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Process options for conversion of *Agave tequilana* leaves into bioethanol

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ABSTRACT

This paper reports on mild acid pretreatment options for the conversion of *Agave tequilana* leaves into composite sugars for ethanol fermentation. The effect of five different pretreatment conditions (time, temperature and acid concentrations) were assessed in terms of cellulose digestibility, hemicellulose solubilisation and lignin content in leaves of 1.25 years old *A. tequilana* plants from Rockhampton and 2.5 year plants from Kalamia. Dilute acid pretreatment and enzyme saccharification of *A. tequilana* leaf bagasse significantly improved total glucose recovery. A recovery of 273 mg/g (70% theoretical) was attained when the bagasse was pretreated with 2.0% H_2SO_4 for 60 min at 121 °C and saccharified with 6% (w/w) CTec 2. *Saccharomyces cerevisiae* efficiently fermented crude *A. tequilana* bagasse and juice hydrolysates within 13 h and 7 h respectively, yielding up to 38.6 g/L and 12.4 g/L. This corresponds to glucose to ethanol conversion rate of 68 and 61% for *A. tequilana* leaf bagasse and juice, respectively. With further developments, including fermentation of C5 sugars and inulinase saccharification of juices (release of fructose), this process could deliver greater yields, reinforcing its potential as a biofuel feedstock.

1. Introduction

The ever-increasing demand for transportation fuels combined with diminishing reserves of fossil fuels compels the production of energy from renewable sources. Biofuel produced from lignocellulosic feedstock is considered to be an exceptional solution owing to its favourable greenhouse gas (GHG) footprint status as a renewable resource and supply (McIntosh et al., 2012). Some *Agave* spp. could serve as potential sources of lignocellulosic biomass. *Agave* spp. are highly efficient in their use of water and require minimal supplementation with water or fertilisers (Holtum et al., 2010). Since they possess the crassulacean acid metabolic (CAM) photosynthetic pathway, they can be grown in arid and semi-arid regions that are unsuited to conventional agricultural crops or lignocellulosic feedstock such as poplar, miscanthus and switchgrass (Li et al., 2012; Somerville et al., 2010). Surprisingly, the agaves have higher average annual productivities ranging from 10–34 Mg/ha as compared to switchgrass (15 Mg/ha) and 11 Mg/ha for poplar wood (Somerville et al., 2010).

There are numerous studies reporting the use of *Agave tequilana* stem (pina) for production of potable alcohol (Tequila) but relatively few on biofuel production from agave bagasse (portion remaining after extracting fructose from the pina) (Caspeta et al., 2014; Hernández-Cortés et al., 2010; Hernández-Salas et al., 2009). The *A. tequilana* stem contain fructans as the storage carbohydrate which constitute more than 60% of the total soluble carbohydrates (Mellado-Mojica and López, 2012). The polyfructose solution obtained from the pulp of milled agave stem can also be hydrolysed with inulin enzymes to produce fructose syrup commonly used as sweeteners in food and beverage industries (Partida et al., 1998). However, the bagasse of both "pina" and the leaves consists of complex structural carbohydrates and lignin which require harsher treatments in order to release the lignocellulosic sugars (Li et al., 2012; McIntosh et al., 2012; Sluiter et al., 2008a). The leaves of mature *A. tequilana* plant are suitable for bioethanol production ecause they contain up to 42% structural carbohydrates and only 12% lignin (Li et al., 2012). In addition, up to 4.4% soluble sugars have been found to be present in the leaf juice (Li et al., 2014). Although the growth rate of *A. tequilana* is slow with long cropping cycle taking up to 5–7 years for maturity (Escamilla-Treviño, 2012), regular harvesting of leaves during this period presents an opportune feedstock for biofuel production.

Efficient utilization of lignocellulosic biomass requires pretreatment to liberate cellulose from its lignin seal and disrupt its crystalline structure before effective enzymatic hydrolysis can take place. This is generally achieved through either chemical or physical

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Table 1
Geographical location, environmental conditions and age of the plant used in the study (Bureau of Meteorology, 2016).

State/country	Geographical location	Average annual rainfall (mm)	Average annual temperature (min–max °C)	Age of the plant (years)
Queensland, Australia	Rockhampton, 23.32°S,150.52°E	811.2	16.7 – 28.4	1.25
Queensland, Australia	Kalamia, 19.58° S,147.41°E	1076.5	17.9 – 29.1	2.5

methods or a combination of both. Reported pretreatment methods used in the hydrolysis of agave bagasse include dilute acid (HCl 1.2–2% v/v) (Hernández-Salas et al., 2009; Saucedo-Luna et al., 2011), dilute alkali (NaOH 2% w/v) (Hernández-Salas et al., 2009) and ionic liquid (1-ethyl-3 methylimidazolium acetate [C2mim][OAc]) (Perez-Pimienta et al., 2013) followed by enzymatic saccharification. A comparative acid–alkali pretreatment study of *A. atrovirens* bagasse showed that dilute acid pretreatment (1.2% HCl) was far less effective than its alkali counterpart (2% NaOH) in producing sugars after enzyme saccharification (Hernández-Salas et al., 2009). The dilute acid treatment yielded between 5–9.9% (w/w) reducing sugars from bagasse of agave pine cone and whole biomass (pinecone + leaves) while the alkaline treatment yielded 12–58% reducing sugar (Hernández-Salas et al., 2009). In contrast, Saucedo-Luna et al. (2011), demonstrated greater yields with the pina bagasse from *A. tequilana* using a dilute acid approach. They pretreated the bagasse with 1–3% H_2SO_4 (w/w) and following enzymatic saccharisation were able to recover 41 g/L fermentable sugar (73.6% theoretical). Similarly, *A. tequilana* stalk bagasse pretreated with a modified Ethanosolv method (combination of water, ethanol and H_2SO_4 at 10% w/v) lead to the theoretical recovery of 91% of the total fermentable sugars (0.51 g/g) following saccharification with cellulase and beta-glucosidase (Caspeta et al., 2014). Also, agave bagasse pretreated with ionic liquid ([C2mim][OAc]) at 160 °C with solid loading of 15% w/w resulted in 45.5% delignification and significant improvement in recovery of sugar, releasing 14 mg/mL from 7 mg/mL (two fold) as compared to untreated material (Perez-Pimienta et al., 2013).

In terms of converting agave bagasse to ethanol, a reported study using dilute acid pretreatment accompanied by enzyme saccharification at 10% w/w solid loading produced 18.3 g/L ethanol equivalent to 0.18 g/g dry bagasse (Saucedo-Luna et al., 2011). In this study, the fermentation was carried out using native yeast *Pichia carribica* (UM-5 strain) which could ferment both hexose and pentose sugars, with an overall theoretical ethanol yield of 56.8% (w/w). However, in another study conducted by Hernández-Salas et al. (2009), alkaline pretreated/enzymatic saccharified agave bagasse yielded only 6.6 g/L ethanol from 56.4 g/L glucose (23% theoretical yield) following fermentation with a non-recombinant strain of *Saccharomyces cerevisiae* (Hernández-Salas et al., 2009). Higher ethanol yields were reported by Caspeta et al. (2014) by enzymatically hydrolysing the bagasse of *A. tequilana* stalk at high-solids loadings following dilute acid pretreatment. Employing an industrial strain of *S. cerevisiae*, a maximum ethanol yield of 0.25 g/g of dry agave bagasse corresponding to 86% of maximum theoretical (0.29 g/g) was attained. Also, two different strains of *S. cereviseae* were used on juice of *A. tequilana* leaves of 2–3 year old plant producing 11.4–13.8 g/L ethanol corresponding to 54–66% theoretical conversion (Corbin et al., 2015).

Reviews of the literature reveal only a small number of studies that may be construed to be associated with the use of *A. tequilana* leaves for biofuel production. These studies are limited to reports on their chemical composition, cellulose characterisation, with one attempt at pretreatment and enzymatic hydrolysis (Corbin et al., 2015; Li et al., 2012,2014) and bioethanol production (Corbin et al., 2015). To the knowledge of the authors, this is the first study assessing the suitability of bagasse derived from *A. tequilana* leaves of different stages of maturity and localities with different environmental conditions (BOM, 2016) as feedstock for bioethanol production (Table 1). Specifically, this paper examines and reports on the major chemical constituents of *A. tequilana* leaf biomass and details the use of dilute acid pretreatment and enzyme saccharification options of *A. tequilana* leaf bagasse. The fermentation potential of recovered sugars was also examined using an ethanol fermenting *S. cerevisiae* strain.

2. Materials and methods

2.1. Materials

The chemicals used in these experiments were of analytical grade obtained from Sigma chemicals Co. (St. Louis MO). Cellulase (Cellic® CTec2) was supplied by Novozymes (Bagsvaerd, Denmark).

2.2. Methods

A. tequilana leaves were tested for their composition at different maturity stages. Five different pretreatment options involving combinations of various temperatures, time and acid concentration were assessed to determine the role of various pretreatment conditions in solubilisation of sugar and production of degradation or inhibitory compounds. Enzyme saccharification of pre-treated material was also assessed for changes in sugar composition in response to pretreatment parameters. Finally, the hydrolysates obtained from enzyme saccharification were tested for bioethanol production.

2.2.1. A. tequilana *leaf processing*

A. tequilana leaf samples were obtained from field trials at Rockhampton (ROK) and Kalamia (KAL), Queensland, Australia. The trials at Rockhampton were established in September 2010 and those of Kalamia were started in June 2009. Mature and fully expanded leaves were sampled randomly through each plot using a long handled pruning device. Sampled leaves were washed with distilled water and cut into approximately 2 cm pieces. These were mixed and a subsample of approximately 1.5 kg fresh weight was dried in oven at 60 °C for 96 h prior to compositional analysis. The dried samples were ground in a laboratory grinder (Mikro Feinmuhle Culatti, Janke and Kunkel GmbH and Co., Staufen, Germany) and sieved. The fraction between 150–530 μm was collected and stored in an air tight container prior to determination of structural carbohydrates and lignin.

The remaining portion of the fresh leaf pieces were blended in a mixer grinder (Bajaj-GX7, IN) to macerate the pieces and put through a juicer (Sunbeam Café Series-JE 8600, Sunbeam Australia) to separate the juice from the bagasse. The bagasse was further squeezed to obtain the remainder of the juice which was then stored at −20 °C for further analysis by HPLC. The residual bagasse was washed with hot distilled water, squeezed and then oven dried at 60 °C for 48 h. The dried bagasse was ground using a rotary mill (Retsch ZM 1000) and sieved. Fractions between 530–1100 μm were retained for subsequent pretreatment experiments.

2.3. Pretreatment

Five pretreatment options were selected with different combination of H_2SO_4 acid concentration (1, 1.5, 2 and 4% v/v), time (30, 60, and 90 min) and temperature (115, 120 and 130 °C) to test their