

Low-Input Fermentations of *Agave tequilana* Leaf Juice Generate High Returns on Ethanol Yields

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Abstract During tequila production, up to 75 % w/w of the *Agave* plant is discarded when leaves are removed from the stem. The discarded leaves represent an extensive amount of unexploited biomass that was used here for bioethanol production in no-input fermentations, where no acid or enzymatic hydrolysis, supplementation of nutrients or standardization of carbohydrate content occur. Ethanol yield from *Agave* leaf juice is unaffected by sterilization but reduced if fermentation is reliant solely on endogenous microorganisms. Non-*Saccharomyces* yeasts, including *Kluyveromyces marxianus* and *Candida akabaneensis*, proved to be more robust than standard *Saccharomyces* spp. and yielded up to 88 % of the theoretical maximum ethanol from leaf juice. Combining leaf and stem juice, as from a whole plant, was predicted to maximize yield at up to 19,439 L/ha of ethanol from mature plants.

Keywords *Agave tequilana* · Ethanol · Fermentation · Non-*Saccharomyces*

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Abbreviations

CAM Crassulacean acid metabolism
TSS Total soluble solids
YPD Yeast extract-peptone-dextrose

Introduction

The advent of plant-based ethanol production using fermentation can be traced back to as early as 6000 BC [1]. Alcoholic fermentation involves the release of energy from carbohydrates by microorganisms, usually under low oxygen conditions, and yields ethanol and carbon dioxide. Historically, the production of alcoholic beverages has been reliant on a consortium of commensal microflora to convert the plant-derived carbohydrates into ethanol [2]. However, these spontaneous (uninoculated) fermentations often yield unpredictable end-products. In more recent times, the industry has optimized the fermentation process to generate more predictable and consistent high value end-products, such as wine, beer and spirits.

In principle, the same techniques apply for the production of alcohol for transportation fuel; however, the goal is not to produce a high value end-product but rather a cheaply produced “drop-in” fuel, which is both profitable and cost competitive with fossil fuels. In recent years, waste from feedstocks used for the manufacture of various beverages has been attracting attention as potential biomass sources for bioethanol and biochemical production [3]. One example of agro-industrial waste attracting attention is *Agave tequilana* plants, which are traditionally used for making tequila in Mexico. In tequila making, only part of this feedstock is converted to alcohol, rendering a substantial amount of plant material being underutilized. The highest producing region in Mexico, Jalisco, alone yields 6.59×10^5 tons of biomass per year [4].

For tequila making, the leaves are discarded and extensive processing is performed upon the stem biomass to generate a fermentable juice. Such processing steps include cooking the stem for extended periods to hydrolyse polymers, milling the cooked stems to extract the juice or “honey”, converting the sugars in the honey to alcohol by fermentation and finally double distilling the fermentation broth to produce pure tequila, which may be aged for a further 3 to 12 months in oak barrels [5]. The fibrous biomass that remains following milling of the stems is commonly referred to as the bagasse. This discarded bagasse contributes 40 % (wet *w/w*) of the stem mass [5]. Thus, under this processing regime, only about 25 % of the above-ground biomass of the *Agave* plants is utilized. In a biofuel context, this processing scheme provides opportunities for converting a substantial amount of aggregated vegetative tissue to ethanol or other low molecular mass alcohols.

To date, available information is related to the fermentation of juice extracted from cooked *Agave* stems, which is reflective of the processes used in the tequila industry (Table 1). *Agave* stem tissue is enriched with fructans, which are soluble polymers of fructose with mainly fructosyl-fructose linkages linked to a terminal glucose molecule that acts as storage polymers in 15 % of flowering plant species [6]. In these published studies, the effects of a range of parameters, including incubation temperatures and differing fermenting microorganisms, have been investigated (Table 1). Standard methods for *Agave* fermentation include sterilizing the juice, spiking it with monosaccharides and nutrients (usually to boost nitrogen content) and/or adding polysaccharide-specific enzymes to optimize fermentation conditions. However, the costs incurred by optimizing the nutrient content of the juice are frequently overlooked [7] and may not be economically viable for the production of cheap biofuel. In addition, little is known about the composition and fermentation of juice extracted from the discarded *Agave* leaves. To obtain a realistic, base-line view of utilizing novel fructan-enriched biomass such as *Agave* for potential large-scale bioethanol production, information about fermentations using no or minimal inputs other than leaf and stem juice is required.

In the present study, ethanol yields achieved from *Agave* leaf juice on a small scale were investigated using different processing methods and fermenting microorganisms. Four different fermentation schemes were used for *Agave* leaf juice, none of which included supplementation or standardization of the carbohydrate or nutrient content. The production of ethanol by native or commercially available fermenting organisms was assayed from raw and sterilized leaf juice substrates and compared with fermentation rates of either pure stem juice or combined stem and leaf juice. The ethanol (g/L) produced is then compared to the theoretical maximum ethanol production to determine the efficiency achieved. The theoretical maximum ethanol production was calculated by measuring the

total sugar content in each substrate and assuming 100 % conversion of monosaccharides to ethanol [8]. This fermentation data was then extrapolated to predict the ethanol that could be produced using *Agave* waste on a large scale. Thus, a toolset of methods and information specific to the small-scale fermentation of *Agave* juice has been developed and is likely to be useful in a commercial context for forecasting the potential yields of bioethanol from novel biomass sources such as *Agave* leaves.

Methods

Collection of *A. tequilana* Plants

A. tequilana plants ($n=3$) were harvested in Ayr, Queensland, Australia (19° 31' 49.9" S; 147° 24' 51.5" E). At the time of harvest, the 4.5-year-old plants were separated into leaves, roots, stem and offshoots, and the fractions were weighed. A commercial shredder (Cutter-Grinder CG03; South Australia, Australia) was used to extract the juice from a subset of each fraction. The juice was collected and transported to the University of Adelaide on dry ice and stored at -20 °C.

Total Carbohydrate Content in *Agave* Juice

The carbohydrate content in raw and pre-treated (autoclaved, trifluoroacetic acid (TFA) or fructanase-treated) *A. tequilana* juice from leaves, stem and offshoots was quantified by hydrophilic interaction chromatography (HILIC) [9]. Briefly, glucose, fructose and sucrose were quantified using a Prevail Carbohydrate ES column (150 × 4.6 mm) (Alltech; IL, USA) on an Agilent 1200 series liquid chromatography instrument equipped with an evaporative light scattering detector (Alltech ELSD 800). The mobile phase was 10 % water (A) and 90 % acetonitrile (B) at a flow rate of 1.0 mL/min. Sample peak areas were compared to calibration curves of standard solutions.

For pre-treatment, juice samples were autoclaved at 121 °C (Tuttnauer 3850 ELC Benchtop Sterilizer; Brinkmann Instruments, NY, USA) or hydrolysed using either TFA or fructanase (Fructan HK-Megazyme: AOAC Method 999.03; International Ireland Ltd., Wicklow, Ireland) as previously described [10]. For acid hydrolysis, juice and acid were mixed in equal proportions to a final concentration of 0.2 M TFA and heated at 80 °C for 1 h. For enzymatic hydrolysis, juice and enzyme were combined 1:1 and incubated at room temperature for 30 min. The enzyme was deactivated by heating 100 °C for 15 min. A sugar recovery standard was carried through the acid hydrolysis to calculate the degradation rates of released monosaccharides, which were used to correct HILIC data.

Table 1 Selected fermenting strains and conditions using *Agave tequilana* as reported in the literature

Substrate	Fermenting strain	Fermentation conditions	Sterilization	Sugar adjustments	Added nutrients	% conversion of carbohydrates to ethanol	Citation
Cooked <i>A. tequilana</i> stem	<i>Kluyveromyces marxianus</i> UMPE-1	20–28 °C/72 h pH 4–5	121 °C for 15 min Filtered	YPD supplemented with high fructose <i>Agave</i> syrup Adjusted to 120 g/L fermentable sugars	Ammonium phosphate (60 mg/L) Ammonium salts (20 mg/L)	94–96 %; <i>K. marxianus</i> 70–76 %; <i>S. cerevisiae</i>	[22]
	<i>Saccharomyces cerevisiae</i> Pan1						
<i>A. tequilana</i> stem juice source from tequila distillery	<i>Kloeckera africana</i> K1	30 °C/80 h pH n/a	121 °C for 15 min Filtered	Adjusted to 100 g/L (reducing sugars)	Ammonium sulfate (220 mg N/L) or Mixture of amino acids, final concentration of 220 mg N/L	49 %; supplemented with ammonium sulfate 94 %; supplemented with asparagine	[43]
	<i>Kloeckera africana</i> K1 <i>Saccharomyces cerevisiae</i> S1	30 °C/24 h pH 4.2	121 °C for 15 min Filtered	Adjusted to 12 °Bx with water (95 g/L reducing sugars)	Ammonium sulfate or diammonium phosphate (1 g/L) or Yeast extract (2 g/L) Ammonium sulfate (1 g/L) Ammonium phosphate monobasic (4 g/L)	78 %; <i>K. africana</i> 92–100 %; <i>S. cerevisiae</i> Higher for fermentations supplemented with N	[17]
Cooked <i>A. tequilana</i> stem (100 °C for 36 h)	<i>Saccharomyces cerevisiae</i> (S1 or S2)	30 °C/length n/a pH 2.5–4 [controlled using NaOH]	121 °C for 15 min	Adjusted to 100 g/L (reducing sugars)		88 %; no significant improvement due to pH, yields improved using aeration	[44]
	<i>Kloeckera africana</i> K1 <i>Kloeckera apiculata</i> K2 <i>Saccharomyces cerevisiae</i> (S1, S2 and S3)	35 °C/72 h pH 4.2	121 °C for 15 min Filtered	Adjusted to 12 °Bx (95 g/L reducing sugars)	Ammonium sulfate (1 g/L)	52 %; <i>K. africana</i> 43 %; <i>K. apiculata</i> 82–90 %; <i>S. cerevisiae</i>	[16]
<i>A. fourcroydes</i> leaf juice combined with industrial molasses ^a	<i>Kluyveromyces marxianus</i> Cicy-Ki and/or <i>Saccharomyces cerevisiae</i>	35 °C/48 h pH 4.7	121 °C for 1 h	Adjusted to 12 °Bx using varying ratios of juice + molasses [sugar concentration in g/L not given]	Ammonium sulfate (1.5 g/L)	5.2 % v/v ethanol; 25/75 mix of <i>K. marxianus</i> / <i>S. cerevisiae</i>	[15]

The definition of stem is used interchangeably with the terms pine, head, must and *piña*. Data was sourced from the following publications: [15–17, 22, 43, 44]

^aNo information was provided regarding the source of molasses

pH, Brix, Total Soluble Solids and Mineral Content

The pH of the juice was determined using a 900-P pH-mv-Temperature metre (TPS Pty Ltd, Australia). Brix readings of the juice samples were measured with a refractometer (Atago Co., Japan). The total soluble solids (TSS) and mineral content of juice samples were determined according to Corbin et al. [9].

Selection of Microorganisms

Fermenting microorganisms were obtained from the United States Department of Agriculture Agricultural Research Service (ARS) Culture Collection, National Center for Agricultural Utilization Research (Peoria, IL, USA) [11]. The yeast strains selected were *Kluyveromyces marxianus* (NRRL 1598), *Pichia kluyveri* (NRRL 17228), *Candida akabianensis* (NRRL 7846) and *Saccharomyces cerevisiae* (NRRL 636 and NRRL 139). The strains were streaked on 2 % w/v agar (yeast extract-peptone-dextrose, YPD) plates containing 1 % w/v yeast extract, 2 % w/v peptone and 2 % w/v D-glucose, and incubated at 28 °C for 48 h. Single colonies were picked and inoculated into the YPD medium and shaken (120 rpm) overnight at 28 °C. Cultures were diluted to an OD₆₀₀ of 0.5 to determine the optimal growth temperature. Serial 5-fold dilutions were spotted on YPD plates and incubated for 24 h at 28, 32, 37 or 42 °C ($n=2$).

Fermentation Conditions

Juice samples were centrifuged at 5000 rpm for 5 min to remove excess leaf tissue. Autoclaving of juice was completed at 121 °C at a maximum pressure of 210 kPa (Tuttnauer 3850 ELC Benchtop Sterilizer; Brinkmann Instruments, NY, USA). Leaf and stem juice were combined in a volume ratio of 3:1 (leaf/stem), to a final volume of 100 mL, representative of the whole plant being fermented simultaneously. At least two replicates were used for each of the inoculated fermentations and three replicates for spontaneous fermentations.

Native microorganisms in raw *Agave* leaf and stem juice were streaked (0.05 mL aliquots) on solid YPD plates and grown at 28 °C. A representative microbial culture of the endogenous species found within the *Agave* juice was made by harvesting these plates using a sterile loop. This formed an inoculum that was used to re-inoculate 100 mL of autoclaved juice samples, representative of juice samples that had become contaminated following sterilization.

The yeasts obtained from the ARS Culture Collection were grown overnight at 28 °C on YPD and a single colony was picked. The single colony was grown in YPD liquid broth (28 °C) in a shaker incubator (120 rpm) and used to inoculate 100 mL of raw juice samples at a cell density of 5×10^6 cells/mL. All fermentations were completed in sterile Erlenmeyer flasks (250 mL in size) with side arm sampling ports sealed

with water-filled airlocks. The fermentation flasks were shaken at 150 rpm and at 28 °C. The yeast cells were removed from the fermentation broth by centrifugation (1 min/10,000×g), and the supernatant was stored at –20 °C until analysis.

Analysis of Substrate and Fermentation Products

The concentrations of organic acids (acetic, citric, lactic and malic acid), glycerol and ethanol in the fermentation broth were quantified by high-performance liquid chromatography (HPLC) as previously described [12]. Analysis was completed using an Aminex HPX-87H cation exchange column (Bio-Rad Laboratories) on a HPLC 1100 series (Agilent Technologies, Germany) with a mobile phase of 2.5 mM sulphuric acid (H₂SO₄). Calibration curves relating concentration to optical density or refractive index were fitted using ChemStation software (Agilent, CA, USA).

Theoretical Maximum Ethanol Production

Theoretical maximum ethanol production was calculated using the assumption that 0.511 kg of ethanol is produced from 1 kg of completely hydrolysed substrate comprising glucose and fructose monomers only [8, 10]. The following mathematical formulae were used to calculate the theoretical maximum ethanol production and conversion efficiency. a = total sugar in TFA-treated juice (monosaccharides g/L); b = ethanol yield coefficient 0.511; c = theoretical maximum ethanol production (g/L); d = observed ethanol yield from fermentations (g/L); and e = % of predicted ethanol production.

$$a \times b = c \quad (1)$$

$$\frac{d}{c} \times 100 = e \quad (2)$$

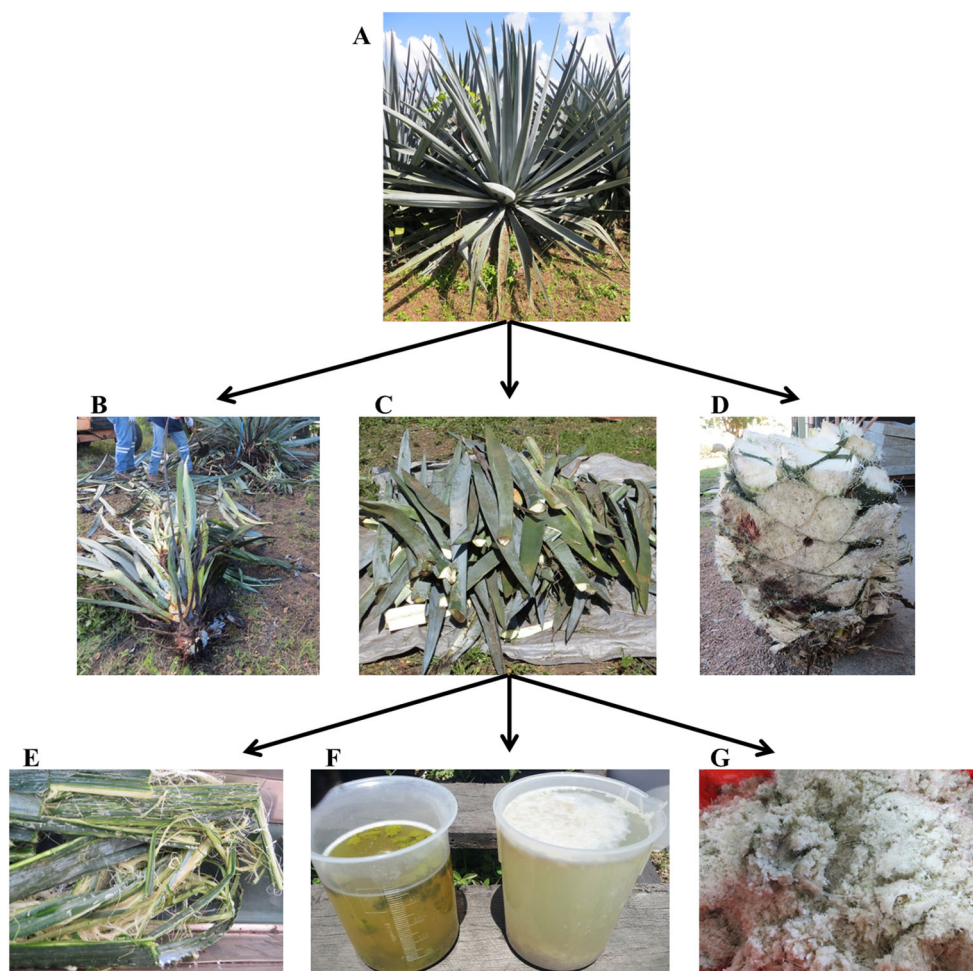
Results and Discussion

Composition of *A. tequilana*

A whole *Agave* plant, including the main unit (mother plant) and its attached offshoots, generated 360 kg of raw biomass that could be used for biofuel production. The harvested biomass was separated into different anatomical fractions (leaves, stem and offshoots) to create multiple production streams from one feedstock (Fig. 1). For 4.5-year-old *A. tequilana* plants, 73 % w/w of the above-ground biomass was attributable to leaves and 27 % w/w to the stem (Table 2). By crushing the leaves, 68 % w/w of the leaf mass could be extracted as a weakly acidic fermentable juice (Table 2).

The acidity of *Agave* juice (pH 4.6–5.0; Online Resource 1) is a characteristic of plants that use the Crassulacean acid

Fig. 1 Diagram outlining the fractions that can be generated from processing of *Agave* plants. Whole units of *Agave* plants (a) were harvested and divided into offshoots (b), leaves (c) and stem (d). Each of these fractions can be further processed to generate a cellulose-enriched fibrous bagasse (e), a fermentable juice (f) and residual biomass (g)



metabolism (CAM) photosynthesis pathway, in which organic acids such as malic acid are stored in the vacuoles of cells during the night [13]. The two abundant organic acids measured by HPLC in the leaf juice were acetic and malic acid (Online Resource 2). High concentrations of weak acids such as acetic acid or low concentrations in the presence of other compounds (i.e. furfural) have been shown to reduce biomass

accumulation and production of ethanol, resulting in a need for superior strain selection [14]. A small percentage of the total mass of the juice was attributed to minerals at 1–2 % w/w (Online Resource 1).

TSS and °Brix have previously been used to estimate sugar content in raw and fermented *Agave* juice [15–18]. The measured carbohydrate content for *A. tequilana* leaf (~9.6 % w/v)

Table 2 Mass distribution and carbohydrate content of *Agave tequilana* plants

	Average mass per plant (kg)	% juice collected per fraction (w/w)	Total soluble carbohydrates in juice (% w/v) ^a	°Brix (w/v)	TSS (% w/v)
Mother plant					
Leaf	163 ± 24	68	6.0	9.6 ± 2.2	9.3 ± 2.1
Stem	60 ± 15	43	17.1	17.3 ± 3.8	17.4 ± 3.7
Offshoots	137 ± 50	27	4.0	7.9 ± 0.3	7.5 ± 0.5

The weight of biomass derived from *Agave tequilana* plants was recorded at the time of harvest (fresh wt; kg). The mother plant (4.5 years old) was separated into leaves, stem and root fractions and the younger plants (offshoots) were removed. The majority of the above-ground mass of mother plants (excluding offshoots) is attributed to the leaves at 73 % w/w. Three different methods were used to determine the carbohydrate content in *Agave* juice: HPLC (Table 3), Brix and total soluble solids (TSS)

^a HPLC

and stem juice (~17.3 % w/v) using the two methods, TSS and °Brix, were comparable (Table 2). However, when juice was analysed using HPLC, it was clear that these methods overestimated the amount of carbohydrates in leaf and offshoot juice (Table 2). This overestimation is likely due to the presence of solutes other than carbohydrates such as calcium oxalate crystals. To quantify the real carbohydrate content and subsequently predict ethanol yields, it is essential that accurate methods are used.

Juice samples were treated with TFA or fructanase to cleave higher molecular weight carbohydrates, predominantly fructans, into monosaccharides for quantification. *A. tequilana* plants are considered to be high accumulators of branched fructans and neo-fructans [19]. However, only 10–15 g/L of the carbohydrates in *A. tequilana* leaf and offshoot juice is attributed to fructans (Table 3). Whilst over 85 % of the carbohydrates in stem juice is complex fructans, many microorganisms cannot ferment these in their native branched forms and thus a hydrolysis or cooking step is required [20]. Although such methods are considered to be efficient for the complete hydrolysis of fructans in both leaf and stem tissue, they can be costly and unfavourable for downstream processing [20, 21]. In addition, fructose is highly unstable relative to other monosaccharides such as glucose, even under moderate pre-treatment conditions, and is converted into compounds, such as hydroxymethylfurfural, which are toxic to fermenting microorganisms [21]. As a result, acid and enzymatic pre-treatments were not further explored in this study.

Alternatively, autoclaving is considered a low-cost, low-input pre-treatment for *Agave* juice. For example, autoclaving increased the measurable monosaccharides in stem juice by 50 g/L (Table 3). In addition, higher

levels of the intermediate hydrolysis product sucrose were present, indicating that partial breakdown of the complex fructans was achieved where one sucrose moiety is liberated per molecule of fructan [21].

In the tequila industry, cooking the stem at elevated temperatures for an extended period is the standard method for fructan hydrolysis, for production of aromatic compounds and for softening of the recalcitrant tissue before milling [5]. Autoclaving can similarly be used to treat the juice prior to fermentation (Table 1), but there are inconsistent reports in the literature regarding the efficiency or necessity of the autoclaving step. Some studies indicate that autoclaving *Agave* juice partially hydrolyses fructans [15]. Other studies imply that autoclaving is employed strictly as a sterilization step and does not modify the structure or composition of carbohydrates in the juice [16], whilst others claim that for complete hydrolysis (98 %) of fructans to occur, the samples must be heated at about 80 °C for more than 25 h [19]. Findings herein indicate that the efficiency of fructan hydrolysis by a simple thermal pre-treatment (autoclaving) is substrate-dependent, as there was no difference in the monosaccharide content between raw and autoclaved leaf juice, but that the amount of monosaccharides in the stem juice increased following autoclaving.

Although immature offshoots contributed a substantial amount of the total biomass (38 % w/w; Table 2), they were at varying stages of development, potentially rendering biomass weights, carbohydrate content and ultimately ethanol yields unpredictable. As a result, it may be more advantageous to harvest only the mother plant and to leave the offshoots to grow for future harvests; thus, offshoots were not further analysed in this study.

Table 3 Analysis of *Agave* juice to quantify total carbohydrate content

Tissue	Treatment of juice	Fructose (g/L)	Glucose (g/L)	Sucrose (g/L)	Total (g/L)
Leaves	Raw	12.7±2.7	25.6±4.9	6.0±4.5	44.3
	TFA	23.9±6.0	35.9±7.8	–	59.8
	Fructanase	22.4±6.8	33.2±10.0	–	55.6
	Autoclaved	15.2±4.4	25.6±4.5	4.6±1.0	45.4
Stem	Raw	6.2±1.2	11.3±3.2	8.0±5.6	25.5
	TFA	129.8±32.5	39.8±9.4	–	169.6
	Fructanase	132.0±33.9	38.7±8.1	–	170.8
	Autoclaved	34.3±2.9	15.7±1.1	25.2±7.2	75.2
Offshoots	Raw	10.4±3.4	15.1±4.6	2.1±0.2	27.6
	TFA	20.9±7.3	17.3±3.6	–	38.2
	Fructanase	22.5±8.1	17.7±3.4	–	40.2
	Autoclaved	12.2±0.6	14.4±4.5	2.7±1.3	29.4

The amount of detectable soluble carbohydrates (glucose, fructose and sucrose) in *Agave* juice (leaf, stem and offshoots) is dependent on the pre-treatment method employed: raw, TFA-treated, fructanase-treated or autoclaved. Italics indicate values derived from an additive calculation. Data are presented as grams per liter; leaves and stem *n* = 3; offshoots *n* = 2

Spontaneous Fermentation of *Agave* Juice Is Induced by Endogenous Species

In commercial tequila production, spontaneous (uninoculated) fermentation is a common practice [22]. However, in a biofuel context, spontaneous fermentations are unlikely to be favourable and partial fermentation of the carbohydrates can occur prematurely during transport of feedstocks to processing facilities, thus reducing ethanol yields (Online Resource 3). Here, the spontaneous fermentation of *A. tequilana* leaf and stem juice was tested by allowing raw leaf and stem juice to ferment for 120 h without the addition of any external microorganisms. Both juice sources gave similar, albeit low, yields of ethanol: 9.0 g/L for leaf juice and 14.6 g/L for stem juice (Fig. 2a). However, the fermentation profiles of these two substrates over 120 h were quite different (Fig. 2a).

The proportion of sugar converted to ethanol was higher in leaf samples (Fig. 2b), which may be attributed to the structural differences of the carbohydrates in the two substrates compared. For example, the majority of the carbohydrates in *Agave* leaf juice are fermentable mono- or oligosaccharides, whereas in stem juice, they are complex fructans (Table 3). The final ethanol yield for both spontaneous fermentations was similar (9–15 g/L), which may be indicative of

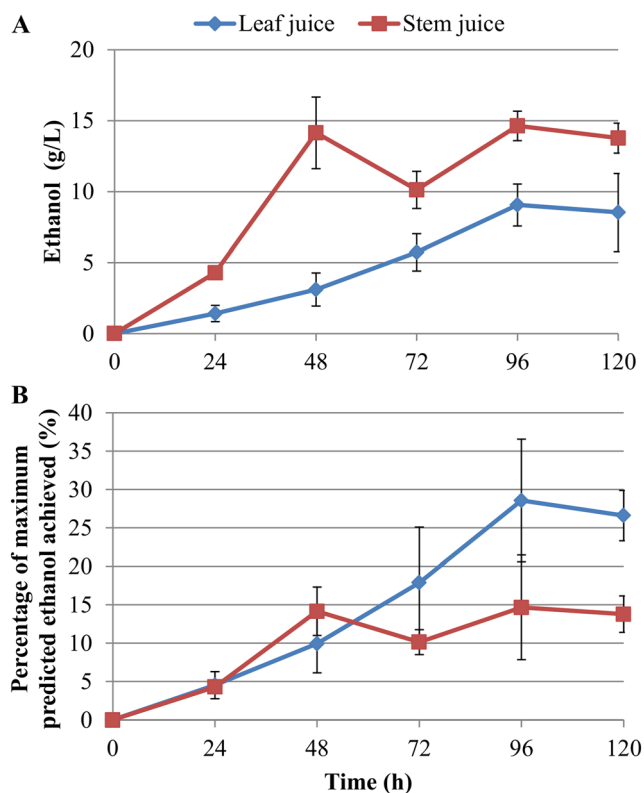


Fig. 2 Spontaneous fermentation of *A. tequilana* juice. Raw *Agave* leaf and stem juice was fermented using only the native organisms present within the substrate; $n = 3$. The production of ethanol (g/L; **a**) was measured over 120 h. The actual ethanol yield achieved was compared to the maximum yield to determine fermentation efficiency (%; **b**)

similarities between the membership of the microbial mixture both at the start and throughout the fermentation. The endogenous microorganisms found within these *Agave* samples may have a low tolerance to ethanol resulting in incomplete fermentation (only 32 and 17 % of the theoretical ethanol maximum was produced for leaf and stem juice, respectively), although other factors may be hindering the conversion of carbohydrates to ethanol.

Spontaneous fermentations are heterogeneous microbiological and biochemical processes in which populations increase and decrease as selective pressures are induced, ultimately resulting in the dominance of species with superior catabolism rates and/or tolerance to the alcoholic end-products [2]. Microorganisms isolated from the early stages of *Agave* stem fermentations have been shown to contain a rich mixture of yeast species. For example, 192 yeasts were identified from alcoholic fermentation of *Agave salmiana* juice when it was streaked on nutrient agar plates [23]. In this study, *Saccharomyces* was found to be the predominant strain at the onset of fermentation (94–98 % occurrence) and remained the most abundant species. However, as fermentations progress, the number of species and their abundance can change. Some studies indicate that the diversity diminishes over the course of the fermentation [24], whereas in other studies, a more diverse mixture of microorganisms was observed due to the introduction of contaminants [23]. A negative interaction between *S. cerevisiae* and non-*Saccharomyces* yeast strains has been proposed, which may be one of the factors contributing to the reduced efficiency of spontaneous fermentations [25].

In addition, active killer yeast (and bacterial) genera, such as *K. marxianus* var. *drosophilum* and *Pichia membranaefaciens*, are frequently present in the spontaneous fermentation of plant biomass, including *Agave*, resulting in sluggish or stuck fermentations [26, 27]. Strains that exhibit these killer properties tend to colonize fermentations early and produce zymocidal substances that can reduce beneficial yeast populations [28]. The rise and fall of populations during the course of the fermentation could explain the sporadic changes in ethanol content observed in the fermentation profiles of unmodified *Agave* leaf and stem juice. Prolonged spontaneous fermentations of *Agave* juice, if not monitored, could therefore inadvertently result in reduced ethanol yields. Such information will be instructive when considering the processing, transport, handling and storage of *Agave* biomass and juice.

Selection of Microorganisms

As microorganisms exhibit a narrow range of tolerances to environmental conditions, identifying fermenting organisms that are particularly adapted to the substrate is crucial. In this work, five novel yeast strains were obtained from the ARS

Culture (NRRL) Collection [11] for fermentation studies (Table 4). The selected yeast had been isolated directly from *Agave*, other succulents or from biomass rich with carbohydrates found in *Agave* such as grape (glucose and fructose) or sugarcane molasses (sucrose). The use of novel microorganisms isolated from a biomass source has been shown to have great potential for the biofuel industry [29]. A recent study showed that fungi growing on a feedstock had higher enzymatic activity and was more efficient at degrading the cell wall components compared to commercial fungus [29]. Furthermore, in this study, it was determined that the most superior fungi identified had not previously been exploited for bioconversion processing, highlighting the importance of trialling novel strains.

All genera of yeast selected have been identified as dominant or secondary yeasts in tequila fermentations or tequila processing facilities [24]. The fermentation performance of three strains had previously been tested (CBS-KNAW Fungal Biodiversity Centre [30]; Table 4). For the other two yeasts, data specific to the strains were not available, but species-level information [30] regarding fermentation performance was considered (Table 4). Of the yeast selected, one strain is known to ferment the primary carbohydrates in *Agave* juice, glucose, sucrose and fructans, *K. marxianus* (*Km1598*) (CBS-KNAW Fungal Biodiversity Centre id: CBS 745 [30]; Table 4). In addition, isolates of *K. marxianus* have been shown to produce fructan-hydrolysing enzymes [31]. The yeast *P. kluyveri* was also selected as it has been reported to display high fermentation efficiency in tequila making [32].

To determine the optimal growth temperature, each yeast was diluted to a standardized cell concentration and spotted on YPD plates incubated at four different temperatures (28, 32, 37 and 42 °C). All yeast strains grew similarly at temperatures at or below 32 °C (Online Resource 4), and thus, 28 °C was

chosen as the optimal temperature for the fermentation experiments. Interestingly, it was found that *K. marxianus* (*Km1598*) tolerated temperatures above 37 °C. This strain was isolated from *Agave* that is able to thrive in arid regions and which can tolerate temperatures above 60 °C [33]. Such information suggests that further analysis of the metagenome of *Agave* may identify novel, superior microorganisms for the production of bioethanol. For example, although less than 1 % of microorganisms present in many natural environments can be cultured in vitro [34], endogenous microorganisms from *Agave* juice exhibited growth over a wide range of temperatures (28–42 °C; data not shown). Yeasts isolated from *Agave* also have the advantage of being tolerant to toxic compounds (i.e. furans) present in the juice and are less likely to become inactive during fermentation as they are more adaptive to the substrate [35].

Comparison of Microbial Strains and Treatment of *A. tequilana* Leaf Juice

In large-scale biofuel production, where rapid and reliable fermentations are essential, the use of robust and reliable pure yeast inocula of known performance is common practice, rather than spontaneous fermentation [2]. Here, five yeasts (Table 4) were selected for the fermentation of raw and autoclaved *Agave* leaf juice. Subsequently, yeast strains with the highest fermentation performance were cultured in autoclaved leaf juice after the re-introduction of endogenous microbes (Table 5).

S. cerevisiae is one of the most widely utilized yeasts for alcoholic fermentations such as wine making and brewing, yet the ethanol yields achieved from *Agave* leaf juice (raw or autoclaved) using two *Saccharomyces* isolates (*Sc636* and *Sc139*) were lower compared with the ethanol yields achieved

Table 4 Source, fermentation capability and assimilation with no ethanol of selected microorganisms for fermentation of *Agave* juice

Source	Accession number	Organism	Fermentation			Assimilation
			Glucose	Sucrose	Inulin	Ethanol
Rotting <i>Agave sisalana</i> leaves	1598 ^a (CBS: 745)	<i>Kluyveromyces marxianus</i>	+	+	+	Growth
Rotting <i>Opuntia stricta</i>	17228 (CBS: 7274)	<i>Pichia kluyveri</i>	+	n/a	n/a	Growth
Jamaica sugarcane molasses	7846 (CBS: 7878)	<i>Candida akabanensis</i>	+	+	n/a	Growth
Molasses	636 (CBS: 1235)	<i>Saccharomyces cerevisiae</i>	+	+	n/a	Growth
Fermenting grapes	139 (CBS: 1539)	<i>Saccharomyces cerevisiae</i>	+	+	n/a	Growth

Five yeasts were used for the fermentation of *Agave* juice. Organisms were sourced from the ARS Culture Collection (NRRL) [11]. Accession numbers for strains in the NRRL database are listed first, followed by the corresponding number in the CBS database. Fermentation and assimilation data for microorganisms were obtained from CBS-KNAW Fungal Biodiversity Centre [30] unless not available (n/a); bold text indicates that data for the specific strain was unavailable but information is provided at the species level

^a 1598 has been reported to secrete fructan-hydrolysing enzymes [31]

Table 5 Ethanol yields from fermentation of *A. tequilana* leaf juice by different microbial strains

Treatment	Microbial strain	Ethanol yield (72 h)	
		Observed yield (g/L)	Proportion of maximum theoretical production (%)
Non-autoclaved + inoculation	<i>Km1598</i>	23.0 ± 3.4	75
	<i>Pk17228</i>	23.4 ± 0.1	77
	<i>Ca7846</i>	24.8 ± 6.0	81
	<i>Sc636</i>	13.7 ± 0.3	45
	<i>Sc139</i>	14.6 ± 2.4	48
Autoclaved + inoculation	<i>Km1598</i>	26.1 ± 6.4	85
	<i>Pk17228</i>	25.7 ± 4.2	84
	<i>Ca7846</i>	26.9 ± 1.9	88
	<i>Sc636</i>	19.5 ± 0.4	64
	<i>Sc139</i>	16.8 ± 5.1	55
Autoclaved + indigenous microbes	<i>Km1598</i> ^a	16.8 ± 1.7	55
	<i>Pk17228</i> ^a	18.9 ± 2.0	62
	<i>Ca7846</i> ^a	18.3 ± 2.0	60

Comparison of observed ethanol yield (g/L) and proportion of the maximum predicted ethanol yield (%) for five different yeast strains when cultured in raw and autoclaved *A. tequilana* leaf juice; $n = 2$. In addition, indigenous microbes were re-introduced into autoclaved leaf juice and fermented using non-*Saccharomyces* yeasts. The theoretical maximum ethanol production was calculated using the total sugars measured in TFA-treated leaf juice samples (59.8 g/L; Table 3)

^a $n = 3$

using the less commonly studied yeasts *Ca7846*, *Km1598* and *Pk17228* (Table 5). The poorer fermentation performance of the *Saccharomyces* isolates is likely to be partially attributable to the high levels of fructose present in the substrate, as *Saccharomyces* yeast species preferentially use glucose (although the level of preference varies) when in a heterogeneous culture with other monosaccharides such as fructose [36]. When glucose is transported across the plasma membrane in these mixed cultures and ethanol is accumulated, the tautomeric equilibrium of fructose is shifted, converting it from fructopyranose to fructofuranose [37]. This shift in conformation has been suggested to lower the rate of fructose transport, further limiting its uptake by the yeast [36]. The accumulation of carbohydrates that are not metabolized by the yeast results in slowed or arrested fermentations, ultimately decreasing ethanol yields. In another report, reduced ethanol yields were reported for *Saccharomyces* inocula when used in *Agave* fermentations if the yeast did not originate from *Agave* [38]. In another fermentation study, it was observed that *S. cerevisiae* was unable to grow in either thermo acid or enzymatically treated *Agave* juice [20]. The authors correlated this inhibition of growth to toxic levels of phytochemicals such as saponins which are produced by *Agave* plants. Interestingly, the saponins did not inhibit the growth or fermentation performance of the yeast *K. marxianus*.

In this study, higher ethanol yields were achieved using non-*Saccharomyces* yeasts than *Saccharomyces* yeasts. The increase in ethanol yields using these strains may be attributed

to their superior ability to degrade fructose and fructan polymers, or possibly their tolerance to compounds inherent to the substrate (i.e. malic acid) or the production of ethanol and inhibitory by-products. For example, *K. marxianus* is known to produce fructan-specific enzymes, which enables the yeast to simultaneously hydrolyse fructans and ferment the liberated monosaccharides [31], resulting in higher ethanol yields (Table 5). Alcohol yields achieved using the non-*Saccharomyces* yeasts *P. kluyveri* and *K. marxianus* were similar, consistent with previous reports [32]. The most efficient isolate used was *Ca7846* (88 % conversion; Table 5). *Candida* species have previously been isolated from *Agave* fermentations [23, 24]; however, this is the first study to investigate its use for bioethanol production and little is known about its fermentation capabilities.

There is no benefit to autoclaving *Agave* leaf juice prior to fermentation. The modest increase in ethanol yields achieved using autoclaved juice (Table 5) is likely due to the slight increase in available monosaccharides following autoclaving (Table 3). No differences between ethanol yields (g/L) or conversion efficiency were observed between non-*Saccharomyces* strains when cultured in raw or autoclaved *Agave* leaf juice (Table 5). In addition, the accumulation and production of organic acids (acetic, citric, acetic and malic acid) and glycerol in non-*Saccharomyces* fermentations of raw and autoclaved juice was similar (Online Resource 2), indicating that acid production and consumption patterns of the yeast are not influenced by autoclaving the juice. However, when endogenous

microorganisms were re-introduced into autoclaved juice substrates, the ethanol yields were lower for all non-*Saccharomyces* yeasts tested than yields from fermentations where endogenous microorganisms were not re-introduced. The strain *Km1598*, which was originally isolated from *Agave sisalana* (Table 4), had the lowest ethanol yield (g/L) in the presence of native microbes (raw and re-introduced) compared with *Ca7846* and *Pk17228*. Overall, the efficiency of the fermentations were influenced more by the yeast species present (Table 5) than by the treatment of the juice. However, there was a clear distinction between the fermentation performance of *Saccharomyces* and non-*Saccharomyces* yeast in both raw and autoclaved leaf juice (Table 5).

The current research has revealed that autoclaving is nonetheless important, but as a means of eliminating contamination. Ethanol yields from fermentations containing native organisms in addition to the selected yeast strains were adversely affected compared to the yeast monoculture experiments. The highest ethanol conversion was observed in the presence of native organisms, 61 % using *Pk17228* (Table 5). However, this value is lower than the rate observed when culturing raw leaf juice (77 % conversion for *Pk17228*) and autoclaved juice without native organisms (84 % of maximum for *Pk17228*).

It is interesting that autoclaved juice supplemented with native organisms and the non-autoclaved juice produced different effects. This reduction in ethanol production is likely due to the compositional and chemical changes that occur as a result of autoclaving, creating an environment in which native microorganisms are not adapted. One means by which autoclaving changes the composition of *Agave* juice is by degrading vitamins and thereby inducing a nutrient-limited environment [16]. Deficient vitamin levels can reduce cell growth rate and biomass production, decrease viability and ultimately affect fermentation performance [39]. In addition, it can result in the degradation of nitrogen-containing compounds (i.e. ammonium), which is an essential macronutrient required by microorganisms for the maintenance of stationary phase fermentation [39].

Current studies suggest that less than 1 % of microorganisms present in many natural environments can be cultured in vitro [34]. Thus, by culturing the endogenous microbes present in *Agave* juice on plates, only a small proportion of the microbial population was represented, and therefore, this inoculum was not identical to the breadth of microorganisms found in raw *Agave* juice. However, as in the tequila industry, even a small diversity of microbial contaminants can have a profound effect on the fermentation performance and end-products generated [24]. Contaminants from *Agave* will inevitably be present in *Agave* processing facilities. The current study provides founding data from which strategies to manage *Agave* storage and handling may be developed.

Fermentation of *Agave* Stem Juice

Microbes that naturally deconstruct plant walls may provide the best enzymes for bioconversion of energy crops [40]. To explore this concept, the fermentation of autoclaved *Agave* stem juice was investigated using *Km1598*, a strain which was originally isolated from *Agave*. A maximum conversion rate of 64 % was achieved by 48 h (Fig. 3a). This conversion rate was lower than previously reported for *K. marxianus* (94–96 % efficiency after 72 h; Table 1), but the stem juice used for this study was not cooked for an extended period to hydrolyse the fructans [22].

More recently, the potential of producing biofuel from whole *Agave* plants has been considered [41]. However, these experiments are still reliant on the use of costly chemical pre-treatments and enzymatic cocktails for hydrolysis of fructans. In our study, leaf and stem juice were combined in a volume leaf/stem ratio of 3:1, which was representative of a scenario where whole plants are crushed as one unit, without the leaves and stem being separated. When stem juice and leaf juice were

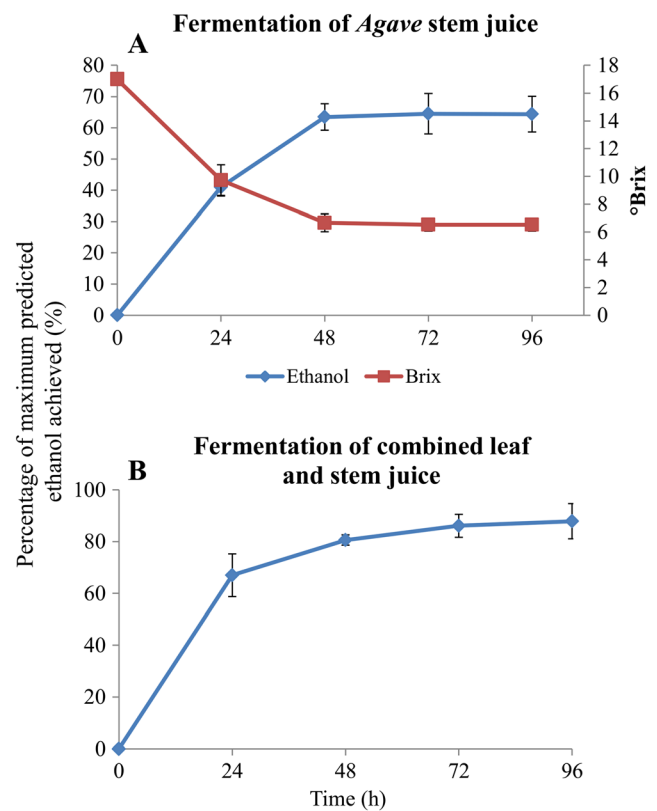


Fig. 3 Conversion rates of total carbohydrates to ethanol using *Agave* stem juice. Kinetic profile of the fermentation of *Kluyveromyces marxianus* (*Km1598*) in autoclaved *A. tequilana* stem juice over time, 96 h, *n* = 3 (a) and in combined leaf and stem juice (b). The autoclaved leaf and stem juice were combined in volumes equivalent to the mass distribution of a whole plant, namely 75 % leaves and 25 % stem, and fermented using *K. marxianus* (b). The fermentation of combined *Agave* juice from stem and leaf yielded 85 % of the predicted maximum ethanol yield

Table 6 Predicted ethanol production from *Agave tequilana*

Biomass	Productivity (L juice/ha/year)	Carbohydrate content (kg/ha/year)	Theoretical maximum ethanol production ^a (L/ha/year)	Observed ethanol yield (%)	Predicted ethanol production ^b (L/ha/year)
Leaf	78,820	4713	3053	45–88	1369–2687
Stem	18,347	3134	2029	64	1299
Whole plant			5082	85	4320

Calculations are based on 4000 *Agave* plants ha⁻¹, assuming a juice density of 1.25 and average plant age of 4.5 years. The productivity and carbohydrate content of *Agave tequilana* are calculated using real-harvest data (Table 2) and HPLC data (Table 3)

^a Theoretical maximum ethanol production assumes 100 % efficiency of carbohydrate conversion to ethanol. Observed ethanol yields were determined in this study to be 45–88 % using leaf juice (Table 5) and 64 % using stem juice (Fig. 3a), and 85 % when juice from the whole plant was used (Fig. 3b). Italics indicate values derived from an additive calculation rather than direct measurement

^b The predicted ethanol production is calculated using figures for the theoretical maximum ethanol production from each fraction and the actual ethanol yields achieved in this study

combined, 85 % conversion to ethanol was achieved (Fig. 3b). This approach is advantageous as it reduces the time and labour required for processing and harvesting of *Agave* plants, potentially reducing production costs.

Ethanol Yield Predictions

The theoretical ethanol yields for *A. tequilana* rival other currently studied lignocellulosic feedstocks [42]. Juice derived from *A. tequilana* stems had a higher proportion of carbohydrates than leaf juice (Table 2), but in total, the juice from the leaves contributed more total carbohydrates (6.6 kg per plant) than the stem (4.4 kg per plant). Therefore, a higher theoretical maximum ethanol production was calculated for *A. tequilana* leaf juice, 3053 L/ha/year relative to stem juice, 2029 L/ha/year (Table 6). Fermentation of juice from whole mother plants in an *Agave* plantation could yield up to 5082 L/ha/year ethanol, leaving the offshoots to keep growing in the field for future harvests. However, the conversion of carbohydrates into ethanol is mediated via a complex chemical and enzymatic pathway, and actual fermentation data is required to validate the estimates of the value of this biomass as a substrate for fermentation.

There was a range in the conversion of carbohydrates to ethanol in the fermentation of *Agave* leaf juice, 45–88 % (Table 5). This correlates to the predicted production of up to 2687 L/ha/year ethanol (Table 6). Therefore, the fermentation of *A. tequilana* leaves, which are currently discarded in the beverage industry, could add significant value to existing *Agave* industries. *Agave* production specifically for biofuel may also be profitable, particularly as *Agave* plants are highly drought tolerant and can be produced in agriculturally marginal regions with limited rainfall [42]. Based on fermentation data from this study, if grown specifically for biofuel production at a density of 4000 plants/ha, the juice derived from 4.5-year-old *A. tequilana* plants (stem and leaf) could produce

ethanol yields of up to 19,439 L/ha. Optimizing processing methods or fermenting microorganisms could increase ethanol yields to their theoretical maximum, 22,870 L/ha.

Conclusion

The data generated in this study challenges current practices in bioethanol production of supplementing and pre-treating *Agave* juice prior to fermentation. Leaf juice substrates do not benefit from autoclaving prior to inoculation, and the selection of superior fermenting organisms is essential for generating high ethanol yields. The best microorganisms studied were isolated from succulents. A third species, *C. akabanensis*, was used for the first time in bioethanol fermentation studies of *Agave* juice, suggesting that further exploration of non-traditional *Saccharomyces* species could improve bioethanol yields. Actual and extrapolated ethanol yields from *Agave* leaf juice confirm that this biomass has significant potential for bioethanol production.

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