Biocatalysis and Agricultural Biotechnology 10 (2017) 148–155

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Evaluation of the quantity and composition of sugars and lipid in the juice and bagasse of lipid producing sugarcane

Haibo Huang[a], Robert A. Moreau[b], Michael J. Powell[b], Zhaoqin Wang[c], Baskaran Kannan[d], Fredy Altpeter[d], Aleel K. Grennan[e], Stephen P. Long[e,f,g], Vijay Singh[c,*]

[a] Department of Food Science and Technology, Virginia Polytechnic Institute and State University, 1230 Washington Street, SW, Blacksburg, VA 24061, USA
[b] Sustainable Biofuels and Co-Products Research Unit, Eastern Regional Research Center, USDA-ARS, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA
[c] Department of Agricultural and Biological Engineering, University of Illinois, 1304 W. Pennsylvania Ave, Urbana, IL 61801, USA
[d] Agronomy Department, Plant Molecular and Cellular Biology Program, Genetics Institute, University of Florida, IFAS, Gainesville, FL, 32611, USA
[e] Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[f] Department of Crop Sciences, University of Illinois at Urbana Champaign, Urbana, IL 61801, USA
[g] Department of Plant Biology, University of Illinois at Urbana Champaign, Urbana, IL 61801, USA

ARTICLE INFO

Keywords:
Lipid
Sugar
Sugarcane
Transgenic
Biofuel
Processing

ABSTRACT

Biodiesel production in the U.S. from vegetable oils has increased substantially during the past decade. However, its further increase is limited by the low amounts of oil produced per hectare from temperate oilseed crops. Recently novel transgenic sugarcane has been developed to accumulate both sugars and lipids in stems, making it a promising dual-purpose feedstock to produce both ethanol and biodiesel. In this study, two lines of the transgenic lipid producing sugarcane (lipid-cane) and the non-transformed sugarcane were characterized and processed. The total lipid concentrations were 0.7%, 0.9% and 1.3% for the non-transformed sugarcane and lipid-cane lines19B and 25 C, respectively. Lipid composition analysis showed that about 31–33% of the total lipids were triacylglycerols, main feedstock for biodiesel production, for the lipid-cane samples, while this value was only 5% for the non-transformed sugarcane. By processing the sugarcane stems with a juicer, about 90% of the sugars and 60% of the lipids were extracted with juice. The extracted sugars in juice were fermented to ethanol and the lipids were later recovered from the fermented juice using organic solvents. The recovered lipids from the fermented juice were 0.3, 0.5 and 0.8 g/100 g dry stem for the non-transformed sugarcane and lipid-cane lines 19B and 25 C, respectively. This study proved the concept of the lipid and sugar coproduction from the novel lipid-cane, which have a potential to make a large-scale replacement of fossil derived fuel without unrealistic demands on land area.

1. Introduction

In the U.S. ethanol production from cereal grain, primarily corn, has increased from 1.6 billion gallons (6.0 billion liters) in 2000 to 13.0 billion gallons (49.1 billion liters) in 2014 (Renewable Fules Association, 2015), in efforts to achieve greater energy independence and reduce carbon emissions. However, further increase is limited by the revised renewable standard (RFS2) mandate, which caps off ethanol production from cereal grains at 15 billion gallons (56.7 billion liters). Although lately much emphasis has been placed on cellulosic ethanol, this alternative technology has not achieved high commercial production levels and still faces many challenges, such as feedstock availability and high conversion costs.

Biodiesel is another promising biofuel that can be easily produced from vegetable oils and requires few inputs. Biodiesel supports engine performance that is generally comparable to that of fossil diesel fuel, with reduced engine emissions of particulates, hydrocarbons, and carbon monoxide (Graboski and McCormick, 1998; Haas et al., 2006). The US biodiesel production increased substantially from 8.5 million gallons (32.0 million liters) in 2001 to 1.4 billion gallons (5.1 billion liters) in 2013 (Energy Information Administration, 2013). In the US, the major feedstock to produce biodiesel is soybean. Although soybean occupied about 79.0 million acres (33.0 million hectares) in the US, second largest planted crop only after corn, the amount of oil produced per hectare was small, between 0.16 and 0.27 t/acre (0.36 and 0.61 MT/ha) (USDA, 2015). Thus if the entire soybean crop was used for

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; ASG, acyl steryl glucoside; DAGAT1-2, diacylglycerol acyltransferase1-2; Lipid-cane, lipid producing sugarcane; MGDG, Monogalactosyldiacylglycerol; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, phosphatidylinositol; PXA1, peroxisomal ABC transporter1; TAG, triacylglycerol

* Corresponding author.
E-mail address: vsingh@illinois.edu (V. Singh).

http://dx.doi.org/10.1016/j.bcab.2017.03.003
Received 26 December 2016; Received in revised form 22 February 2017; Accepted 6 March 2017
Available online 07 March 2017
1878-8181/ © 2017 Elsevier Ltd. All rights reserved.

biodiesel production, the total biodiesel production is only about 12.6–21.3 million ton 13.9–23.5 million MT), which is only about one tenth of the US distillate fuel oil consumption at 170 million ton (155 million MT) (Energy Information Administration, 2015). With the limited arable land, it is important to consider with the rapid progress of plant bioengineering to develop more productive crops that can accumulate oil and be grown on poorer soils and land that would not compete with the major food and feed crops (Long et al., 2015).

Recently, metabolic engineering strategies have been proven successful in generating and accumulating triacylglycerol (TAG), the vegetable lipid precursors of biodiesel, in plant vegetative tissues in model plants species, *Arabidopsis* and *tobacco* (Chapman et al., 2013; James et al., 2010; Vanhercke et al., 2014a, b). By co-expressing of three genes (i.e. WRINKLED1, diacylglycerol acyltransferase1-2 (DAGA T1-2), and Oleosin1) involved in TAG production, a substantial amount of TAG can be accumulated in sugarcane stems (Zale et al., 2016). There are several advantages of accumulating lipids in sugarcane. Firstly, sugarcane is one of the most productive crops in terms of converting sunlight energy into chemical energy that can be stored in the plant through its effective use of C4 photosynthesis. The sugarcane yield of stem can be as high as 180 – 220 MT/ha (at about 70% moisture), much higher than the average yield of soybean at about 2–3 MT/ha (USDA, 2015). The ultra high yield of sugarcane indicates that accumulation of lipids in sugarcane even at low levels can lead to a large amount of lipid production per hectare of land area (Huang et al., 2016). Secondly, sugarcane is less demanding in fertilizers for growing and its by-products (i.e. bagasse) can be burned to generate heat to support sugarcane-fuel process; therefore, fuels derived from sugarcane have a life-cycle greenhouse gas emission that is less than one eighth of that of fossil fuels (Long et al., 2015; Yan et al., 2012). Thirdly, sugarcane itself is an excellent feedstock for ethanol production due to high amount of sugars in stems. Therefore, the developed lipid producing sugarcane (lipid-cane) can be a promising dual-purpose biofuel crop: sugars are used to produce ethanol and lipids are used to produce biodiesel.

Although the lipid-cane holds promising properties for biodiesel and ethanol production, there are several questions remaining before it can be promoted to commercial production: 1) What are the sugar and lipid profiles in this newly developed lipid-cane? 2) Are the lipids and sugars extractable from the lipid-cane? Therefore, the objectives of this study were to 1) characterize the sugar and lipid compositions of the lipid-cane; 2) quantify the sugar and lipid distribution in the extracted juice and remained bagasse with the current existing sugarcane process. This is the first investigation of characterization and processing of the novel lipid-cane for biofuel (ethanol and biodiesel) purpose.

2. Materials and methods

2.1. Lipid producing sugarcane samples

The transgenic lipid producing sugarcane (lines 25 C and 19B) were developed by co-expressing WRINKLED1, DGAT1-2, Oleosin1 in concert with the RNAi suppression of ADP-glucose pyrophosphorylase (AGPase) and a subunit of the peroxisomal ABC transporter1 (PXA1), and they were grown at the University of Florida Plant Science and Education Center in 2014 under USDA-APHIS permit number 13–299-101r. The non-transformed sugarcane cultivar CP 88–1762 was grown in the same way at the University of Illinois. Sugarcanes were planted on April 10th, 2014 and harvested on November 4th, 2014, thus the time duration of sugarcane in field is 208 days. All chemicals and laboratory reagents, unless stated otherwise, were analytical grade and were purchased from Fisher Scientific Inc. (Springfield, NJ).

2.2. Sugar analysis

After sugarcane harvesting, fresh stem samples were cut into pieces with about 2 cm in length and then ground with dry ice for 1 min with a blender (Osterizer, Boca Raton, Florida). The moisture content of the ground samples was measured by placing them at 103 °C for 24 h (ASABE Standard, 2012). The sugar analysis was conducted based on a previous study (Chen et al., 2014). Ground samples (~1 g dry weight) in triplicate were placed in 100 ml screw capped Pyrex glass bottles containing 20 ml of deionized water each. Then the bottles were loosely capped and autoclaved at 121 °C for 15 min (Napco model 9000D, Thermo Fisher Scientific, Waltham, MA). The resulting extract solutions were analyzed for sucrose, glucose, and fructose using high performance liquid chromatography (HPLC) equipped with a refractive index detector (Model 2414, Waters Corporation, Milford, MA). The column used was an Aminex HPX-87P (Biorad, Hercules, CA), which was eluted with pure water at 85 °C and 0.6 ml/min. The total sugars were calculated by summing the amounts of sucrose, glucose and fructose in the ground sample.

2.3. Lipid analysis

For the lipid analysis, ground samples were extracted using a modification of a previous method (Fang and Moreau, 2014; Hara and Radin, 1978). About 1*g* of the ground sample (dry weight) was mixed with isopropanol (10 ml) and hexane (15 ml) in a 50-ml screw top tube. The mixture was homogenized for 1 min for two times with a homogenizer (LabGen 700, Cole Parmer, Vernon Hills, IL) at 5000 rpm. The slurry was shaken with a wrist action shaker (HB-1000 Hybridizer, UVP LLC, Upland, CA) for 10 min at room temperature. Then 16 ml of sodium sulfate solution (6.7%, w/v) was added, and then shaken for 10 min. The mixture was centrifuged for 20 min at 200 rpm, and the top phase was carefully transferred with a pipette to a screw capped tube and the solvent was evaporated under a gentle stream of nitrogen and the mass was weighed on an analytical balance. The total lipid content in sugarcane stem is based on the assumption that a 100% lipid yield was obtained with isopropanol and hexane solvent extraction as assumed in a previous study (Fang and Moreau, 2014).

After weighing, dried samples were re-dissolved 10 mg/ml in 85:15 chloroform-methanol solvents. For nonpolar lipid analyses, a portion of the sample was transferred to mini-vials to include about 1 mg of sample. These were dried again under nitrogen, and re-dissolved 1 mg/ml in hexane with 0.01% BHT. Nonpolar lipid HPLC analyses were performed using a LiChrosorb 5 DIOL column employing the method of Moreau et al. (Moreau et al., 1996). With this method, the non-polar lipids, including triacylglycerols, free palmitic acid, linoleic acid, linolenic acid, stanol and sterol, were measured. For polar lipid analyses, samples were dissolved in chloroform-methanol with 0.01% BHT. Polar lipid HPLC analyses were performed on a Agillent Hewlett-Packard model 1100 HPLC, with auto sampler and a UV–visible detector (Agilent Technologies, Avondale, PA). For the non-polar analyses, Hewlett-Packard model 1050 HPLC was used. The column was a LiChrosorb 5 DIOL with 3 mm in diameter and 100 mm in length (Chrompack, Raritan, NJ). The analyzed polar lipids included acyl steryl glucoside (ASG), monogalactosyldiacylglycerol (MGDG), and phospholipids including phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI). The details of the lipid analysis were described elsewhere (Moreau et al., 1996, 2003).

2.4. Juice and bagasse separation

The flow diagram of sugarcane process is shown in Fig. 1. After being cut into small pieces (~2 cm in length), 250 g of sugarcane stems were processed using a juicer (Model: BJE510XL, Briville Inc., Torrance, CA) to separate juice and bagasse. After that, the bagasse was soaked with 50 ml of hot water (60 °C) and re-processed to extract the remaining juice. The soaking and extraction procedure was repeated four times to mimic the sugarcane industrial milling process to maximize sugar and lipid extraction from sugarcane stems, as milling

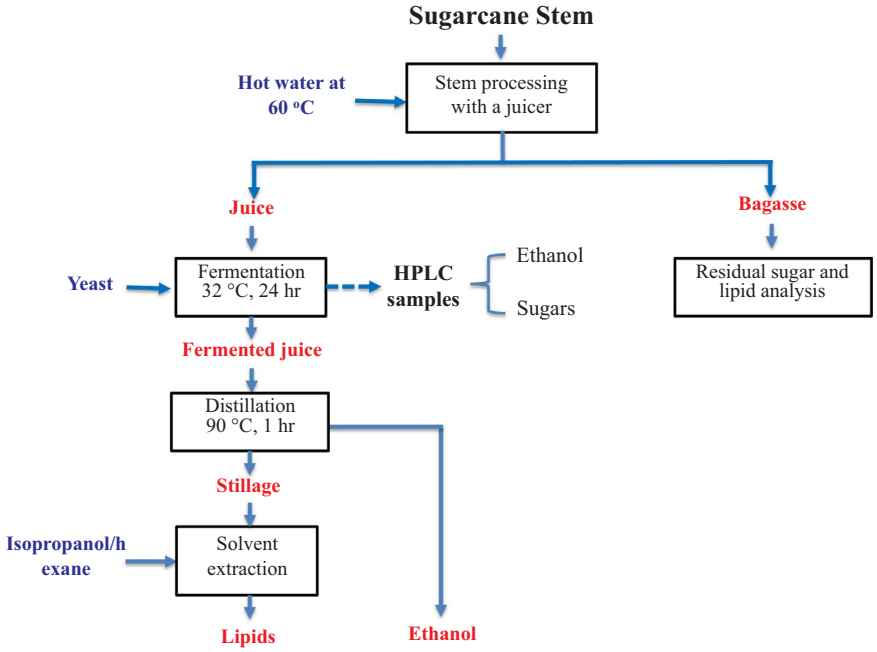

Fig. 1. Flow diagram of sugarcane process to produce ethanol and lipids. Sugarcane is cut and processed to separate juice and bagasse. Juice is fermented for 24 h by yeast to produce ethanol. After distillation, isopropanol and hexane is applied to extract lipids from stillage.

trains typically have four to six mills in the tandem to sequentially mill sugarcane stems for juice extraction (Bonomi et al., 2011). After juice extraction, the volume of juice was measured using a graduated cylinder. The wet bagasse was dried in a forced-air oven at 49 °C for 48 h. The dried bagasse was weighed and analyzed for sugar and lipid concentrations with the methods described in previous sections. The lipid contents in juice were calculated by comparing the amount of lipids in the sugarcane stems and in the bagasse. The sugar and lipid extraction efficiencies from the sugarcane stems were calculated by the following equations as provided in the literature (Xie et al., 2011):

$$\textit{Sugar extraction efficiency}\ (\%) = \frac{\textit{Total sugars in juice}}{\textit{Total sugars in stem}} \times 100\% \quad (1)$$

$$\textit{Lipid extraction efficiency}\ (\%) = \frac{\textit{Total lipids in juice}}{\textit{Total lipids in stem}} \times 100\% \quad (2)$$

2.5. Juice fermentation to convert sugars to ethanol

Commercial Red® yeast stock culture (Lesaffre yeast corporation, Milwaukee, WI) was prepared by adding 5 g of yeast dry powder into 25 ml deionized water and incubated at 32 °C in a shaking water bath for 25 min (Chen et al., 2014). After incubation, 1 ml of prepared yeast culture was added into a 50 ml of juice sample in a Pyrex™ bottle to start fermentation. Fermentation was conducted at 32 °C for 24 h in an incubator (Innova® 42, New Brunswick Scientific corporation, Enfield, CT). Fermentation was monitored by taking 1 ml of samples at 0, 4, 8, 12 and 24 h and measuring sugar and ethanol concentrations using HPLC with at least two determinations at each sampling time (Huang et al., 2015b). Ethanol production was calculated by multiplying the final ethanol concentrations by the volume of the broth after 24 h fermentation.

2.6. Lipid recovery with organic solvents

The fermented juice (broth) was heated to 90 °C for 1 h to distill ethanol (Fig. 1). After distillation, the volume of the stillage was about 20 ml. The lipids in the stillage were recovered by solvent extraction. The basic steps of the extraction included adding 10 ml of isopropanol and 15 ml of hexane, homogenizing for 1 min for two times, and then shaking for 10 min at room temperature. After that, 16 ml of sodium sulfate solution (6.7%, w/v) was added and then shaken for 10 min. The mixture was centrifuged and the top phase was carefully transferred with a pipette to a pre-weighed tube. The solvent was evaporated under a gentle nitrogen stream and the dried lipids were weighed. The lipid recovery efficiency were calculated as

$$\textit{Lipid recovery efficiency}\ (\%) = \frac{\textit{weight of recovered lipids}}{\textit{Total lipids in stem}} \times 100\% \quad (3)$$

2.7. Data analysis

All experiments, except for the lipid composition analysis, were carried out in triplicate. The values reported are the means ± standard deviation. Means of sugar and lipid concentrations, and extraction efficiencies were compared using Tukey's HSD (honestly significant difference) test (R software, version 3.2.0, www.r-project.org). The level selected to show statistically significant difference was 5% ($P < 0.05$).

3. Results and discussion

3.1. Sugar and lipid compositions of sugarcanes

Sugar content analysis showed that the non-transformed sugarcane and lipid-cane lines 19B and 25 C had total sugar concentrations of 37.0, 56.1 and 53.8 g per 100 g dry stem, respectively (Table 1). The non-transformed sugarcane had lower sugar concentrations than the two lipid-canes (19B and 25 C); however the sugar concentrations of all three sugarcanes were within the range of values reported in a previous literature (Lingle et al., 2010). The sugar profiles of the lipid-canes (19B and 25 C) were similar to those of the non-transformed sugarcane. For all three sugarcanes, sucrose content was the highest, accounting for more than 85% of the total sugar content, and glucose and fructose contents were relatively low (Table 1). This observation was in agreement with previous reports (Bonomi et al., 2011; Yusof et al., 2000) that sucrose was the dominant constituent of total sugars in

Table 1
Sugar concentrations in stem, juice and bagasse of the non-transformed sugarcane, the transgenic lipid-cane 19B, and 25 C.

		Non-transformed sugarcane	Lipid-cane 19B	Lipid-cane 25C
Stem	Total sugars (w/w%)	37.0 ± 0.3 c	56.1 ± 0.2 a	53.8 ± 0.6 b
	Sucrose (w/w%)	34.3 ± 0.2 c	50.2 ± 0.3 a	45.6 ± 0.4 b
	Glucose (w/w%)	0.9 ± 0.1 c	3.1 ± 0.1 b	4.3 ± 0.0 a
	Fructose (w/w%)	1.8 ± 0.1 c	2.8 ± 0.2 b	3.9 ± 0.1 a
Juice	Total sugars (w/v%)	6.9 ± 0.3 c	12.8 ± 0.6 a	10.4 ± 0.5 b
	Sucrose (w/v%)	6.1 ± 0.3 c	12.3 ± 0.5 a	9.5 ± 0.7 b
	Glucose (w/v%)	0.4 ± 0.0 ab	0.3 ± 0.1 b	0.5 ± 0.1 a
	Fructose (w/v%)	0.4 ± 0.0 a	0.2 ± 0.0 b	0.4 ± 0.1 a
Bagasse	Total sugars (w/w%)	10.3 ± 1.9 a	11.2 ± 2.7 a	14.8 ± 0.7 a
	Sucrose (w/w%)	9.3 ± 1.7 a	9.4 ± 2.5 a	11.9 ± 0.3 a
	Glucose (w/w%)	0.4 ± 0.0 c	0.9 ± 0.1 b	1.3 ± 0.2 a
	Fructose (w/w%)	0.7 ± 0.2 b	1.0 ± 0.2 b	1.7 ± 0.2 a

Note: values are means ± standard deviations. Values within one row without same letter were significantly different at $P < 0.05$.

sugarcane juice. It is worthy to mention that study samples were processed at high temperatures (i.e. 121 °C autoclaving and 85 °C HPLC mobile phase), and it is possible that these high temperatures caused hydrolysis of some of the sucrose. To test this hypothesis, a control experimental with sucrose in water was set up, and approximately 3% of sucrose was hydrolyzed to glucose and fructose during the thermal process (Fig. A1, Appendix A).

The total lipid concentrations in the stems of non-transformed sugarcane and lipid-canes lines 19B and 25 C were 0.66, 0.93 and 1.34 g per 100 g dry stem, respectively (Table 2), confirming that the genetically modified lipid-canes accumulated significantly higher lipid concentrations compared to the non-transformed sugarcane. The major non-polar and polar compounds of the total lipids are listed in Table 3. TAG is the desired and valuable compounds that can be used as feedstock to produce biodiesel by transesterification process. For the non-transformed sugarcane, the amount of TAG was found to be only 5% of the total extracted lipids, indicating that TAG expression in the non-transformed sugarcane was quite low. For the lipid-cane lines 19B and 25 C, the amounts of TAG were between 31% and 33% of the total lipids. Although 31–33% TAG purity of the total lipids seems low, these results are similar to TAG purity in other lipid producing biomass, such as algae and seaweed (Cheung, 1999; Halim et al., 2011). In these studies, the amounts of TAG were found to be between 23% and 44% of the total extracted lipids. Besides TAG, free palmitic acid, free linolenic acid, and sterol were also present at different levels (Table 3). The result shows that the transgenic lipid-cane (19B and 25 C) had substantial lower linolenic acid (C18:3) concentrations compared to the non-transformed sugarcane. This observation is in line with a previous study that showed decreased linolenic acid concentrations in transgenic tobacco leaves with the similar transgenic approaches (Vanhercke et al., 2014a). Each sample likely contained some free oleic acid, however, this peak was integrated into the palmitic acid peak in HPLC analysis.

Some polar lipids were also present in the total lipids (Table 3). Most of the polar lipids in stems were cell membrane components, such as ASG, MGDG, PE, PC and PI. Comparison of the major polar lipids in the three sugarcane samples showed that there were no remarkable differences in the composition of polar lipids. For example, the ASG components were between 2% and 3% of the total extracted lipids in all three sugarcanes. This result makes senses because the overexpressing genes (WRINKLED1 and DGAT1-2) in transgenic lipid-cane are only involved in the fatty acids and TAG manipulation, but not intend to change the composition of membrane lipids. It is worth noting that other lipid compounds were not identified via the current HPLC method used; therefore, the summed non-polar and polar lipids were below 100%. Also it is believed that significant levels of chlorophyll were also present because of the dark green appearance of the extracted lipids.

Table 2
Total lipid concentrations in stem, juice and bagasse of the non-transformed sugarcane, the transgenic lipid-cane 19B, and 25 C.

	Non-transformed sugarcane	Lipid-cane 19B	Lipid-cane 25C
Stem (w/w%)	0.66 ± 0.05 c	0.93 ± 0.02 b	1.34 ± 0.09 a
Juice (w/v%)	0.08 ± 0.01 c	0.12 ± 0.01 b	0.19 ± 0.02 a
Bagasse (w/w%)	0.62 ± 0.10 c	0.85 ± 0.13 b	1.19 ± 0.04 a

Note: values are means ± standard deviations. Values within one row without same letter were significantly different at $P < 0.05$.

Table 3
Nonpolar and polar lipid components of the total extracted lipids from stems (weight percent, wt% of total lipid).

	Lipids	Non-transformed sugarcane	Lipid-cane 19B	Lipid-cane 25 C
Non-polar lipids	Triacylglycerols (w/w%)	4.76	30.90	33.10
	Palmitic Acid, free (w/w%)	4.78-[a]	4.91	3.50
	Linoleic Acid, free (w/w%)		0.26	0.33
	Linolenic Acid, free (w/w%)	4.43-[a]	1.87	1.66
	Stanol (w/w%)		0.35	0.37
	Sterol (w/w%)	5.42	3.19	3.07
Polar lipids	Acyl Steryl glucoside (w/w%)	2.39	2.26	2.25
	Monogalactosyldiacylglycerol (w/w%)	3.25	2.24	2.07
	Phosphatidylethanolamine (w/w%)	1.95	2.38	2.95
	Phosphatidylcholine (w/w%)	2.00	0.60	1.23
	Phosphatidylinositol (w/w%)	3.31	3.34	3.20

[a] undetected.

3.2. Sugar and lipid extraction with juice

After quantifying total sugar and lipid content in sugarcane stems, the stems were processed to extract and quantify sugars and lipids with juice. There are two separate components produced in the processing step: juice and bagasse, where juice is the main product and bagasse is the by-product. During the process, majority of the sugars were extracted with juice and some sugars remained in bagasse. Results reveal that each 100 g of dry bagasse contained 10.3–14.8 g of total sugars, of which 9.3–11.9 g was sucrose (Table 1). And the total sugar concentrations in extracted juice were 6.9–12.8 g per 100 ml of juice (Table 1). By comparing the amounts of sugars distributed in juice and

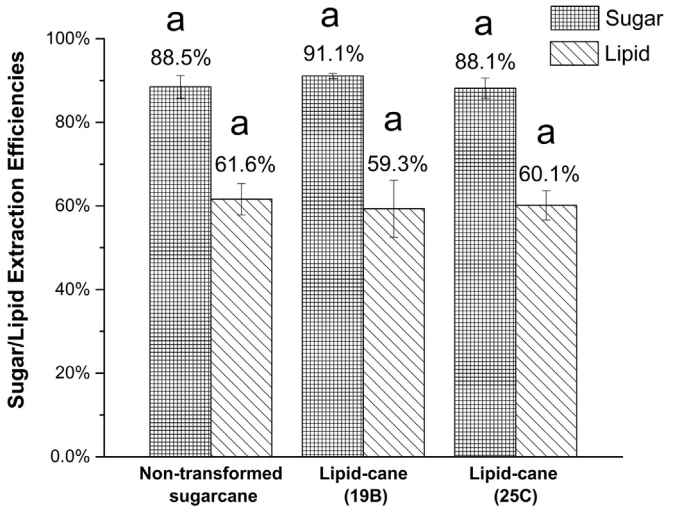

Fig. 2. Sugar and lipid extraction efficiencies for non-transformed sugarcane, lipid-cane 19B, and 25 C. Values within one category without common letter were significantly different at $P < 0.05$. Error bars represent standard deviations of three replicates.

bagasse, the sugar extraction efficiencies with juice were close to 90% (Fig. 2). The result implies that lipid expression in lipid-cane does not interfere with the sugar extraction during the process, since the sugar extraction efficiencies were not significantly different between the transformed and non-transformed sugarcanes. The sugar extraction efficiency at 90% is very close to the values obtained in sugarcane processing plants. In modern sugarcane plants, where more powerful roller mills are used in sequence to squeeze sugars out of the stems with hot water, the sugar extraction efficiency can reach 90–95% (Bonomi et al., 2011; Dias et al., 2011; Goldemberg, 2008).

The lipid concentrations in juice were quite low (Table 2), because significant amounts of hot water were added during the juice extraction process, which increased the volume of the juice and diluted the lipid concentrations in juice. By comparing the amounts of lipid distributed in juice and bagasse, the lipid extraction efficiencies for the three sugarcanes were between 59.3% and 61.6%, with remaining about 40% of lipids in bagasse. This result verifies our hypothesis that lipids in sugarcane stems can be extracted with juice by mechanically squeezing stems. But it also indicates that the developed method is not sufficient enough to extract lipids from the lipid-cane stems, especially considering that the lipid extraction efficiency in a typical soybean processing plant is about 90% with organic solvent (hexane) extraction (REMCO and Desmet ballestra, 2005). This could be due to several reasons. Firstly, the lipid concentrations in current lipid-cane stems at the current stage are still low, making the lipid extraction difficult. Some of our recent transgenic material (lipid cane varieties) has 5% TAG and about 10% total fatty acid in laboratory stage (Zale et al., 2016; Huang et al., 2015a). Higher lipid concentration is expected to result in higher extraction efficiency. Secondly, the lipid bodies in stems are protected by structural proteins (i.e. Oleosins), making them difficult to be extracted by squeezing with hot water. While hexane extraction of lipids from the bulky and wet lipid-cane stems are probably not economically favorable, new technologies could be developed and applied to improve the lipid-extraction efficiency, such as proteinase-assisted aqueous extraction (Dickey et al., 2011; de Moura et al., 2008) and supercritical carbon dioxide extraction (Halim et al., 2011). Nonetheless, lipid extraction efficiency at about 60% is a good baseline for future improvement.

3.3. Fermentation of sugarcane juice to produce ethanol

The extracted juice was subjected to the fermentation process to convert sugars to ethanol. For the juice extracted from the non-transformed sugarcane, the ethanol concentration was 3.5% (v/v) after 24 h fermentation (Fig. 3a). The final ethanol concentration for lipid-canes 19B and 25 C were higher, due to the higher initial sugar concentrations in the extracted juice. The sugar concentrations of the juice from the non-transformed sugarcane were about 6.9% (w/v), whereas the sugar concentrations of the juice from the lipid-canes 19B and 25 C were 12.8% and 10.4% (w/v), respectively (Table 1). During fermentation, the sucrose concentrations decreased rapidly to a negligible level in the first 4 h, during which the glucose and fructose concentrations increased at different levels. This increase was due to sucrose being hydrolyzed to glucose and fructose by extracellular invertase produced by yeasts (Novick and Schekman, 1979). Hydrolyzed glucose and fructose were transported into the yeast cell and converted to ethanol (Chen et al., 2013; Koschwanez et al., 2011). After 24 h fermentation, all sugars in juice were depleted, except for the non-transformed sugarcane juice. For the non-transformed sugarcane, a small amount of fructose (0.5%, w/v%) remained in the juice at the end of fermentation. Since the sugarcane juice contains several sugars, the preference of the yeast to a specific sugar affects the fermentation efficiency (Chen et al., 2013). According to the previous literature, when present in equal amounts, glucose was utilized twice as fast as fructose (D'Amore et al., 1989). Similar results were also observed in our study (Fig. 3b–d), that glucose was consumed faster than fructose by yeasts.

3.4. Mass balance of sugar and lipids during process

Fig. 4 shows the sugar and lipid mass distributions during the processes of the three sugarcanes based on 100 g of dry stem. The overall lipid recovery efficiencies (the mass of recovered lipids after fermentation over the mass of total lipids in stems) from the sugarcane stems were 43%, 61%, and 56% for non-transformed sugarcane and lipid-canes 19B and line 25 C, respectively. The process of recovering lipids in this study is very similar to the process of recovering oil in the corn dry grind ethanol industry, where corn oil is recovered from thin stillage after fermentation and distillation. It was reported that about 30–60% of corn oil is recovered in the corn dry grind ethanol industry with different processing technologies.

(US Grains Council, 2015.), which is similar to the overall lipid recovery efficiencies obtained in our study.

For the non-transformed sugarcane, 100 g of dry stems contained 37.0 g of sugar and 0.7 g of total lipids. After the processing step, 89% (32.9 g) of the sugars, and 60% (0.4 g) of the lipids were extracted with the juice, with the rest of them remaining in the bagasse. During fermentation, the extracted sugars were converted to 13.9 g of ethanol by yeasts. After fermentation and distillation, 0.3 g of lipids was recovered from the fermented juice using the organic solvents (hexane and isopropanol). For lipid-cane 19B, 29.2 g of ethanol and 0.5 g of lipids were obtained from 100 g of dry stem. For lipid-cane 25 C, 23.6 g of ethanol and 0.8 g of lipids were obtained from 100 g of dry stem. These data proved our original idea that the novel lipid-cane can be used as a dual biofuel crop: the sugars in juice are used to produce ethanol, the recovered lipids from juice can be used as feedstock for biodiesel production, and the remaining bagasse can be burned to produce heat and electricity.

In the US, the sugarcane yield in the most productive zone (Hawaii) can be as high as 54–66 dry MT/ha, though this value decreased to 23–33 dry MT/ha in the southern states of the US (Florida, Louisiana, and Texas). If the dry matter yield at 33 MT/ha was applied, the lipid-cane 19B and 25 C can provide approximately 0.17 and 0.26 MT lipids per hectare with the current technology. This number is not far away from the current lipid production from soybean at 0.36 and 0.61 MT/ha (USDA, 2015). With new transgenic lines having 10% total lipids (Zale et al., 2016; Huang et al., 2015a), 2.0–3.3 MT/ha lipid production potential can be achieved. Therefore, with the increased lipid accumulation in lipid-cane and improved lipid extraction efficiency in the

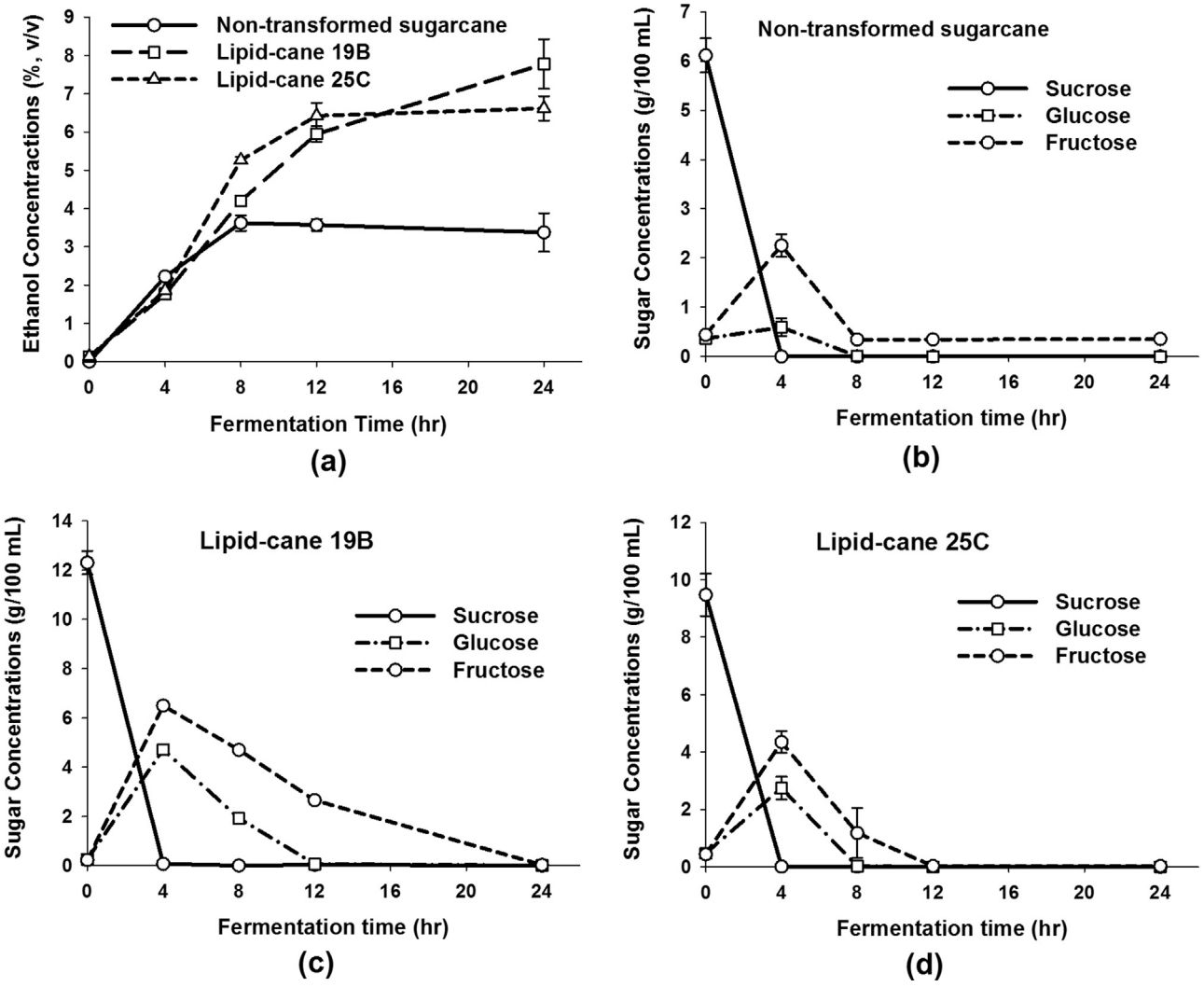

Fig. 3. Sugarcane juice fermentation profile. (a) ethanol concentrations during fermentation; (b) sugar concentrations during fermentation of juice from the non-transformed sugarcane; (c) sugar concentrations during fermentation of juice from the lipid-cane 19B; (d) sugar concentrations during fermentation of juice from the lipid-cane 25 C. Error bars represent standard deviations of three replicates.

future, lipid-cane could have a big impact on the lipid production with the limited land resource.

4. Conclusions

In summary, our study reveals that the transgenic lipid-cane has significantly higher lipids compared to the non-transformed sugarcane. TAG was the most dominant lipid in lipid-cane stems through the composition analysis. Results showed that both sugars and lipids in the lipid cane are extractable from the lipid-cane stems by the developed process. The sugar extraction efficiency was high at 90% by the repeated hot water extraction. Compared to sugars, the lipid extraction efficiency was lower at 60%, indicating that improved processes should be developed to increase the lipid extraction efficiency. This study showed that the transgenic lipid-cane could be a potential dual-purpose feedstock for both ethanol and biodiesel production, but it also needs some future improvements to be commercialized, such as increasing lipid concentrations in stems and improving lipid extraction efficiency.

Acknowledgements

The information, data, or work presented herein was funded in part by the Advanced Research Projects Agency-Energy (ARPA-E), U.S. Department of Energy, under Award Number DE-AR0000206. The authors thank Mrs. Wei Liu for her great help on sugarcane sample preparations and HPLC sample analysis.

Appendix A

Sucrose hydrolysis under thermal processing

The objective of this test was to investigate the sucrose hydrolysis during the thermal treatment. In the test, 20 g/L of sucrose was prepared by dissolving 1 g of sucrose in a 50-ml solution in 100 ml screw capped Pyrex glass bottles. The sucrose solution was autoclaved at 121 °C for 15 min (Napco model 9000D, Thermo Fisher Scientific, Waltham, MA). The autoclaved solution was then analyzed for sucrose, glucose, and fructose using high performance liquid chromatography (HPLC) equipped with a refractive index detector (Model 2414, Waters Corporation, Milford, MA). The column used was an Aminex HPX-87P (Biorad, Hercules, CA), which was eluted with pure water at 85 °C and 0.6 ml/min. The test was conducted in three replicates. The result showed that about 3% (0.58 g/L out of 20 g/L) sucrose were hydrolyzed into glucose and fructose during the thermal treatment (Fig. A1).

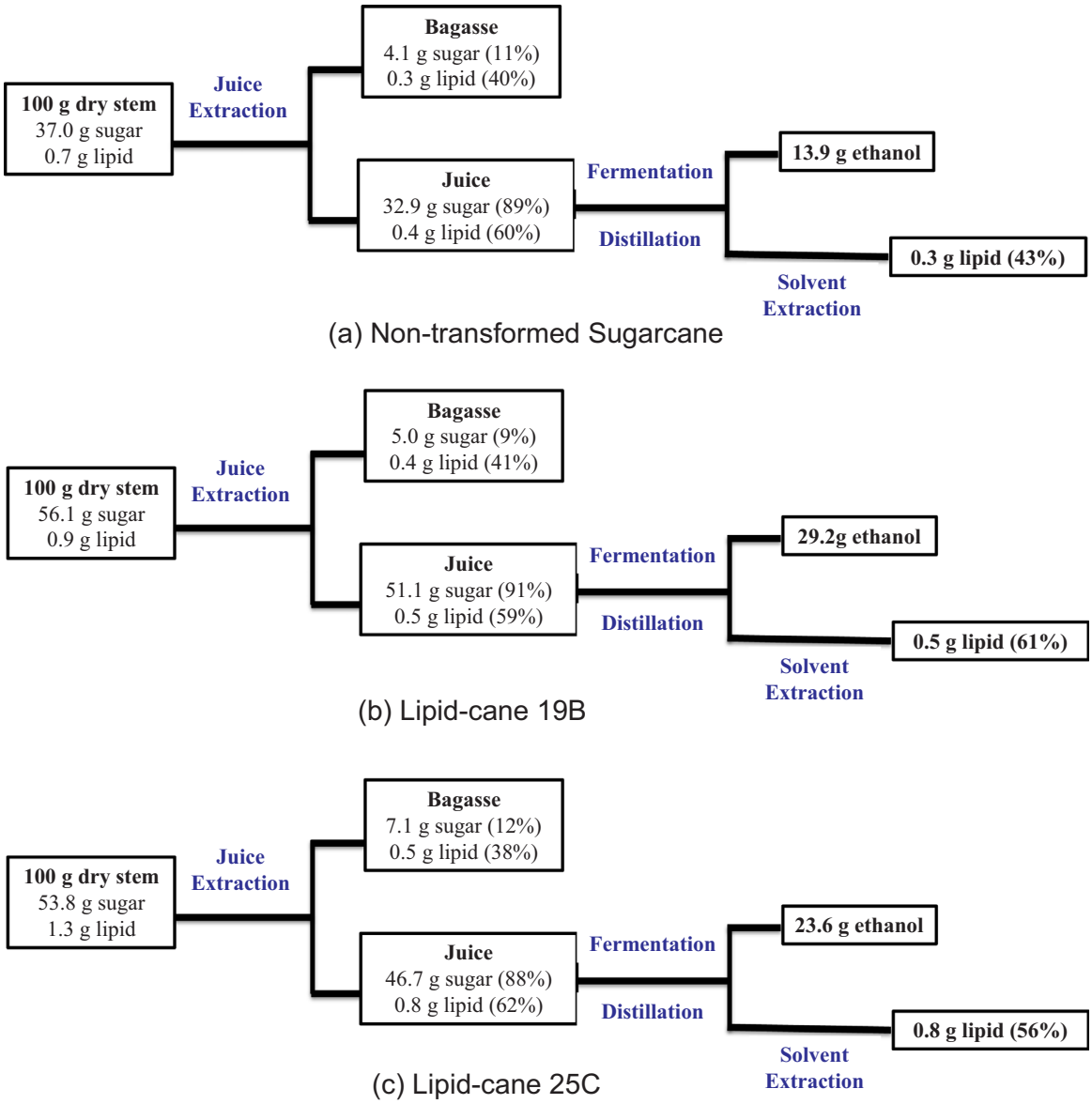

Fig. 4. Mass balances of sugar and lipids during the processes of sugarcanes.

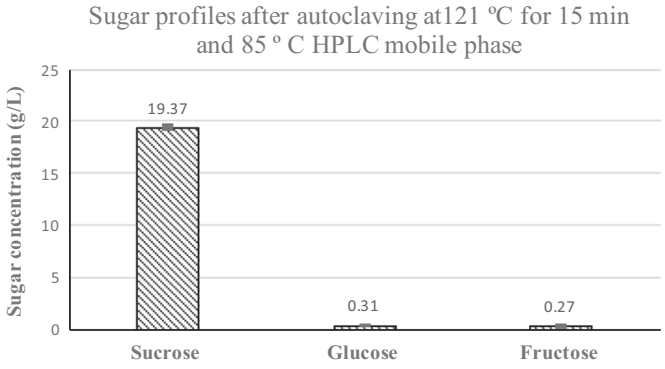

Fig. A1. Sugar profiles of sucrose solution (20 g/L) after 121 °C autoclaving and 85 °C mobile phase.

References

ASABE Standard, 2012 ANSI/ASAE S358.3 Moisture Measurement -Forages. American Association of Agricutural and Biologicla Engineers, St. Joseph, MI.

Bonomi, A., Mariono, A.P., de Jesus, C.D.F., Franco, H.C.J., Cunha, M.P., de Souza Dias, M.O., Chagas, M.F., Cavalett, O., Mantelatto, P.E., Filho, R.M., Junqueira, T.L., de Fátima Cardoso, T., 2011. Virtual Sugarcane Biorefinery Report. 〈http://www.scribd.com/doc/108970905/Virtual-Sugarcane-Biorefinery-Report-2011〉 - scribd(acessed 15.11.02).

Chapman, K.D., Dyer, J.M., Mullen, R.T., 2013. Commentary: Why don't plant leaves get fat? Plant Sci. 207, 128–134.
Chen, M.-H., Dien, B.S., Vincent, M.L., Below, F.E., Singh, V., 2014. Effect of harvest maturity on carbohydrates for ethanol production from sugar enhanced temperate × tropical maize hybrid. Ind. Crop. Prod. 60, 266–272.
Chen, M.-H., Kaur, P., Dien, B., Below, F., Vincent, M.L., Singh, V., 2013. Use of tropical maize for bioethanol production. World J. Microbiol. Biotechnol. 29, 1509–1515.
Cheung, P.C., 1999. Temperature and pressure effects on supercritical carbon dioxide extraction of n-3 fatty acids from red seaweed. Food Chem. 65, 399–403.
D'Amore, T., Russell, I., Stewart, G.G., 1989. Sugar utilization by yeast during fermentation. J. Ind. Microbiol. 4, 315–323.
Dias, M.O., da Cunha, M.P., Maciel Filho, R., Bonomi, A., Jesus, C.D., Rossell, C.E., 2011. Simulation of integrated first and second generation bioethanol production from sugarcane: comparison between different biomass pretreatment methods. J. Ind. Microbiol. Biotechnol. 38, 955–966.
Dickey, L.C., Johnston, D.B., Kurantz, M.J., McAloon, A., Moreau, R.A., 2011. Modification of aqueous enzymatic oil extraction to increase the yield of corn oil from dry fractionated corn germ. Ind. Crop. Prod. 34, 845–850.
Energy Information Administration, 2013. Biodiesel production capacity and production. 〈http://www.eia.gov/biofuels/biodiesel/production/?src=Petroleum-f7〉 (acessed 15.11.02).
Energy Information Administration, 2015. Sales of Distillate Fuel Oil by End Use. 〈https://http://www.eia.gov/dnav/pet/pet_cons_821dst_dcu_nus_a.htm〉 (acessed 15.11.02).
Fang, X., Moreau, R.A., 2014. Extraction and demulsification of oil from wheat Germ, barley Germ,germ, and rice bran using an aqueous enzymatic method. J. Am. Oil Chem. Soc. 91, 1261–1268.
Goldemberg, J., 2008. The Brazilian biofuels industry. Biotechnol. Biofuels 1, 1–7.
Graboski, M.S., McCormick, R.L., 1998. Combustion of fat and vegetable oil derived fuels in diesel engines. Prog. Energy Combust. Sci. 24, 125–164.
Haas, M.J., McAloon, A.J., Yee, W.C., Foglia, T.A., 2006. A process model to estimate biodiesel production costs. Bioresour. Technol. 97, 671–678.
Halim, R., Gladman, B., Danquah, M.K., Webley, P.A., 2011. Oil extraction from microalgae for biodiesel production. Bioresour. Technol. 102, 178–185.
Hara, A., Radin, N.S., 1978. Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. 90, 420–426.
Huang, H., Long, S.P., Singh, V., 2015a. Ultra-Oil Producing Sugarcane and sweet sorghum. AOCS INFORM 26, 278–282.
Huang, H., Long, S., Singh, V., 2016. Techno-economic analysis of biodiesel and ethanol co-production from lipid-producing sugarcane. Biofuels. Bioprod. Biorefin. 10 (3), 299–315.
Huang, H., Qureshi, N., Chen, M.-H., Liu, W., Singh, V., 2015b. Ethanol production from food waste at high solids content with vacuum recovery technology. J. Agric. Food Chem. 63, 2760–2766.
James, C.N., Horn, P.J., Case, C.R., Gidda, S.K., Zhang, D., Mullen, R.T., Dyer, J.M., Anderson, R.G., Chapman, K.D., 2010. Disruption of the Arabidopsis CGI-58 homologue produces Chanarin–Dorfman-like lipid droplet accumulation in plants. Proc. Natl. Acad. Sci. 107, 17833–17838.
Koschwanez, J.H., Foster, K.R., Murray, A.W., 2011. Sucrose utilization in budding yeast as a model for the origin of undifferentiated multicellularity. PLoS Biol. 9, e1001122.
Lingle, S.E., Johnson, R.M., Tew, T.L., Viator, R.P., 2010. Changes in juice quality and sugarcane yield with recurrent selection for sucrose. Field Crops Res. 118, 152–157.
Long, S.P., Karp, A., Buckeridgec, M.S., Murphyf, D.J., Onwona-Agyemang, S., Vonshakh, A., 2015. Feedstocks for Biofuels and Bioenergy. In: Souza, G.M., Victoria, R.L., Joly, C.A., Verdade, L.M. (Eds.), Bioenergy and Sustainability: Bridging the Gaps. Scientific Committee on Problems of the Environment (SCOPE), Paris, pp. 302–336.
Moreau, R.A., Powell, M.J., Hicks, K.B., 1996. Extraction and quantitative analysis of oil from commercial corn fiber. J. Agric. Food Chem. 44, 2149–2154.
Moreau, R.A., Powell, M.J., Singh, V., 2003. Pressurized liquid extraction of polar and nonpolar lipids in corn and oats with hexane, methylene chloride, isopropanol, and ethanol. J. Am. Oil Chem. Soc. 80, 1063–1067.
de Moura, J.N., Campbell, K., Mahfuz, A., Jung, S., Glatz, C., Johnson, L., 2008. Enzyme-assisted aqueous extraction of oil and protein from soybeans and cream de-emulsification. J. Am. Oil Chem. Soc. 85, 985–995.
Novick, P., Schekman, R., 1979. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. 76, 1858–1862.
REMCO, Desmet_ballestra,2005. Feasibility study and preliminary business plan for a Michigan soybean crush plant, soybean oil refinery and/or biodiesel production plant in Gratiot County or other Michigan sites. 〈http://www.michigan.gov/documents/MDA_Biodiesel_REMCO_Exec_Sum_153502_7.pdf〉 (acessed 15.11.02).
Renewable Fules Association, 2015. Monthly US fuel ethanol production demand. 〈http://tinyurl.com/me8qhkb〉 (acessed 15.11.02).
US Grains Council, 2015. Chapter 2: Ethanol Production and its Co-Products Dry-Grind and Wet Milling Processes, in: DDGS User Handbook, online book. 〈http://www.grains.org/buyingselling/ddgs/ddgs-user-handbook〉 (acessed 15.11.02).
USDA, 2015. US soybean acres and yields. Available at: 〈http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/market-outlook/usda-soybean-baseline,-2010-19.aspx〉 (acessed 15.11.02).
Vanhercke, T., Petrie, J.R., El Tahchy, A., Singh, S.P., Liu, Q., 2014b. Processes for producing hydrocarbon products. US Patents: 8,735,111B2 (2014).
Vanhercke, T., El Tahchy, A., Liu, Q., Zhou, X.R., Shrestha, P., Divi, U.K., Ral, J.P., Mansour, M.P., Nichols, P.D., James, C.N., 2014a. Metabolic engineering of biomass for high energy density: oilseed-like triacylglycerol yields from plant leaves. Plant Biotechnol. J. 12, 231–239.
Xie, M., Dunford, N.T., Goad, C., 2011. Enzymatic extraction of wheat germ oil. J. Am. Oil Chem. Soc. 88, 2015–2021.
Yan, S., Chen, X., Wu, J., Wang, P., 2012. Ethanol production from concentrated food waste hydrolysates with yeast cells immobilized on corn stalk. Appl. Microbiol. Biotechnol. 94, 829–838.
Yusof, S., Shian, L., Osman, A., 2000. Changes in quality of sugar-cane juice upon delayed extraction and storage. Food Chem. 68, 395–401.
Zale, J., Jung, J.H., Kim, J.Y., Pathak, B., Karan, R., Liu, H., Chen, X., Wu, H., Candreva, J., Zhai, Z., 2016. Metabolic engineering of sugarcane to accumulate energy-dense triacylglycerols in vegetative biomass. Plant Biotechnol. J. 14, 661–669.