barrera et al. 2016). The determination of

cellulose found that the fiber in both *Agave* species were counted for more than 60% cellulose levels. As reported in previous work (Medina-Morales et al. 2011), the untreated sample after physical treatment contained cellulose of $23.48 \pm 0.509\%$ in *A. atrovirens*, and $35.26 \pm 0.113\%$ in *A. salmiana*. Subsequently, material was given an alkaline treatment and the cellulose content was analyzed after alkali treatment to 67.12 ± 0.296 and $61.25 \pm 0.098\%$ in *A. atrovirens* and *A. salmiana*, respectively. This change in the amount of cellulose may be due to interference of the variety of compounds in *Agave* leaves, along with ligno-cellulose. The alkaline treatment removed these compounds and mostly cellulose was left (Medina-Morales et al. 2011).

Pretreatment and enzymatic hydrolysis

In the pretreatment of *A. salmiana* conducted with plain water, the best saccharification results were obtained in leaves-fibers pretreated for 30–45 min and no difference in hydrolysis results of alkali and acid pretreatment of 15 min (Fig. 1). The 30-min procedure in aqueous treatment could be selected because it represents less energy expenditure and high accumulation of sugars liberated by enzymatic hydrolysis. The enzymatic hydrolysis of fibers after aqueous pretreatment allowed an accumulation of 529.44 mg/g of sugars.

A similar phenomenon took place in pretreated *A. atrovirens* as indicated by hydrolysis results (Fig. 2). The highest accumulation of sugars were detected using the aqueous treatment. Therefore, it is justified that process with less expenditure of energy will be used for further pretreatments. The highest sugar concentration was of 300.55 mg/g, which confirmed enzyme had a better action on pretreated *Agave* leaf-fibers for hydrolysis. It is worth noting that, this high sugar concentration, aside from the glucose liberated by enzymatic hydrolysis, fructose can be released by the pretreatments. In later experiments, instead of reducing sugars, glucose was quantified as the sugar liberated by enzymatic hydrolysis.

In both cases, enzyme-blank saccharifications showed lower sugars liberated by the average amount of 24.54 mg/g (2.45%) in the case of *A. atrovirens*, and 32.46 mg/g (3.24%) in *A. salmiana*. These values are well below from the sugars liberated in process using enzymes. The aqueous pretreatment was found suitable for processing *Agave* leaves-material by increasing cellulose susceptibility to enzymatic hydrolysis, this result is in agreement with previous reports (Barrera et al. 2016; Moxley et al. 2012). The pretreatment performed with strong acids can have consequences of production of toxic compounds that usually inhibit ethanologenic microorganisms such as *Saccharomyces cerevisiae* (Baeyens et al. 2015). Moreover,

Fig. 1 Release of reducing sugars after conducting hydrolysis of fibers from three pretreatments of *A. atrovirens*

Fig. 2 Release of reducing sugars after conducting hydrolysis of fibers from three pretreatments of *A. salmiana*

the use of diluted acid significantly reduces the formation of inhibitors (Arenas-Cárdenas et al. 2016). In the alkaline pretreatment, solubilization of hemicellulose, especially xylan occurs (Samanta et al. 2012). These reactions cause the lignocellulosic material to swell and thus cellulose becomes more accessible. The controlled pretreatments can promote the removal of hemicellulose from fibers, enhancing the accessibility of cellulose to enzymatic hydrolysis (Vallejos et al. 2015). A disadvantage of alkali

pretreatment is that some compounds can be formed, such as catechol and 4-hydroxybenzaldehyde, depending of the nature of lignocellulosic material (Pedersen and Meyer 2010). The procedure of pretreatment carried out with water or autohydrolysis, promotes the hydrolysis with no addition of chemicals (acid or alkali), which saved the process-cost and avoided the effluent generated as washings of treated fibers after acidic and alkaline pretreatments (Vallejos et al. 2015). The only disadvantage of aqueous

Table 3 Plackett–Burman cellulose enzymatic hydrolysis yield in the evaluated conditions

Treatment	Temperature	Stirring	pH	Enzyme	Substrate	Surfactant	Fiber origin	Cellulose hydrolysis (%)
A	50	50	4.6	1000	0.4	0.03	<i>A. salmiana</i>	11.27 ± 1.31
B	46	150	5.0	1000	0.4	0.03	<i>A. atrovirens</i>	10.65 ± 2.98
C	46	50	5.0	2000	0.4	0.01	<i>A. salmiana</i>	12.02 ± 0.94
D	50	150	4.6	2000	0.4	0.01	<i>A. atrovirens</i>	25.43 ± 1.31
E	46	150	4.6	1000	0.6	0.01	<i>A. salmiana</i>	3.71 ± 0.38
F	50	50	5.0	1000	0.6	0.01	<i>A. atrovirens</i>	9.01 ± 1.00
G	50	150	5.0	2000	0.6	0.03	<i>A. salmiana</i>	11.82 ± 0.99
H	46	50	4.6	2000	0.6	0.03	<i>A. atrovirens</i>	18.19 ± 2.88

treatment is usually as this process run at neutral pH, it requires higher temperatures and, therefore, in current work a typical-temperature autoclave was operated for aqueous pretreatment, because avoiding high temperatures (150–180 °C), toxic compounds would be produced in lesser amounts (Arenas-Cárdenas et al. 2016). The results in Figs. 1 and 2 concluded that for later processes, pretreatments of leave-fibers with water or autohydrolysis would be the procedure of choice, as the amounts of sugar released are high, and pretreatment is with low cost, convenient to perform and very eco-friendly for the environment.

Selection of hydrolysis parameters and variables

By using experimental designs it is possible to determine how the system parameters affect the process, and based on this, identify the most adequate conditions to achieve the highest possible yield of glucose by enzymatic cellulose hydrolysis. According to the results from Plackett–Burman design (Table 1), the highest contribution to cellulose enzymatic hydrolysis was achieved by enzyme loading (31.3%), followed by fiber species (23.3%), amount of substrate (15.9%), pH (14.4%), temperature (12.4%), surfactant (1.7%) and agitation (1.1%). Last two factors were of no significance, whether increasing or decreasing their levels would not have effect on cellulose enzymatic hydrolysis. Each treatment (coded A to H) yielded a certain amount of cellulose hydrolysis, a minimum of 3.71 in treatment E to a maximum 25.43% in D (Table 3). *A. atrovirens* fibers were more susceptible to enzymatic hydrolysis than *A. salmiana*, yielding higher levels of cellulose hydrolysis. According to research from other investigators, there are certain features that can make cellulose less recalcitrant to hydrolysis. High moisture content in biomass such as used in this work could have high amorphous mass fractions, more susceptible to depolymerization (Yang et al. 2015).

By using a Box–Behnken design, the response detected from enzyme-dosage, substrate and pH-levels, was

evaluated for cellulose hydrolysis and amount of glucose liberated from pretreated *Agave* leaves-fibers. The design with experimental-matrix allowed a better experiment management and result interpretation. The evaluated factors were arranged by levels. Two levels were high and low as +1 and −1, and a midpoint value was shown as 0. Glucose levels varied from 157 to 310 mg/g, which equals to 23, 39 and 46.18%, respectively (Table 2). Lower levels of pH and substrate promoted lower glucose yields. The highest yield was obtained with a medium enzyme level (4000 U/L) instead of using higher level 6000 U/L. This could be attributed to the fact that with medium enzyme levels, pH of 4.6 and high level of substrate, it reached an equilibrium allowing enzyme to depolymerize available cellulose. The high tested pH level would increase glucose-release if high levels of substrate were used. The highest glucose levels (Fig. 3) were determined when a pH 4.6 was used. As it was expected, lowering pH decreased cellulose performance for releasing glucose.

Many reported cellulases in specialized databases are listed in pH range of 4–5 (Singhania et al. 2010). In the Fig. 4 a zone of maximum glucose accumulation can be observed. In this step, by keeping medium levels of enzyme loading and high pH-levels, the maximum accumulation of glucose was observed. The treatment matrix used allowed finding optimal conditions for glucose-release. In the process, a maximum of 310 mg of glucose/g of fibers was the result of the conditions marked as treatment C (Table 2), this effect is given by the enzyme level in Fig. 3c.

After defining the optimal conditions for hydrolysis, an experiment was carried out until 96 h. Comparing the results from a 96 h of hydrolysis under a previous reported work (Medina-Morales et al. 2011), there was a difference of 161.14 mg/g of glucose on hydrolysis yield where the maximum value was 285.17 mg/g. It is important to take into account that where an optimization design could propose the best conditions for a certain process, the duration of reaction-time as an important factor could be exploited for increasing the hydrolysis yields. Research work

Fig. 3 Surface graphic showing the effect of enzyme and pH (a), pH and substrate (b) and enzyme and substrate (c)

Fig. 4 Glucose accumulation under Box–Behnken resulting conditions at 96 hours of enzymatic hydrolysis showing correlation values with (solid line) calculated values and (diamonds) experimental values in each graphic. R^2 values are: **a** 0.988, **b** 0.995 and **c** 0.985

regarding enzymatic cellulose-depolymerization reports that these processes are subject of finding the most adequate conditions to achieve maximum cellulose degradation (Pihlajaniemi et al. 2015). In this case, an exploratory design was applied, which derived in pointing the most significant factors and treatments that yielded certain glucose levels. The PB design did provide the most adequate conditions for enzymatic hydrolysis, the Box–Behnken design best evaluated the results.

Cellulase-enzyme complex is composed of endo β -1,4 glucanases, exo β -1,4 glucanases (cellobiohydrolases) and β -1,4 glucosidases (Ogeda et al. 2012; Kuhad et al. 2016). The third component of cellulase has great importance in the process because is responsible for effective glucose-release. As long as β -1,4 glucosidase is available in reaction-system, there will be no by-product-limitations in hydrolysis, such as cellobiose inhibition (Li et al. 2017). If high levels of enzyme are added to the hydrolysis system, the yields may increase (Zhang and Cai 2008), but the oligomer-levels must be kept at medium levels. According to calculation, 46.32% of cellulose content was enzymatically hydrolyzed. Comparing these results to other lignocellulosic materials, there have been hydrolysis yields of 32% from rice (Saha and Cotta 2008), 36% from olive tree biomass (Cara et al. 2008). Using low solids concentration, cellulose degradation was better with low accumulation of fermentable sugars as a disadvantage (Medina-Morales et al. 2011). Considering that 96 h is a considerable amount of time, the presence of agents such as a surfactant, such as the one used in this work, could grant higher stability to the enzymes and achieve higher saccharification yields (Tabka et al. 2006; Sánchez-Ramírez et al. 2017).

After the results of 96-h hydrolysis using BB design conditions for maximum cellulose hydrolysis and glucose accumulation, a mathematical equation determined the time of maximum hydrolysis. In this case, the mathematical model gave a time of 28 h of hydrolysis, where amounts of glucose between 24 and 36 h were of 259.21 and 296.29 mg/g, respectively. In Fig. 4, it is clear that after 24 h of hydrolysis, rate of glucose-release was lower, compared to early-hours in same system. Long-duration processes are expensive, which are less attractive at industry levels; so, with mathematical tools, more precise selection criteria should be applied to ensure the optimal development of a process to be operated at larger scale.

Using the results of the 96-h hydrolysis experiment, a mathematical equation was applied to estimate the time of hydrolysis in which the maximum yield could be achieved. In addition, correlation between calculated and experimental values were estimated. As shown in Fig. 4, mathematical modeling was done using the most adequate

enzymatic hydrolysis conditions. Each experiment yielded its respective $\text{Glu}_{\max}$ in mg of glucose per gram of substrate (mg/g) and K in hours (h). In Fig. 4a, the $\text{Glu}_{\max}$ value of 433.26 mg/g was obtained with a K of 18.51 h. In Fig. 4b the 484.47 mg/g and 21.92, respectively, and in 4c the values obtained were of 496.81 mg/g and 22.97. Correlation coefficients were calculated being values of 0.988, 0.995 and 0.985 on Fig. 4a, b and c, respectively. High correlation was observed between experimental and theoretical results of enzymatic hydrolysis. Considering the obtained values for both variables, an average value can be established as 471.52 mg/g and a K value of 21.14 h. These results helped to establish theoretically the highest yield under the specified conditions and prediction of glucose yields using same parameters as used in this experiment. Comparing the maximum hydrolysis yield from the Box–Behnken design, glucose released from *Agave*-fibers was improved from 310.9 to 414.85 mg/g, which is 46.18 and 61.8% of depolymerized (pretreated) cellulose. The increase in cellulose hydrolysis in the system, compared with the time used in experimental designs, may be attributed to the fact of longer time for enzymatic hydrolysis of cellulosic fibers, which accumulated more glucose (Medina-Morales et al. 2011).

In case of cellulose enzymatic hydrolysis, the highest theoretical values in BB design, were obtained experimentally as 54.88, 59.67 and 60.74% (Fig. 5a, b, c). The highest calculated values in same systems were of 67.56, 76.20 and 78.65%. The average values of the highest cellulose hydrolysis ($\text{Cel}_{\max}$) were of $74.13 \pm 5.8\%$ and the K value of $25.68\% \pm 3.1$. The three replicates of the experiment, being modeled with the same equation (Eq. 2), showed similar behavior and high correlation values of 0.982, 0.996 and 0.98. Similar Box–Behnken design strategy has been successfully applied in biotechnology and biochemistry field (Rodríguez-Durán et al. 2011; Sepúlveda et al. 2012).

Conclusions

Enzymatic hydrolysis evaluated by Plackett–Burman showed that enzyme loading, pH, substrate quantity, using *Agave* leave-fibers, were the most influential factors in the process. By Box–Behnken design, the conditions of 0.4 g of fibers, pH of 4.6 and 6000 U/L resulted in the highest glucose accumulation by cellulose-depolymerization. Box–Behnken design conditions, compared to the Plackett–Burman, cellulose degradation reached 61.81 and 42.39%, respectively. Overall conclusion is that the aqueous pre-treatment proved to be more effective for enzymatic hydrolysis for extended period of time, which allowed the

Fig. 5 Apparent cellulose hydrolysis with correlation values under Box-Behnken design conditions at 96 hours of enzymatic hydrolysis with (solid line) calculated values and (diamonds) experimental values in each graphic. R^2 values are: **a** 0.982, **b** 0.996 and **c** 0.98

enzyme complex to achieve a larger amount of hydrolyzed cellulose.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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