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Separate Hydrolysis and Fermentation of Sugarcane Tops for Bioethanol Production using Yeasts from Thai Liquor Producer and Commercial Sources

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ABSTRACT

Separate hydrolysis and fermentation (SHF) of sugarcane tops (SCT) for bioethanol production appears viable and economically feasible, especially in view of ongoing global energy demands. SCTs were pretreated with different hydrolytic substrates such as base (NaOH), acid (H_2SO_4), and the α-amylase enzyme before being subjected to alcoholic fermentation using distinct yeasts from both commercial and northern Thai liquor (local) sources. The properties of SCT such as biomass composition and physicochemical attributes were investigated. The levels of reducing sugar and bioethanol yields were analyzed during the pretreatment and fermentation stages, respectively. The experimental results showed that hydrolysis with H_2SO_4 and the α-amylase enzyme yielded a greater amount of reducing sugars compared to NaOH, with reducing sugar content ranging from 28.56-40.10 mg/mL. On the fourteenth day of fermentation, diverse yeasts led to the highest bioethanol yield in the case of commercial yeast (4.29-4.92%) while fermentation with a local yeast resulted in a comparatively lower yield. Notably, the SHF processes involving H_2SO_4 and α-amylase substrates along with fermentation using commercial yeast, exhibited the most promising potential for converting SCT into bioethanol. This study presents preliminary findings on the separate hydrolysis and fermentation processes of sugarcane tops for bioethanol production. By the way, expanding and increasing the scale in the future presents a feasible opportunity.

1. INTRODUCTION

The increasing use of non-renewable fuels coupled with their continuous depletion has become a major concern in recent years. Biofuels derived from renewable sources through biorefineries have recently been adopted to address this challenge [1]-[3]. The term bioethanol is used to define the amount of ethanol (ethyl alcohol; C_2H_5OH) that can be used as a pure fuel or blended with gasoline and other fuels. It is produced from the fermentation of sugar originating from a variety of sources [4]. The potential of producing bioethanol from agro-residues presents an ecologically friendly avenue, encompassing four stages: pretreatment, hydrolysis, fermentation, and distillation. Lignocellulosic biomass, which includes agro-residues, grasses, sawdust, wood chips, wheat straw, sugarcane bagasse, cotton stalk, and soft bamboo, among others, is suitable for use as feedstock and is economically feasible for bioethanol production [5]-[7]. Sugarcane tops (SCT) or trash from sugarcane (*Saccharum officinarum*) are the most interesting agro-residues owing to their large annual production during a harvest season. It contains about 40-45% cellulose, 25-30% hemicellulose, and 15-19% lignin, offering the potential for several value-added products such as oligosaccharides, oil-based petrochemical, bioethanol, xylan, lignin, furfural, *etc.* [2].

Bioethanol production from SCT necessitates pretreatment, such as enzymatic, acid, or base hydrolysis. For instance, pretreating sugarcane leaves through a combination of acidic and enzymatic hydrolysis involving dilute sulfuric acid and cellulase enzyme has been explored. The use of amylase enzyme in the process of breaking down polysaccharides in SCT is also crucial. The primary polysaccharides present in raw cane sugar are dextran and starch. The growing point and the leaves at the tops exhibit higher concentrations, but mature cane shows a lower starch content. SCT starch is composed of two components,

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namely amylose and amylopectin, both of which are glucose polymers [8]. The use of *Saccharomyces cerevisiae* (*S. cerevisiae*) in the fermenting batch resulted in a peak bioethanol yield of 4.71 g/L or 8.00% (w/w) after 24 h [9]. Proper pretreatment may increase the concentration of fermentable sugar after hydrolysis, thereby improving the efficiency of the whole process. Other efficient microbes and genetically modified microbes may also amplify their capabilities [6]. In the past, a great deal of effort has been focused on genetically engineered microbes such as *Escherichia coli*, *Zymomonas mobilis*, and *Schefferosomyces stipitis* to efficiently ferment sugars like xylose into bioethanol [10]. Non-conventional yeast strains such as *Schefferosomyces (Pichia) stipites* have been successfully employed for bioethanol production from brown seaweed [11]. Some microbial communities like *S. cerevisiae*, *Penicillium chrysogenum* VS4, *Kluyveromyces marxianus* TISTR5177 necessitate a three-step conversion process involving i) production of cellulolytic and xylanolytic enzyme, ii) enzymatic saccharification of delignified biomass, and iii) fermentation of monosaccharides to bioethanol [2], [12]-[14]. A multi-functional microbe like *K. Marxianus* (a thermo-tolerant yeast) is capable of co-fermenting both C5 and C6 sugars and can endure temperatures of 42-45°C. Hybrid strains, genetically engineered, or co-culture of two strains have been developed for demanding fermentation tasks [15]. The potential for multi-bioactivity and their functions can be traced as fingerprints in by-products, which were identified in vinasse [16].

Separate hydrolysis and fermentation (SHF) have been defined as the process of hydrolysis carried out separately from the fermentation step. This approach presents a means to enhance efficiency for the pretreatment of lignocellulosic materials [17]. SHF encompasses two discrete units: first, degradation of lignocellulosic material into reducing sugars, followed by fermentation into bioethanol. Furthermore, conventional processing involves the mixture of lignocellulosic and non-lignocellulosic substances, including lignocellulose, polysaccharides, starch, carbohydrates, and sugar. The material exhibits the capacity to produce dual enzymatic hydrolysis processes such as a simultaneous pretreatment, which can be utilized to degrade both lignocellulose and polysaccharides into reducing sugars [18]. The enzymatic hydrolysis can be carried out at 45-50°C, and fermentation at a slightly lower temperature of 30-37°C. Alternatively, robust hydrolysis involving acids and bases demands higher temperatures (90-110°C) to break the intermolecular ester bonds between lignin and hemicellulose polymers, causing delignification/ depolymerization and improving the digestibility of polysaccharides in subsequent stages [13]. The option to adopt yeast strains for bioethanol production, particularly local yeast from northern Thai liquor producers is now open. A fermenting starter referred to as "Look-pang" derived from starch and herbal components is utilized. This mixture involves amylase-produced fungi of the *Rhizopus* and *Amylomycetes genera, along with* alcohol-produced yeasts such as *Saccharomyces* and *Endomycopsis* [19]. In the Look-pang environment, wild strains of *S. cerevisiae* and other alcohol-produced yeasts can flourish. These wild strains of *S. cerevisiae* thrive in alkali conditions and are capable of overwintering while sporulating [4].

The potential and intriguing properties of SHF application, involving various hydrolysis substrates and fermentation with both commercial and wild yeast strains for SCT, warrant thorough investigation. Bioethanol production and efficiency were observed under different conditions. Descriptive statistical analysis was performed on the observed data using an open-source program.

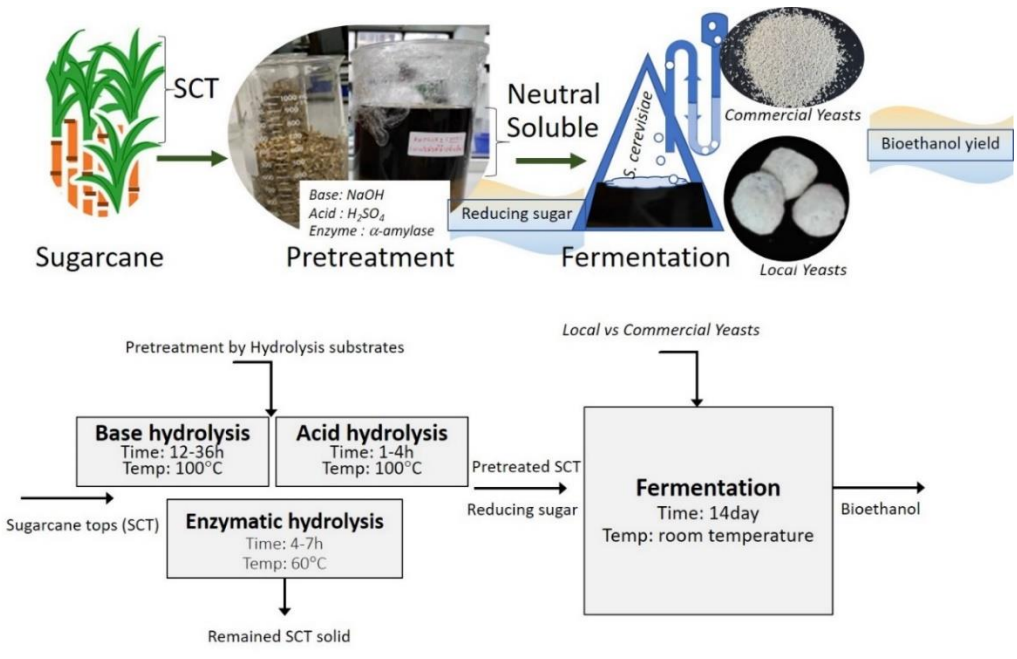

Fig. 1. The experimental design of this work.

2. METHODOLOGY

2.1 Sugarcane Tops

SCTs were sourced from the field at coordinates 16.730288 and 100.193214, Thapho sub-district, Mueang, Phitsanulok, Thailand during the crop year 2021 harvest season. The sugarcane (variety K84-200) was mechanically harvested while still green, and chopped into uniform lengths of 2-5 cm. After that, the whole biomass and powder-like starch of SCT were dried at 60°C for 72 hours, put in plastic bags, and kept at 4°C. The biomass compositions, namely cellulose, hemicellulose, and lignin contents were determined using methods commonly applied in pulp and paper testing [9].

2.2 Characterization

The physical and chemical characteristics of the SCTs were assessed via proximate and ultimate analyses. The moisture content was measured by calculating the different masses before and after drying samples in an oven at 105°C overnight. Volatile matter (VM) was measured after heating the SCT sample in a covered crucible within a muffle furnace at 950°C for 7 minutes following the ASTM D3175 standards. After the VM determination, the sample in an uncovered crucible was heated at 750°C for 4 hours to determine ash content (on a dry basis). Fixed carbon (FC) was calculated by subtracting the preceding parameters from a percentage as per the formula:

$$FC\ (\%) = 100 - [\text{moisture}\ (\%) + VM\ (\%) + \text{ash}\ (\%)] \quad (1)$$

The ultimate analysis was performed in duplicates using a TrueSpec Micro elemental analyzer (Leco CHNS628, USA). The carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) contents of samples were determined and the oxygen (O) content was calculated based on the dry ash-free basis using the equation:

$$O\ (\%) = 100 - [C\ (\%) + H\ (\%) + N\ (\%) + \text{ash}\ (\%)] \quad (2)$$

In addition, the high heating value (HHV) content was calculated based on the ultimate analysis outcomes described by Nhuchhen and Afzal (2017) using the formula [20]:

$$HHV = 32.7934 + 0.0053C2 - 0.5321C - 2.8769H + 0.0608CH - 0.2401N \quad (3)$$

2.3 SHF via Different Hydrolysis Substrates

SCT was subjected to hydrolysis utilizing several substrates, namely sodium hydroxide (NaOH, Gammago, Thailand), sulfuric acid (H_2SO_4, Gammago, Thailand), and the α-amylase enzyme (Reace biotechnology, Thailand), respectively. All the chemicals were of commercial quality. For NaOH hydrolysis, 50 g of SCT was mixed with 2.5 M NaOH, 500 mL with an SCT: NaOH ratio of 1:10 (g/mL), followed by reflux extraction at 100°C for durations of 12, 18, 24, 30, and 36 h. For H_2SO_4 hydrolysis, 500 mL of 5% H_2SO_4 was mixed with 50 g of SCT, followed by reflux extraction at 100°C for durations of 1, 2, 3, and 4 h. The α-amylase enzymatic hydrolysis was conducted by mixing 20 g of SCT with 10 mL of deionized water and adjusting the pH value to 6. Then, 0.2% (by weight of SCT) of α-amylase enzyme was added to the mixture, followed by the saccharification step and incubation at 60°C, for 4, 5, 6, and 7 h. The reactor was kept at room temperature prior to analyses of the reducing sugar content. Acidic and alkaline solutions were used to neutralize hydrolyzed SCT samples to pH ~5. By-products were removed using nylon filter and the samples were kept at room temperature before they were fermented. The quantity of reducing sugar was determined using a UV-visible spectrometer (Metash, UV-5100, China).

2.4 Fermentation with Different Yeasts

Bioethanol production from hydrolyzed SCT was investigated using two yeast types such as a strain of *S. cerevisiae* from baker's yeast (Saf-instant®, France) and a wild strain derived from the Look-pang (Surasakthong community enterprise, Phrae, Thailand), a local Thai liquor producer. Three processes of SHF pretreatment gave the optimized condition of glucose content in the range of 32-40 mg/mL. The yeasts were used in bioethanol fermentation at a constant concentration of 10% (by weight of the hydrolyzed SCT). The batch ethanol fermentation process was carried out at ambient temperature for 14 days to avoid complete inhibition of the bioreactor and ensure continued fermentation of ethanol [21]. Aliquots were sampled for analysis of reducing sugars and alcohol content using an ebulliometer.

3. RESULTS AND DISCUSSION

3.1 Physical and Chemical Properties of SCT

The results of biomass composition are presented in Table 1. SCT samples had different values of cellulose and hemicellulose contents. Specifically, the leaf sample demonstrated cellulose and hemicellulose levels approximately 1.83 and 1.53 times higher than the top sample, respectively. Both sugarcane leaves and tops exhibited lignin content ranging from, 12.22-19.60% with average numbers of 17.75±1.78% and 15.19±2.82%, respectively. In principle, cellulose and hemicellulose constituted about 15-30% and 15-35% of the dry weight of the total primary plant cell wall, respectively. Cellulose is strong, crystalline, and hydrolysis-resistant. In contrast, hemicellulose has an amorphous and random structure, possessing lower strength. It can be hydrolyzed using dilute acid, base, or hemicellulose enzymes. Lignin is a complex organic polymer that is generally found in barks and wood, with content varying between woody (27-32%) and herbaceous (14-25%) plants [12], [19].

The physical and chemical properties of the SCT sample compared with bagasse biomass are shown in Table 2. The SCT sample exhibited higher moisture content and FC than the bagasse sample. Previously, a bagasse sample was found to have low moisture content and FC [20]. Ash content and VM of the SCT sample

were 5.42% and 60.87%, respectively. Chemical attributes of the SCT sample related to its comparatively lower calorific value with C and H elements contributing to a heat of combustion of 17.72 MJ/kg (HHV) compared to bagasse's 18.52 MJ/kg (HHV). C and H elements, pivotal energetic components of lignocellulosic biomass significantly influenced sugar conversion capability during hydrolysis [22]. In addition, the SCT sample featured a low S content of 0.15% dry weight, which benefits utilization by reducing concerns about sulfur residues. Conversion of S by yeast to sulfurous off-flavors such as hydrogen sulfide (H_2S) during fermentation can inhibit substrates, leading to delayed fermentation [23], [24].

Table 1. Compositions in sugarcane leaves and SCT.

Item	Sugarcane leaves*	SCT
Cellulose (% dry basis)	37.14±1.63	20.23±2.86
Hemicellulose (% dry basis)	23.07±2.08	15.11±1.77
Lignin (% dry basis)	17.75±1.78	15.19±2.82

**Composition analysis of the sugarcane leaves [9].*

Table 2. Properties of sugarcane leaves and SCT.

Item	Bagasse*	SCT
Physical properties (%)		
Moisture	7.85	11.82
Ash	7.75	5.42
VM	70.42	60.87
FC	13.98	21.89
Chemical properties (%)		
C	44.90	41.28
H	5.90	5.19
N	0.84	0.98
O	40.75	52.40
S	0.31	0.15
HHV (MJ/kg)	18.52	17.72

**The data was derived from sugarcane bagasse [22].*

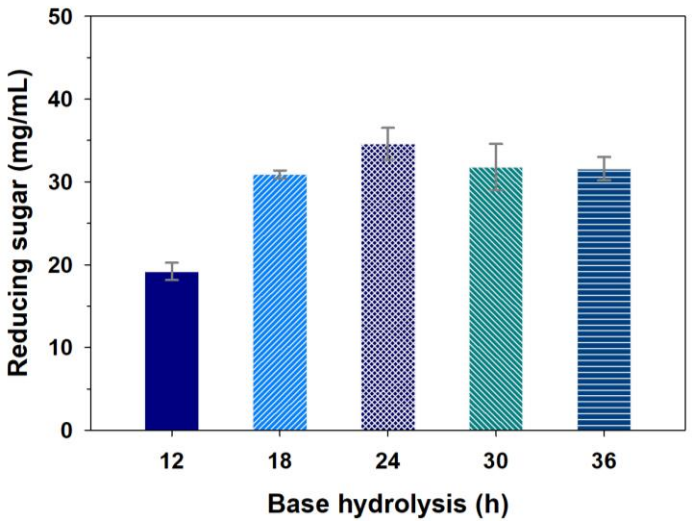

Fig. 2. Reducing sugar content from NaOH hydrolysis.

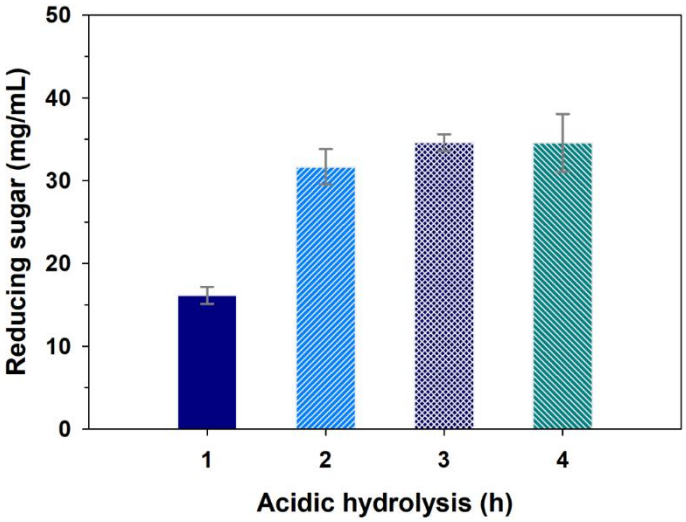

Fig. 3. Reducing sugar content from H_2SO_4 hydrolysis.

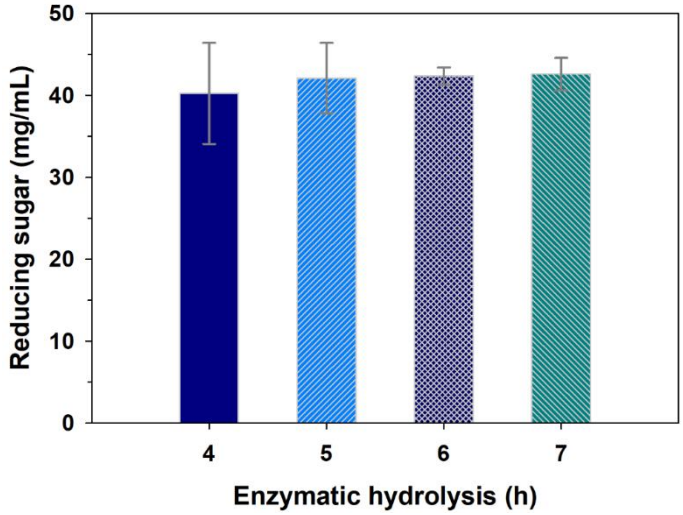

Fig. 4. Reducing sugar content from enzymatic hydrolysis.

3.2 Pretreatment of SCT via Hydrolysates

The hydrolysis method influenced the final bioethanol yield and fermentation efficiency of lignocellulosic biomass. The hydrolysis process released common chemicals from the lignocellulosic biomass such as glucose, xylose, pentose, and hexose, alongside inhibitors like furfural, hydroxymethyfurfural, 4-hydroxybenzaldehyde, vanillin, and syringaldehyde (called fermentable sugar or reducing sugar), which are important for yeast fermentation [9], [15]. The reflux extraction and filtration techniques could be employed to mitigate the inhibition of these compounds. The results of SCT hydrolysis pretreated using base, acid, and enzyme are shown in Figures 2 to 4. NaOH hydrolysis at 100°C for 36 h showed an increase in reducing sugar content at 18 h, reaching a maximum at 24 h, followed by a slight decrease at 30 h (Figure 2). A shorter incubation time was found in the acidic hydrolysis of SCT with H_2SO_4. That is the complete hydrolysis lasted just 4 hours. The strong acid (H_2SO_4) plays a role in the conversion of lignocellulosic biomass into reducing sugar. The maximum amount of reducing sugar from H_2SO_4 was observed at the third hour of incubation as shown in Figure 3. The use of α-amylase enzyme in enzymatic hydrolysis resulted in positive trends, especially after 4 hours of incubation, as shown in Figure 4. The inclusion of powdered starch in the raw SCT had a beneficial effect on the efficiency of the hydrolysis process particularly in terms of simultaneous saccharification as shown by the results of the reducing sugars. Concentrations of reducing sugar released in all tests ranged from 34.60-42.58 mg/mL and providing sufficient content for bioethanol fermentation in future studies. Bouaziz *et al.*, (2020) reported enzymatic hydrolysis (*Penicilium ocitanis* Pol6 strain) of a harder hydrolyzed lignocellulosic biomass such as date seeds. The optimal time for achieving reducing sugar yield was after 12 h following incubation and the amount of sugar released was 56.10 mg/mL with a 37.40% hydrolysis efficiency [25].

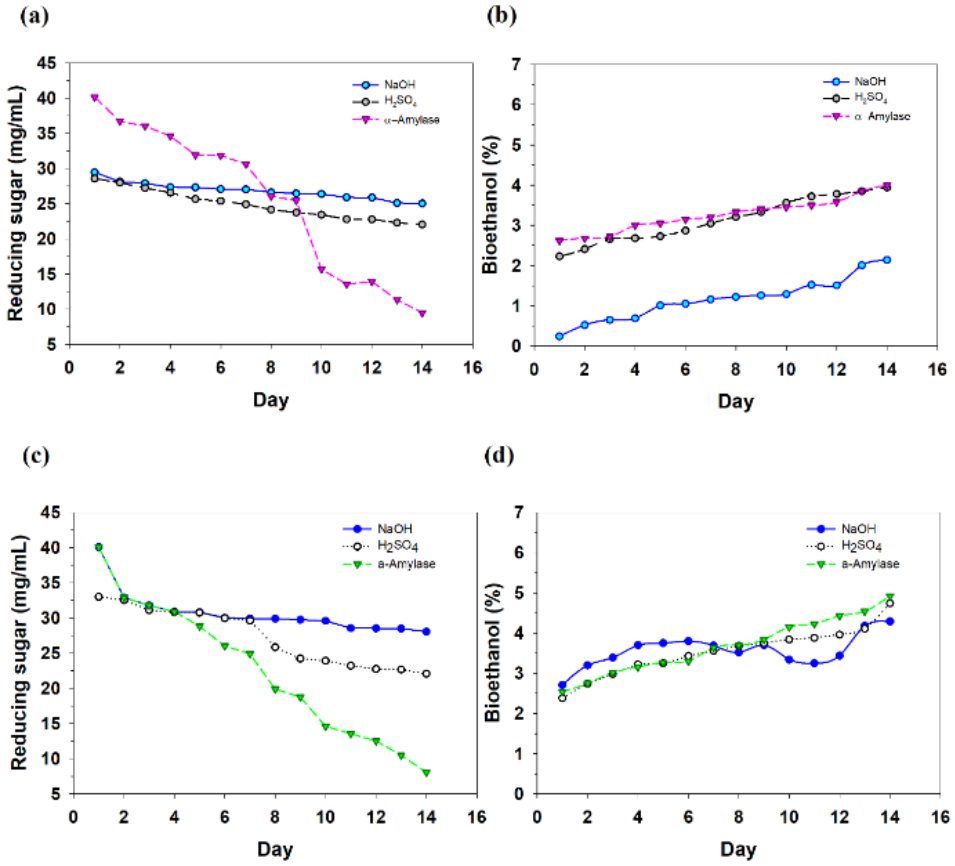

Fig. 5. Reducing sugar remained in reactors and bioethanol yields (a, b) fermented by local yeast and (c, d) fermented by commercial yeast.

Table 3. Bioethanol yields produced from SHF of SCT.

Item	NaOH	H_2SO_4	Enzyme
Commercial yeast			
Initial pH	5.88	4.28	4.40
Final pH	4.58	5.17	4.60
Sugar consumption (%)	29.97	33.14	79.89
Bioethanol yield (%)	4.29	4.74	4.92
Local yeast			
Initial pH	4.65	4.29	4.88
Final pH	5.21	5.31	5.35
Sugar consumption (%)	15.01	22.90	76.38
Bioethanol yield (%)	2.14	3.94	4.00

3.3 SHF of SCT with Different Yeasts

Bioethanol can be produced from every part of sugarcane or (cane) polysaccharides such as glucans (cellulose and β-glucans), hemicelluloses (xyloglucans and heteroxylans), and pectins after passing through the SHF process [25]. In this study, SCT hydrolysates were subjected to different strains of *S. cerevisiae* from local and commercial yeasts, which were the dominant reducing sugar fermenters. The analysis, as shown in Table 3, compared the efficiency of lignocellulose conversion across different strains of *S. cerevisiae* through SHF processes. The finding in the tests suggested that the enzymatic method proved superior to the acidic and alkaline techniques (i.e., enzymatic > acidic > base), with local and commercial yeasts achieving conversion efficiency with the values of sugar consumption rate of 76.38% and 79.89%, 22.90% and 33.14%, and 15.01% and 29.97%, respectively. It is important to emphasize that enzymes and acidic breakdown worked best when the pH level was slightly acidic, between 4.28 and 4.40, for the fermentation of hydrolyted SCT. In all cases, the bioethanol yield from SCT substrates using commercial yeast ranged from 4.29 to 4.92%, while the local yeast yielded between 2.14 and 4.00%. As seen in Figures 5a and 5b, local

yeast from northern Thai liquor sources exhibited promising bioethanol production through enzymatic and acidic hydrolysis but produced significantly lower bioethanol yields through base hydrolysis. The results of bioethanol production by a commercial yeast are illustrated in Figures 5c and 5d. The optimal condition was successfully achieved through enzymatic α-amylase hydrolysis, yielding a bioethanol content of 4.92% within a bioreactor over a 14-day fermentation period. A similar trend was found in the acidic hydrolysis of H_2SO_4. Moreover, NaOH hydrolysis demonstrated a favorable trend during the initial 1 to 8 days of fermentation. The experimental outcomes indicated that the commercial yeast outperformed the local yeast in terms of bioethanol yield. The local yeast could potentially consist of a mixture of various species, which might not be well-suited for the demanding conditions of lignocellulosic fermentation. The complexity of fermenting sugarcane tops, which contain substantial quantities of cellulose, hemicellulose, lignin, and non-lignocellulose poses a challenge. However, some monosaccharides (mostly pentose sugars) are more difficult to ferment to bioethanol than hexose sugars (glucose). This underscores the significance of characterizing the sugar composition within a given plant biomass, a critical factor for optimizing its suitability for cellulosic bioethanol production [26]. An early analysis provides valuable information on the characteristics, production qualities, and the different potential of yeast sources with regards to further investigation.

4. CONCLUSION

Three hydrolysis substrates such as base, acid, and enzyme were employed for the pretreatment of SCT under different SHF conditions. The pretreated substrates were fermented using two yeast strains from local and commercial sources. The experimental results showed that the amount of reducing sugar content depended on the hydrolysis substrates utilized. Among the varied conditions tested, enzymatic hydrolysis with the α-amylase enzyme emerged as the most efficient method for breaking down carbohydrates (polysaccharides) from starch of SCT biomass into fermentable sugars. Enzymatic and acidic hydrolysis were suitable for SHF with a local yeast, whereas commercial yeast proved adaptable to all SHF conditions. A known genotype yeast strain from a commercial source, specifically *S. cerevisiae*, proved suitable for SCT bioethanol production. By selecting the correct hydrolysis substrate, local yeast from a local liquor producer can be used instead of commercial yeast. This implementation provided knowledge and data for transforming waste from sugarcane industry into products with enhanced value. This method not only improves cost effectiveness and enables manufacturing at the local scale, but also establishes a basis for future expansion procedures.

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NOMENCLATURE

C	carbon	%
C_2H_5OH	chemical formula of ethyl alcohol	
FC	fixed carbon	%
H	hydrogen	%
HHV	high heating value	MJ/kg
N	nitrogen	%
O	oxygen	%
S	sulfur	%
SCT	sugarcane tops	
SHF	separate hydrolysis and fermentation	
VM	volatile matter	%

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