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Evaluating the Potential of Newly Developed Energy Cane Clones for First- and Second-Generation Ethanol Production

Sutticha Na-Ranong Thammasittirong [1,2,*], Prasert Chatwachirawong [3], Kedwarin Khemdee [1] and Anon Thammasittirong [1,2]

[1] Department of Science and Bioinnovation, Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[2] Microbial Biotechnology Unit, Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[3] Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
* Correspondence: sutticha.n@ku.ac.th; Tel.: +663-428-1105; Fax: +663-435-1402

Abstract: The rapid increases in fuel ethanol demand and food security concerns have driven the need for diverse feedstocks in the ethanol production process. Energy cane is an energy crop that is an ideal sustainable biofuel feedstock. The present study evaluated ethanol production of the juice and bagasse of two newly developed energy cane clones, TByEFC08-0035 and TByEFC10-0004. The results of the chemical composition analyses of the juice and bagasse samples revealed that the two energy cane clones contained high contents of both sucrose (15.36–17.95%) and fiber (13.44–24.16%). The maximum ethanol concentrations from the juice on a laboratory scale (87.10 g/L) and on an agronomic scale (1211.76 kg/ha) were recorded for TByEFC10-0004 fermented with a new isolate *Kluyveromyces marxianus* SJT83, whereas the maximum ethanol concentrations from bagasse on a laboratory scale (9.81 g/L) and on an agronomic scale (790.68 kg/ha) were reached with TByEFC08-0035 fermented with *Scheffersomyces shehatae* TTC79. The total ethanol yields from the juice and bagasse samples per cultivation area of both energy cane clones were in the range 1294.23–1469.14 kg/ha, being 1.70–1.93 and 1.08–1.23 times higher than the control energy cane Biotec2 variety and the commercial sugar cane Khon Kaen3 variety, respectively. This study revealed the potential of the energy cane clones TByEFC08-0035 and TByEFC10-0004 currently being developed as sugar and lignocellulose substrates for first- and second-generation ethanol industry applications.

Keywords: energy cane; ethanol production; sugar feedstock; lignocellulose feedstock; energy crop; biorefinery

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1. Introduction

Due to the worldwide energy crisis and concerns regarding global warming from the use of fossil fuels, ethanol is a promising biofuel that can mitigate emissions, especially of greenhouse gases. Ethanol plays a major role as a renewable fuel because it can be economically produced from a variety of carbohydrate feedstocks and easily blended into gasoline. The global ethanol supply is mainly produced from sugar and starch feedstocks. The sugar-based substrates can be converted directly to ethanol using microbial fermentation. Sugar cane contains readily fermentable sugars, allowing an easy and economical ethanol production process. Furthermore, sugar cane is an important industrial crop that is widely used as an important source for sugar, ethanol and electricity [1,2]. However, the rapid increase in biofuel demand has raised concerns about the security of feedstock for food and biofuel production. This global problem has encouraged the exploration of alternative renewable feedstocks for sustainable ethanol production, either from non-edible crops or from lignocellulose biomass.

Energy cane is a hybrid of commercial sugar cane and its wild ancestors. Compared with sugar cane, energy cane has a higher fiber content and reduced fertilizer and water input requirements, while being resistant to disease and harsh environmental conditions so that it can be grown on a marginal land area without competing with food production [1,3,4]. The growing interest in bioenergy in recent decades has driven several sugarcane breeding programs worldwide to develop energy cane varieties for bioenergy application. In the United States, several energy cane varieties have been released for use as a biofuel feedstock, including L 79-1002 [5], Ho 00-961 [6] and Ho 02-113 [4], which were developed by the USDA-ARS Sugarcane Research Unit working cooperatively with the Louisiana State University Agricultural Center and the American Sugarcane League of the USA. In Brazil, a Brazilian company, BioVertis/GranBio, has been developing energy cane varieties under the name Vertix®, with 11 varieties registered in Brazil [7]. Other work on improving energy cane varieties has been reported in several countries, for example Argentina [8], India [9] and Japan [10].

In Thailand, the energy cane series "TByEFC" (Tiphuyae and Banyang Energy and Forage-cane Clone) has been developed by researchers in the Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen campus. A high-fiber energy cane Biotec2 (TByEFC01-0009) variety was bred in 2001 and released for use as feed and bioenergy feedstocks [11]. The juice and bagasse from four clones of the energy cane series TByEFC (TByEFC04-1155, TByEFC04-1208, TByEFC05-1558 and TByEFC09-0098) were successfully fermented into ethanol [12]. The current study investigated two new clones of the energy cane series TByEFC (TByEFC08-0035 and TByEFC10-0004) and reported their great economic advantage (high sugar and fiber contents) over the control varieties to produce ethanol either by first- or second-generation processes. The chemical compositions of their juice and bagasse, and ethanol production from both energy cane clones were evaluated and compared with the energy cane Biotec2 variety (control of energy cane) and commercial sugar cane Khon Kaen3 variety (control of sugar cane). Ethanol production from energy cane juice was performed using a new yeast isolate, *Kluyveromyces marxianus* SJT83, with benefits for ethanol production from cane juice. *Saccharomyces cerevisiae* ND48, a potential sucrose-fermenting yeast that produced high ethanol from energy cane juice [12], was used as a control strain for ethanol production from energy cane juice. Ethanol production from the lignocellulosic part of the energy cane was performed using *Scheffersomyces shehatae* TTC79, which was previously reported to be an excellent xylose-fermenting yeast with high lignocellulosic inhibitor tolerance [13]. Ethanol production from the juice and bagasse were reported on a laboratory scale and an agronomic scale.

2. Materials and Methods

2.1. Materials

The field experiment was conducted at the Field Crops Unit, Kasetsart University, Kamphaeng Saen campus, Thailand. The soil at the planting site was a silt loam. A randomized complete block design with 4 replications was used. Two energy cane clones (TByEFC08-0035 and TByEFC10-0004), the energy cane Biotec2 variety (control of energy cane) and the commercial sugar cane Khon Kaen 3 variety, developed by Khon Kaen Field Crops Research Center, Khon Kaen, Thailand (control of sugar cane), were harvested at 8 months from the second ratoon after planting. Ten cane stalks in the 3 middle rows were sampled for yield determination by manually cutting at ground level and topping at their natural break point. The cut cane stalks were immediately stripped of leaves and crushed twice in a small local roller press. The juice was filtered through cheesecloth to remove plant residues and kept at −20 °C for further studies. The cane, sugar and fiber yields were determined as described by Thammasittirong et al. [12].

2.2. Physical and Chemical Compositions of Energy Cane Juice

Total soluble solids (TSS, °Brix) in the energy cane juices were measured using a refractometer (N.O.W.; Tokyo, Japan). Sugars in the juice were determined using a high-performance liquid chromatography (HPLC) system (Water 600E; Waters Corp, Milford, MA, USA) equipped with a refractive index detector. The filtered samples were separated using a sugar pak I column operating at 85 °C with deionized water as a mobile phase at a flow rate of 0.5 mL/min [12,13]. The free amino nitrogen (FAN) content in the juice was determined using the ninhydrin official method with glycine as the standard [14].

2.3. Chemical Composition of Energy Cane Bagasse

The chemical composition of energy cane bagasse was determined following the standard methods of the National Renewable Energy Laboratory (NREL) with slight modification as described by Thammasittirong et al. [12]. The sugar, furfural, 5-hydroxymethyl furfural (HMF) and acetic acid concentrations were analyzed using HPLC according to Senatham et al. [13].

2.4. Isolation and Screening of Yeasts for Energy Cane Juice Fermentation

Yeasts were isolated from the sugarcane juice samples and from soil samples from a sugar cane field in Nakhon Pathom province, Thailand. Isolation was performed using an enrichment technique as described by Sripodok et al. [15]. Screening for sucrose-fermenting yeast strains was performed in two steps. First, the sucrose-fermenting yeasts were screened using the Durham tube method. A loopful of 24 h yeast extract peptone sucrose (YPS) agar-grown culture (10 g/L yeast extract, 20 g/L peptone, 20 g/L sucrose, 15 g/L agar) was inoculated in 10 mL YPS medium in a test tube containing a Durham tube and incubated at 37 °C for 36 h. The yeast isolates that generated CO_2 gas that filled the whole Durham tube within 18 h were then selected for the screening of high ethanol-producing strains in the sugar cane juice model medium. The medium was prepared as described by Ohara et al. [16] with slight modification of the composition (180 g/L sucrose, 45 g/L glucose, 10 g/L yeast extract, 3 g/L malt extract and 20 g/L peptone, pH 5.0). The sugar cane juice model medium was inoculated with yeast cells to provide an initial cell concentration of 5×10^5 cells/mL. The flasks were incubated at 37 °C with shaking at 100 rpm for 48 h. Samples were withdrawn every 12 h to measure the cell density at 600 nm using a UV-Vis spectrophotometer (GENESYS™ 10S UV–vis; Thermo Scientific; Waltham, MA, USA). The ethanol and residual sugar concentrations were determined using HPLC as described above.

2.5. Yeast Identification and Phylogenetic Tree Analysis

Total DNA was extracted from the yeast culture using the method described by Gonzalez-Mendoza et al. [17]. For molecular characterization of the selected yeast isolate, the D1/D2 domain of the 26S rRNA and ITS regions were amplified using PCR with the primers NL1 and NL4, and ITS1 and ITS4, respectively. The PCR reactions and conditions were performed according to Namnuch et al. [18] with slight modification by using an annealing temperature of 55 °C. The DNA sequences were compared to the sequence of related species published in GenBank using the BLASTN program. The D1/D2 domain and ITS sequences were deposited in GenBank under accession numbers OP781886 and OP781903, respectively. Multiple-alignment of the sequences was carried out using the Clustal W software and a phylogenetic tree was constructed using the MEGA X software [19].

2.6. Ethanol Production from Energy Cane Juice

Ethanol was produced from energy cane juice using the newly isolated yeast *K. marxianus* SJT83, with *S. cerevisiae* ND48 (with high ethanol production from sucrose [12]) as a control strain. Energy cane juice medium without nutrient supplementation (pH 5.0) was inoculated with yeast cells to provide an initial cell concentration of 5×10^5 cells/mL. The culture was incubated at 37 °C on a rotary shaker at 100 rpm for 72 h. Samples were withdrawn every 12 h and analyzed for ethanol, residual sugar and cell density [12].

2.7. Preparation of Energy Cane Bagasse Hemicellulosic Hydrolysate Using Dilute Acid Hydrolysis

The milled bagasse was dried in an oven at 60 °C for 24 h to remove any moisture. The dried milled bagasse was soaked in 1% H_2SO_4 at a solid-to-liquid ratio of 1:10 for 30 min at room temperature, and then autoclaved at 121 °C for 30 min. The hydrolysate was separated from the solid fraction using filtration and neutralized with CaO to pH 5.5. Then, the hydrolysate was detoxified using activated charcoal [12].

2.8. Ethanol Production from Energy Cane Bagasse

The hydrolysate medium, adjusted to pH 5.5 and supplemented with 5 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L peptone and 5 g/L yeast extract, was inoculated with *Sch. shehatae* TTC79 (with high lignocellulosic inhibitor tolerance [13]) to provide an initial yeast cell concentration of 1×10^7 cells/mL. The culture was incubated at 30 °C on a rotary shaker at 100 rpm for 72 h. Samples were withdrawn every 12 h and analyzed for ethanol, residual sugar and cell density [12].

2.9. Statistical Analysis

The data were subjected to analysis of variance using the SPSS 16.0 statistical package (SPSS Inc.; Chicago, IL, USA). Data were expressed as a mean ± standard error. Values with $p < 0.05$ were considered significantly different.

3. Results and Discussion

3.1. Chemical Composition of Energy Cane Juice and Bagasse

The chemical composition of the juice was characterized as shown in Table 1. Energy cane juice from TByEFC08-0035 and TByEFC10-0004 contained higher levels of TSS and total sugars compared to the control energy cane Biotec2. Energy cane juice from TByEFC10-0004 had the highest levels of TSS (19.1 °Brix) and total sugar (190.63 g/L), with values close to the control sugar cane Khon Kaen3 (21.1 °Brix and 224.00 g/L, respectively). The current study highlighted the successful genotypic improvement of energy cane to yield a high level of sugar juice, especially TByEFC10-0004, compared to the control varieties and earlier reports, as shown in Table 1. The major sugar component of energy cane juice was sucrose, followed by glucose and fructose. The highest sucrose content (17.95%) was measured in the juice of the energy cane TByEFC10-0004 (Figure 1a). Fanelli at al. [20] reported that the sucrose contents in the juices of the energy canes VIGNIS 3 and VG1126 were 12.4% and 9.0%, respectively. Energy cane L79-1001L juice contained 9.6% sucrose [2]. The sucrose contents in the juices of the energy canes Ho 02-113 and HoCP 72-114 were 9.0% and 10.1%, respectively [21]. Generally, energy cane juice contains a lower proportion of sucrose compared to sugar cane juice. The percentage ratios of sucrose in the juices of both energy cane clones in the current study were high (in the range 85.29–94.18%) compared to in the control energy cane Biotec2 (67.05%), as shown in Figure 1b. Notably, the energy cane TByEFC10-0004 had a high percentage ratio of sucrose (94.18%), which was similar to the value for the commercial sugar cane Khon Kaen3 (96.74%). A high proportion of sucrose content to reducing sugars is a desired characteristic of cane juice for efficient recovery in the sugar crystallization process during sugar production [10]. These results suggested that the juices of the new energy cane clones could be a promising source of sugar-rich juice used as feedstock in fuel ethanol production and sugar production.

FAN is a soluble nitrogenous compound required for yeast growth and fermentation [22]. The FAN values of both the energy cane clones were in the range 117.55–127.23 mg/L, while the control varieties contained FAN in the range 99.07–99.52 mg/L (Table 1). Similar FAN levels were detected in the juices of four energy cane clones in the range of 74.90–184.74 mg/L, which were considered adequate values for ethanol production using *S. cerevisiae* ND48 without any nutrient supplementation in the juice [12]. In contrast to the FAN value reported for sorghum juice, this alternative biofuel feedstock contained a much lower FAN concentration range (19–36 mg/L) [23]. The current results suggested the juices of the energy canes in the

current study could be used as raw material for ethanol production without exogenous nutrient supplementation, resulting in a favorable economic impact for industrial ethanol production.

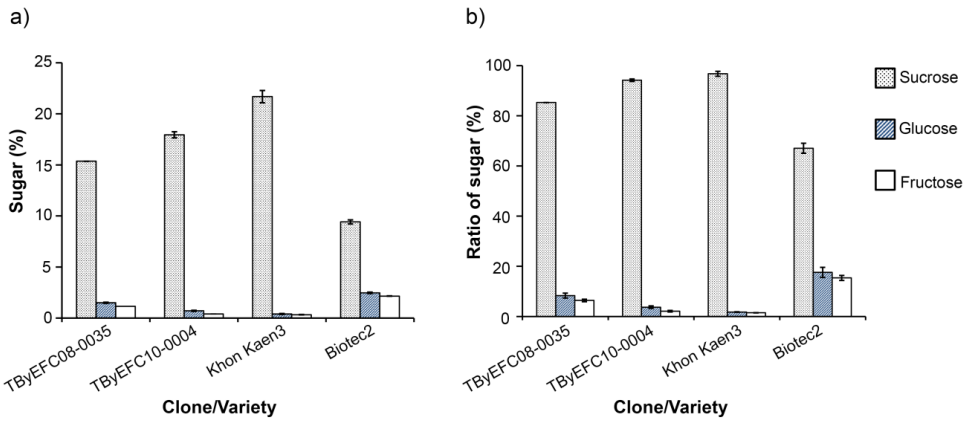

Figure 1. Sucrose, glucose and fructose contents (**a**) and percentage ratio of sugar (**b**) in juice from energy cane clones and control varieties.

Table 1. Chemical composition of juice from energy cane clones and control varieties.

Clone/Variety	TSS (°Brix)	Total Sugars (g/L)	FAN (mg/L)	Reference
TByEFC08-0035	18.0 ± 0.0 ^{c}	180.15 ± 2.00 ^{c}	127.23 ± 2.40 ^{a}	In this study
TByEFC10-0004	19.1 ± 0.3 ^{b}	190.63 ± 1.20 ^{b}	117.55 ± 1.22 ^{b}	In this study
Khon Kaen3 ^{A}	21.1 ± 0.1 ^{a}	224.00 ± 2.73 ^{a}	99.07 ± 0.38 ^{c}	In this study
Biotec2	15.0 ± 0.0 ^{d}	140.50 ± 0.27 ^{d}	99.52 ± 0.41 ^{c}	In this study
TByEFC04-1208	18.4 ± 0.6	179.72 ± 3.60	120.24 ± 3.29	[12]
TByEFC04-1155	16.3 ± 0.7	154.48 ± 0.83	74.90 ± 2.16	[12]
VG11-X1	16.1	NR	NR	[24]
Vx12-0015	16.3	NR	NR	[25]
Vertix 1	NR	85	NR	[26]
INTA05-3116	13.5	NR	NR	[8]
NCo310 ^{A}	15.6	NR	NR	[25]
LK92-11 ^{A}	19.0 ± 0.8	187.40 ± 2.03	136.92 ± 1.25	[12]

Different lowercase superscript letters in a column indicate significant differences between the clones ($p < 0.05$). Values represent mean ± standard deviation from three independent experiments. NR: Not reported. ^{A} Sugar cane.

The chemical compositions of the bagasse from the two clones of energy cane are shown in Table 2. The contents of cellulose, hemicellulose and lignin of the two energy cane clones were in the ranges 37.15–44.25%, 23.30–28.11% and 13.10–15.10%, respectively, which were similar to the ranges for other energy canes and sugar canes [2,8,12,27]. Thus, the lignocellulosic part of the energy canes could be considered suitable for biorefining applications. Compared to the energy cane TByEFC10-0004, the energy cane TByEFC08-0035 had higher contents of fiber (24.16%) and cellulose (44.25%), which were closer to the values of the control energy cane Biotec2 (31.30% and 48.05%, respectively). The high fiber and cellulose contents of the energy cane TByEFC08-0035 are favorable features for ethanol production from lignocellulosic biomass.

The analyses of the chemical compositions of the juice and bagasse revealed that the energy cane clones TByEFC08-0035 and TByEFC10-0004 had a high level of sucrose (15.36–17.95%), TSS (18.0–19.1 °Brix) and fiber (13.44–24.16%). Fanelli et al. [20] reported that the sucrose and fiber contents of the energy cane VIGNIS 3 were 12.4% and 13.9%, respectively, whereas VG1126 contained 9.0% sucrose content and 17.3% fiber. Kane et al. [8] reported that the TSS and fiber values of the energy canes INTA 05-3116 and INTA 05-3118 were in the ranges 13–13.5 °Brix and 23–25%, respectively. The Brix and fiber values of the sugar cane varieties LCP85-384 and NA 78-724 were in the ranges 17.0–18.5 °Brix and 11–14%, respectively. The energy cane L79-1001L contained sucrose (9.6%) and fiber

(26.7%) [2]. The differences in the chemical compositions of the juice and bagasse in energy cane are very important, since they suggest different fields of application. The current results suggested that both the energy cane clones would be excellent for biorefining and sugar production purposes. Regarding biorefining applications, TByEFC10-0004 would be a more suitable feedstock for ethanol production from juice, whereas TByEFC08-0035 would be more suitable for cellulosic ethanol production and electricity generation. In addition, the chemical compositions of the juice and bagasse samples in the current study showed an advantage for cultivar improvement to generate more suitable energy cane clones for specific purposes and industrial applications.

Table 2. Chemical composition and fiber content of raw bagasse from energy cane clones and control varieties.

Clone/Variety	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Fiber (%)
TByEFC08-0035	44.25 ± 0.90 ^{b}	23.30 ± 0.75 ^{c}	13.10 ± 0.20 ^{d}	24.16 ± 1.79 ^{b}
TByEFC10-0004	37.15 ± 0.49 ^{c}	28.11 ± 0.36 ^{a}	15.10 ± 0.47 ^{b}	13.44 ± 1.25 ^{c}
Khon Kaen3	37.78 ± 0.32 ^{c}	28.63 ± 0.72 ^{a}	13.85 ± 0.43 ^{c}	13.78 ± 1.17 ^{c}
Biotec2	48.05 ± 1.10 ^{a}	25.81 ± 0.70 ^{b}	16.09 ± 0.18 ^{a}	31.30 ± 0.40 ^{a}

Different lowercase superscript letters in a column indicate significant differences between the clones ($p < 0.05$). Values represent mean ± standard deviation from three independent experiments.

3.2. Agronomic Characteristics of Energy Cane

The two new energy cane clones, TByEFC08-0035 and TByEFC10-0004, had higher juice and sugar yields than the energy cane Biotec2, as shown in Table 3. TByEFC10-0004 had the highest juice yield (13,912.50 L/ha) and sugar yield (2.65 t/ha), which were approximately 2.50 and 3.35 times higher than for the energy cane Biotec2 and approximately 1.49 and 1.26 times higher than for the sugar cane Khon Kaen3, respectively. Although TByEFC10-0004 had lower levels of TSS and total sugars compared to the commercial sugar cane Khon Kaen3, this energy cane clone had a higher juice yield and cane yield per area. Thus, TByEFC10-0004 had a higher value of sugar yield per area. The combination of high juice yield and sugar yield suggested that the energy cane TByEFC10-0004 could be an efficient feedstock for ethanol production from juice. The chemical composition of the bagasse and the fiber content of the energy cane TByEFC10-0004 were similar to the sugar cane Khon Kaen3 (Table 2). Nevertheless, the energy cane TByEFC10-0004 had the ability to produce higher fiber and cane yields per cultivation area (Table 3), being an even more efficient source of lignocellulose per planted area than the commercial sugar cane variety.

Table 3. Agronomic characteristics of energy cane clones and control varieties.

Clone/Variety	Juice Yield (L/ha)	Sugar Yield (t/ha)	Fiber Yield (t/ha)	Cane Yield (t/ha)
TByEFC08-0035	6213.00 ± 115.20 ^{c}	1.12 ± 0.02 ^{c}	9.04 ± 0.03 ^{b}	34.51 ± 1.31 ^{a}
TByEFC10-0004	13,912.50 ± 298.00 ^{a}	2.65 ± 0.02 ^{a}	3.56 ± 0.12 ^{c}	18.34 ± 0.40 ^{c}
Khon Kaen3	9347.00 ± 102.55 ^{b}	2.10 ± 0.04 ^{b}	2.90 ± 0.27 ^{d}	14.95 ± 0.61 ^{d}
Biotec2	5585.00 ± 43.00 ^{d}	0.79 ± 0.01 ^{d}	11.56 ± 0.56 ^{a}	30.40 ± 1.21 ^{b}

Different lowercase superscript letters in a column indicate significant differences between the clones ($p < 0.05$). Values represent mean ± standard deviation from three independent experiments.

The energy cane TByEFC08-0035 had the highest yields of fiber (9.04 t/ha) and cane (34.51 t/ha) per cultivation area, which were close to the values for the energy cane Biotec2 (11.56 t/ha and 30.40 t/ha, respectively). The values of fiber and cane yield of TByEFC08-0035 were approximately 3.12 and 2.30 times higher than those for the commercial sugar cane Khon Kaen3. Another report showed that the fiber and cane yields from the energy canes TByEFC04-1155, TByEFC04-1208, TByEFC05-1558 and TByEFC09-0098 were in the range 5.82–16.20 t/ha and 28.82–85.88 t/ha, respectively [12]. The average fiber and cane yields of three sugar cane varieties (HoCP 96-540, L 99-233 and L 99-226) and one energy cane variety (L 79-1002) planted on three dates (August, September and October) were in the ranges 15.9–18.9 t/ha and 102.6–120.7 t/ha, respectively [28]. The energy canes

Top 6 RB had reported fiber yield of 31.2 t/ha [1] and the energy canes Ho 02-113 and HoCP72-114 had reported cane yields of 89 t/ha and 63 t/ha, respectively [21]. The fiber and cane yields of the energy cane Ho 00-961 were 16.3 t/ha and 92.9 t/ha, respectively [6]. The lower agronomic yields per unit area of the energy canes in the current study were due to experiments being conducted with the second-ratoon crop and under unusual weather conditions (heat and drought). Previous studies have reported that agronomic yields tend to drop for the second-ratoon crop compared to the planted and first-ratoon crops [28,29]. Other factors affecting the cane productivity and quality were cane variety, the interactions of the crop cycle and cane variety, soil type, climate, planting date and harvest time [20,29–32]. Thus, it is difficult to directly compare agronomic yields between studies. Nevertheless, the current results clearly indicated the successful generation of new potential energy cane clones compared to the control varieties. The energy cane TByEFC08-0035 had higher yields of juice, sugar and cane than the control energy cane, while TByEFC10-0004 had higher yields of juice and sugar than both the control varieties, and higher cane and fiber yields than the control sugar cane variety in the same plantation. Further experiments are required to evaluate the agronomic productivity of plant and ratoon harvests.

3.3. Ethanol Production of Energy Cane

3.3.1. Selection of Yeast Strain for Ethanol Production from Juice

Yeast isolates were initially screened for their sucrose fermentation abilities using the Durham tube method to select efficient yeast strains capable of ethanol production from energy cane juice containing sucrose as the major sugar source. Among 82 yeast isolates, 15 isolates could fill the Durham tube completely with CO_2 gas within 18 h. These ethanologenic yeasts with high sucrose fermentation ability were further screened for their fermentation abilities in sugar cane juice model medium. The results of shake-flask fermentation indicated that isolate SJT83 produced the highest ethanol concentration (111.52 g/L). Therefore, the yeast isolate SJT83 was selected to examine its fermentative capacity to produce a high amount of ethanol from energy cane juice compared to the control strain *S. cerevisiae* ND48. The kinetic parameters during the fermentation were determined and are shown in Figure 2. The yeast isolate SJT83 produced ethanol at a faster rate and higher yield, with more rapid growth than the control strain. The maximum ethanol production of 115.20 g/L was observed for SJT83 at 36 h and for the control strain of 107.35 g/L at 48 h, corresponding to productivity levels of 3.20 g/L/h and 2.24 g/L/h, respectively. These results revealed that the new isolate SJT83 was more effective at ethanol production using the sugar cane juice model medium compared to the control strain *S. cerevisiae* ND48.

Based on the nucleotide sequence and phylogenetic analyses of the D1/D2 domain of the 26S rDNA gene and the ITS gene, this isolated yeast strain was identified as *Kluyveromyces marxianus* (Figure 3). The D1/D2 domain and ITS sequences were deposited in the GenBank database under accession numbers OP781886 and OP781903, respectively. Recently, *Kluyveromyces marxianus*, a non-conventional yeast, has been considered as a feasible alternative to *S. cerevisiae* as an ethanol producer and *K. marxianus* has been reported to utilize various low-cost substrates, including sugar cane juice, for ethanol production [33,34]. A yeast strain capable of high ethanol production and productivity has the benefits of reducing the overall fermentation time and the energy input involved in distillation [34,35]. Thus, *K. marxianus* SJT83 had advantages regarding economical ethanol production.

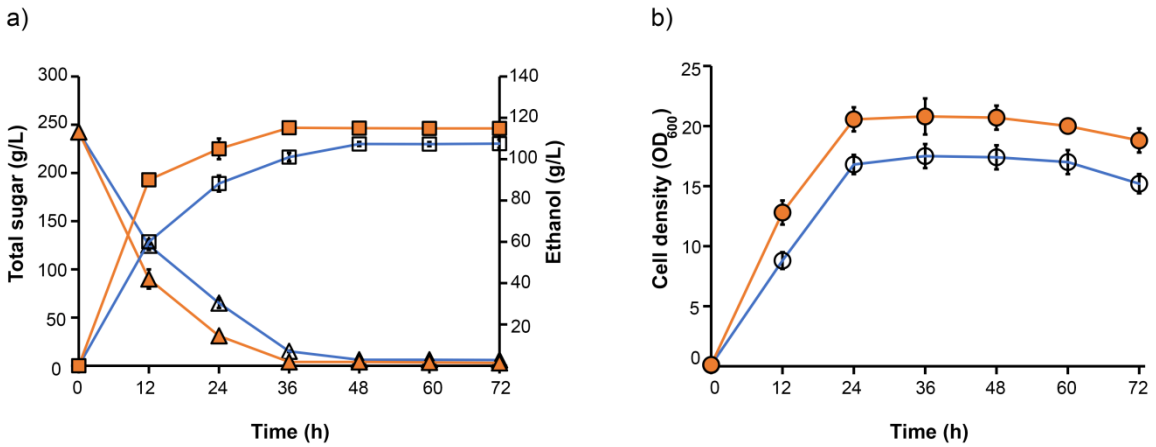

Figure 2. Time course of ethanol production and sugar assimilation (**a**) and cell growth (**b**) of yeast isolate SJT83 (filled symbol) and *S. cerevisiae* ND48 (open symbol) in sugar cane model medium, with total sugar (triangle), ethanol (square) and cell density (circle).

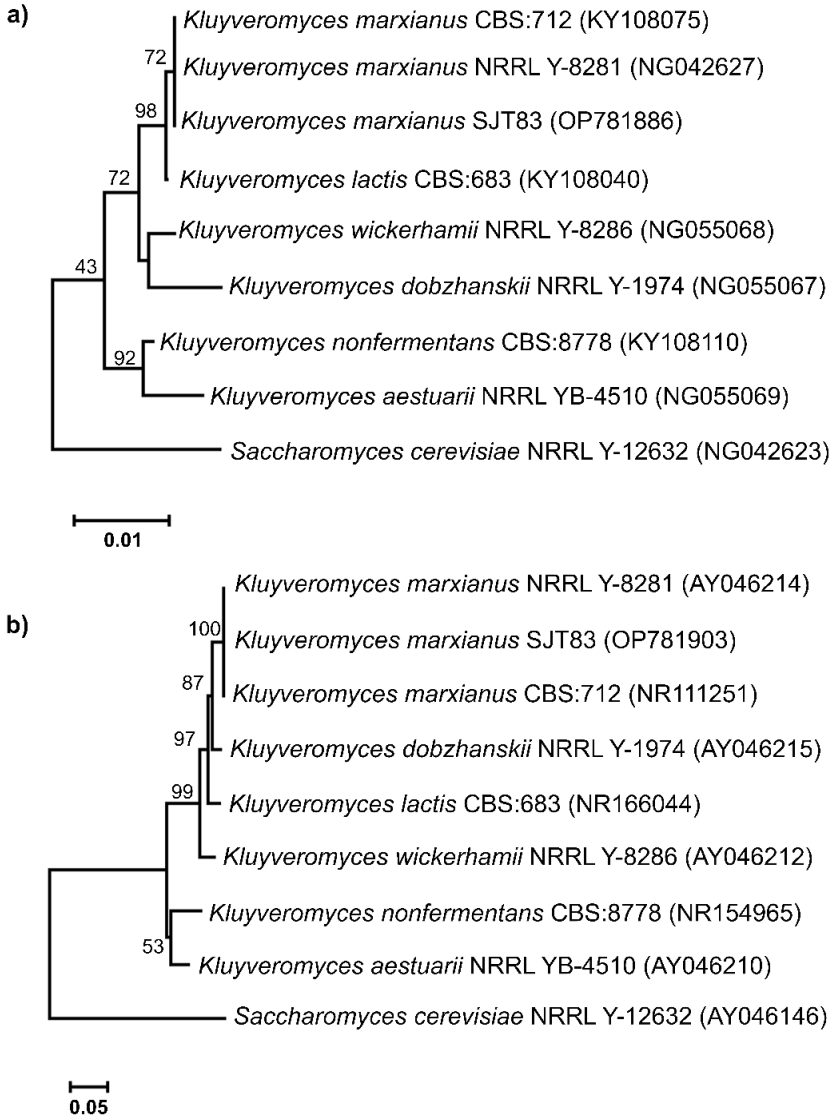

Figure 3. Phylogenetic tree resulting from analysis of D1/D2 domain of 26S rDNA gene (**a**) and ITS gene (**b**) of *K. marxianus* SJT83.

3.3.2. Ethanol Production of Energy Cane Juice and Bagasse

The current study considered ethanol production from energy cane using two different categories: first- and second-generation biofuels. Ethanol was produced from the energy cane juices using the new isolate *K. marxianus* SJT83, with *S. cerevisiae* ND48 being used as the control strain. There were significant differences in ethanol production by energy cane clones and yeast strains. The results correlated with the initial sugar concentration in each of the energy cane juice clones and the ethanol producing capacity of the yeast strain. The maximum ethanol concentration was recorded in the juice of TByEFC10-0004 fermented using *K. marxianus* SJT83 (87.10 g/L) followed by fermentation using *S. cerevisiae* ND48 (81.44 g/L). *K. marxianus* SJT83 achieved the maximum ethanol productivity of 3.63 g/L/h, corresponding to 94.69% of the theoretical yield, whereas those values from *S. cerevisiae* ND48 were 2.26 g/L/h and 88.54%, respectively (Table 4). These results suggested that the new isolate *K. marxianus* SJT83 could be superior to *S. cerevisiae* ND48 for application in ethanol production from energy cane juice or other sucrose-containing juices.

Table 4. Ethanol production by *K. marxianus* SJT83 and *S. cerevisiae* ND48 from juice of energy cane clones and control varieties.

Clone/Variety	Ethanol [A] (g/L)	Productivity (g/L/h)	Ethanol Yield [B] (g_p/g_s)	Theoretical Yield [C] (%)
K. marxianus SJT83				
TByEFC08-0035	81.05 ± 1.78 ^{c}	3.37 ± 0.07 ^{b}	0.46 ± 0.01 ^{a}	91.68 ± 2.00 ^{ab}
TByEFC10-0004	87.10 ± 1.27 ^{b}	3.63 ± 0.05 ^{a}	0.48 ± 0.00 ^{a}	94.69 ± 1.38 ^{a}
Khon Kaen3	99.15 ± 4.03 ^{a}	2.75 ± 0.11 ^{c}	0.48 ± 0.02 ^{a}	93.28 ± 3.79 ^{ab}
Biotec2	62.20 ± 1.41 ^{d}	2.60 ± 0.06 ^{c}	0.45 ± 0.01 ^{a}	88.85 ± 2.02 ^{b}
S. cerevisiae ND48				
TByEFC08-0035	76.50 ± 2.83 ^{b}	2.12 ± 0.08 ^{b}	0.45 ± 0.02 ^{a}	87.55 ± 3.24 ^{ab}
TByEFC10-0004	81.44 ± 2.20 ^{b}	2.26 ± 0.11 ^{b}	0.45 ± 0.02 ^{a}	88.54 ± 4.41 ^{ab}
Khon Kaen3	95.05 ± 4.17 ^{a}	2.64 ± 0.11 ^{a}	0.46 ± 0.02 ^{a}	90.74 ± 3.98 ^{a}
Biotec2	59.00 ± 2.55 ^{c}	2.46 ± 0.10 ^{a}	0.43 ± 0.02 ^{a}	82.28 ± 2.64 ^{b}

Different lowercase superscript letters in a column indicate significant differences between the clones fermented with each yeast strain ($p < 0.05$). Values represent mean ± standard deviation from three independent experiments. [A] The maximum ethanol concentrations produced by the yeast strain. [B] Ethanol yield (g_p/g_s) is calculated as the ethanol accumulation divided by the glucose consumed. [C] The theoretical yield of ethanol is 0.511 g_p/g_s from glucose; the theoretical yield is calculated as the ethanol yield multiplied by 100 and divided by 0.511.

Dilute acid hydrolysis is a fast, simple and efficient method to release sugar from lignocellulosic biomass [36]. In the current study, the obtained fermentable sugars in the hemicellulosic hydrolysate after detoxification process were xylose (11.25–14.08 g/L), glucose (5.98–9.52 g/L) and arabinose (5.16–8.95 g/L). During acid hydrolysis, the low release of glucose primarily contributed to the recalcitrant cellulose structure compared to the hemicellulose structure [37,38]. The bagasse hydrolysate also contained acetic acid (5.20–5.48 g/L), HMF (1.09–1.12 g/L) and furfural (0.16–0.19 g/L). The maximum ethanol concentrations of TByEFC08-0035 and TByEFC10-0004 fermented using *Sch. shehatae* TTC79 were in the range 9.25–9.81 g/L (Table 5). The fermentable sugar concentration is positively correlated with ethanol production. Several factors affect the sugar types and concentrations released from the lignocellulosic biomass, including biomass type and hydrolysis method and conditions [36,39]. Investigation of other hydrolysis methods and optimization of the hydrolysis conditions are required to further improve the conversion of cellulose and hemicellulose into fermentable sugars for enhancing ethanol yield.

Table 5. Ethanol production by *Sch. shehatae* TTC79 from bagasse of energy cane clones and control varieties.

Clone/Variety	Ethanol [A] (g/L)	Productivity (g/L/h)	Ethanol yield [B] (g_p/g_s)	Theoretical Yield [C] (%)
TByEFC08-0035	9.81 ± 0.27 [a]	0.27 ± 0.00 [a]	0.46 ± 0.01 [a]	90.98 ± 2.49 [a]
TByEFC10-0004	9.25 ± 0.64 [ab]	0.26 ± 0.02 [a]	0.44 ± 0.03 [a]	86.12 ± 5.92 [a]
Khon Kaen3	9.85 ± 0.92 [a]	0.27 ± 0.02 [a]	0.45 ± 0.04 [a]	87.62 ± 8.18 [a]
Biotec2	8.10 ± 0.50 [b]	0.23 ± 0.01 [a]	0.42 ± 0.03 [a]	82.99 ± 5.07 [a]

Different lowercase superscript letters in a column indicate significant differences between the clones ($p < 0.05$). Values represent mean ± standard deviation from three independent experiments. [A] The maximum ethanol concentrations produced by the yeast strain. [B] Ethanol yield (g_p/g_s) is calculated as the ethanol accumulation divided by the glucose and xylose consumed. [C] The theoretical yield of ethanol is 0.511 g_p/g_s from glucose and 0.511 g_p/g_s from xylose; the theoretical yield is calculated as the ethanol yield multiplied by 100 and divided by 0.511.

Due to the value of sugar, the juice, fiber and cane yields per cultivation area are directly associated with ethanol yield; thus, a combination of ethanol production on a laboratory scale with agronomic productivity is important for economic production. The current results revealed that the highest ethanol production from juice was reached with TByEFC10-0004 (1211.76 kg/ha), which was 3.49 and 1.31 times higher than the control energy cane Biotec2 and sugar cane Khon Kaen3, respectively (Table 6). The highest ethanol yield from bagasse was produced by TByEFC08-0035 (790.68 kg/ha), which was 1.91 and 2.97 times higher than the control energy cane Biotec2 and sugar cane Khon Kaen3, respectively. The total ethanol yields from the juice and bagasse for both energy cane clones were in the range 1294.23–1469.14 kg/ha, whereas the control energy cane Biotec2 and sugar cane Khon Kaen3 yielded approximately 761.05 kg/ha and 1192.72 kg/ha, respectively. The highest total ethanol yield (1469.14 kg/ha) was obtained from the energy cane TByEFC10-0004, which was 1.93 and 1.23 times higher than those of the control energy cane Biotec2 and sugar cane Khon Kaen3, respectively. Thammasittirong et al. [12] reported that the highest overall ethanol yields from juice and bagasse of the planted energy canes TByEFC04-1155, TByEFC04-1208, TByEFC05-1558 and TByEFC09-0098 were in the range 1179.84–3923.48 kg/ha, while the ethanol yields obtained from the control commercial sugar cane LK92-11 variety was 3570.15 kg/ha. Among the energy cane clones, TByEFC04-1155 showed the highest ethanol yield from juice (2697.00 kg/ha), which was slightly lower than for the commercial sugar cane LK92-11 (3087.94 kg/ha). Unfortunately, no other similar data are available in the published literature for ethanol production from the juice and bagasse of energy cane on a laboratory scale in combination with their agronomic productivity. The low ethanol yield in the current study was related to the lower agronomic yield of the second-ratoon crop and the unusual weather conditions, as mentioned above. Nevertheless, the current study highlighted the successful improvement of energy cane containing high sucrose and fiber contents. The energy canes TByEFC08-0035 and TByEFC10-0004 provided significantly more total ethanol production from both juice and bagasse per cultivation area compared to the control energy cane and sugar cane varieties. Further investigation is required of the agronomic productivity and ethanol production of plant crops and ratoon crops.

Table 6. Ethanol production on laboratory scale and agronomic scale from juice and bagasse of energy cane clones and control varieties.

Clone	Ethanol Production on Laboratory Scale			Ethanol Production per Cultivation Area		
	Juice (g/L)	Bagasse (g/L)	Total (g/L)	Juice (kg/ha)	Bagasse (kg/ha)	Total (kg/ha)
TByEFC08-0035	81.05 ± 1.41 [c]	9.81 ± 0.43 [a]	90.85 ± 0.98 [c]	503.56 ± 10.54 [c]	790.68 ± 7.81 [a]	1294.23 ± 18.34 [b]
TByEFC10-0004	87.10 ± 2.83 [b]	9.25 ± 1.06 [ab]	96.35 ± 3.89 [b]	1211.76 ± 33.74 [a]	257.38 ± 5.06 [d]	1469.14 ± 28.68 [a]
Khon Kaen3	99.15 ± 3.26 [a]	9.85 ± 0.92 [a]	109.00 ± 4.17 [a]	926.80 ± 6.02 [b]	265.98 ± 1.12 [c]	1192.72 ± 7.14 [c]
Biotec2	62.20 ± 2.83 [d]	8.10 ± 0.71 [b]	70.30 ± 3.54 [d]	347.41 ± 3.41 [d]	413.64 ± 6.56 [b]	761.05 ± 3.51 [d]

Different lowercase superscript letters in a column indicate significant differences between the clones ($p < 0.05$). Values represent mean ± standard deviation from three independent experiments.

4. Conclusions

Early-generation energy cane varieties tend to have high fiber and biomass productivity but are unable to match the sucrose content of commercial sugar cane. The current investigation succeeded in improving two energy cane clones with high sucrose content, especially the energy cane TByEFC10-0004. The agronomic productivity per cultivation area results demonstrated that the energy cane TByEFC10-0004 was superior in sugar and juice yields, while the energy cane TByEFC08-0035 was superior in fiber and cane yields. The total ethanol yields from juice and bagasse per cultivation area of the energy canes TByEFC10-0004 and TByEFC08-0035 were higher than those for the control energy cane Biotec2 and sugar cane Khon Khaen3. The two newly developed energy cane clones could be considered as effective green alternative sources of both sugar and lignocelluloses for sustainable and economic ethanol production.

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