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Research Article

Lignocellulosic Bioethanol Production of Napier Grass Using *Trichoderma reesei* and *Saccharomyces cerevisiae* Co-Culture Fermentation

Thirawat Mueansichai[a], Thaneeya Rangseesuriyachai[b,*], Nuttha Thongchul[c,d], Suttichai Assabumrungrat[e]

[a] *Department of Chemical and Materials Engineering, Faculty of Engineering, Rajamangala University of Technology Thanyaburi, Pathumthani 12110, Thailand*

[b] *Department of Civil Engineering, Faculty of Engineering, Rajamangala University of Technology Thanyaburi, Pathumthani 12110, Thailand*

[c] *Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330, Thailand*

[d] *Research Unit in Bioconversion/Bioseparation for Value-Added Chemical Production, Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330, Thailand*

[e] *Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok 10330, Thailand*

Abstract. Bioethanol from agricultural waste is an attractive way to turn waste into added value that will solve the problem of food competition and waste management. Napier grass is a highly productive and effective lignocellulosic biomass, which is an important substrate of the second-generation biofuels. In addition, several processes are required in the production of ethanol from lignocellulosic materials; thus, co-culture fermentation can shorten the production process. This experimental research utilizes *Trichoderma reesei* and *Saccharomyces cerevisiae* co-culture fermentation in the bioethanol production of Napier grass using simultaneous saccharification and fermentation technology. To improve ethanol yield, Napier grass was pretreated with 3% (w/w) sodium hydroxide. An orthogonal experimental design was employed to optimize the Napier grass content, mixed crude co-culture loading, and incubation time for maximum bioethanol production. The results showed that pretreatment increased cellulose contents from 52.85% to 82%. The optimal fermentation condition was 15 g Napier grass, 15 g mixed crude co-culture, and 7 days incubation time, which maximizes the bioethanol yield of 16.90 g/L. Furthermore, the fermentation was upscaled 20-fold, and experiments were performed with and without supplemented sugar using laboratory-scale optimal fermentation conditions. The novelty of this research lies in the use of a mixed crude co-culture of *T. reesei* and *S. cerevisiae* to produce bioethanol from Napier grass with the maximum bioethanol concentration of 25.02 and 33.24 g/L under unadded and added sugar conditions and to reduce operational step and capital costs.

Keywords: Biothanol; Napier grass; *Trichoderma reesei; Saccharomyces cerevisiae*

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1. Introduction

The Sustainable Development Goals (SDGs) are the world's shared plan to achieve a better and more sustainable future quality of life. Affordable and clean energy is one of the 17 goals for sustainable community development. In addition, the use of clean energy can also achieve the climate action goal to solve the current climate change problem, which is another goal of SDG. Bioethanol is renewable energy made from biomass or agricultural by-products, resulting in a clean emission during combustion. Thailand is abundant in energy plants, including grasses, which can be excellent feedstocks for a variety of high-value products, including bioethanol. Unlike the first-generation ethanol that relies on sugar crops, the second-generation bioethanol utilizes lignocellulosic materials (energy plants) and agricultural wastes to mitigate food insecurity (Restiawaty *et al.,* 2020; Sudiyani *et al.,* 2016; Menegol *et al.,* 2016; Sanford *et al.,* 2017).

Napier grass (*Pennisetum purpureum*) typically grown as animal feed, is considered a lignocellulose material, which has a long lifespan with high crop yields and year-round harvest. It has been known as an energy plant and is a promising alternative for bioethanol production

* Corresponding author: thaneeya.r@en.rmutt.ac.th

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because of high crop productivity, ubiquity, abundance, and diverse applications. Napier grass has high nutritional benefits containing 30.9% carbohydrates, 27% proteins, 14.8% lipids, 18.2% total ash, and 9.1% fiber (as dry weight) (Sawasdee & Pisutpaisal, 2014). Napier grass is a high-carbohydrate biomass known to be a precursor to ethanol conversion. Moreover, this plant contains approximately 45%–50% of cellulose, 30%–32% hemicellulose, and 18%–22% lignin (Kommula *et al*., 2013; Reddy *et al*., 2014). Previous studies have used Napier grass as raw material to produce bioethanol through a variety of processes, enzymes, and pretreatment methods. Kongkeitkajorn *et al*. (2020) evaluated the ethanol production potential from Napier grass with the different pretreatment methods and ethanol production processes by using *Saccharomyces cerevisiae* and *Scheffersomyces shehata*e, a xylose-fermenting yeast. They found that alkaline pretreatment with sodium hydroxide (NaOH) using separate hydrolysis and fermentation (SHF) processes showed the best condition with high ethanol yields (44.7 g/L). Tsai *et al*. (2018) reported that Napier grass could be converted to ethanol production of 0.143 g/g raw material by applying simultaneous saccharification and fermentation (SSF) with dried yeast (*S. cerevisiae*) and cellulase (CTec2) after pretreatment with an alkaline solution.

Bioethanol production involves two main processes: hydrolysis and fermentation. There are many methods for hydrolysis: enzymatic, acid, and base hydrolysis (Kusmiyati *et al*., 2016; Adekunle *et al.,* 2016). In conventional SHF, hydrolysis is initially performed to convert cellulose into sugars for subsequent fermentation. However, SHF suffers from multiple operational units and relatively high energy consumption (Alfani *et al*., 2000; Cotana *et al*., 2015). On the other hand, SSF integrates hydrolysis and fermentation into one single operational unit. The advantages of SSF are relatively low investment and operational costs (Wingren *et al*., 2003). As reported by previous literatures (Dahnum *et al.,* 2015; Xu *et al.,* 2015), SSF is superior to SHF because of higher ethanol production efficiency. The superior performance is attributable to lower glucanase and cellobiohydrolase in SSF than in SHF (Banka *et al*., 2015; Loaces *et al.,* 2017). Burman *et al*. (2019) found that acid mine drainage pretreatment achieved a final ethanol concentration of 14.43 g/L for SHF and 14.83 g/L for SSF.

Enzymes play an important role in the bioethanol production of lignocellulose materials, including grass. Specifically, fungi are responsible for converting cellulose into monosaccharides during hydrolysis. In practice, fermentation using whole cells of microorganisms is cheaper than using commercial enzymes. Commonly used cellulolytic fungi are mutant strains of *Trichoderma reesei* (Gusakov *et al*., 2011). Meanwhile, yeast converts sugars into ethanol during fermentation (Ariyanti and Hadiyanto, 2013). Common yeast strains for bioethanol production include *S. cerevisiae, Scheffersomyces stipitis,* and *Schizosaccharomyces pombe* (Azhar, *et al.,* 2017). In addition, Siwarasak *et al*. (2012) utilized the co-culture of *T. reesei* and *S. cerevisiae* in the SSF process for ethanol production of various sugar crops. Meanwhile, *Escherichia coli* and *S. cerevisiae* were the example of the co-culture system used in the bioethanol production of Napier grass (Yasuda et al., 2014), *T. reesei, Aspergillus niger* and *Zymomonas mobilis* (Liu *et al.,* 2017), *S. cerevisiae* and *Pichia stipites* (Wongwatanapaiboon *et al*., 2012), *Bacillus sp*. and *Klebsiella oxytoca* (Tran *et al*., 2013), and *Aspergillus niger* and *S. cerevisiae* (Eliana *et al*., 2014). However, there is less data on the use of *T. reesei* and *S. cerevisiae* mixed-strain fermentation in the bioethanol production of Napier grass. As mentioned above, there are various influential factors in identifying the independent variable for the potential of ethanol production. The number of trials to cover all factors needs to be large. The optimization of important parameters such as substrate, enzyme, and interaction time duration should be taken into account. An effective statistical model based on experimental design is necessary to use for analyzing the variable amount and the effect of the interaction parameters on ethanol yields. Orthogonal array design is one of the factorial designs of experiment (DOE) methods, which deals with minimum numbers of experiments and optimizes the parameters in the ethanol process at a time by using the orthogonal design table and statistical analysis (Akhtar *et al.,* 2017). Moreover, orthogonal design data can be used to consider for scaling up to increase interest in the industrial application. Sharma *et al.* (2019) reported the utilization of the Taguchi orthogonal array design, kinetics, and modeling to scale up and optimize ethanol production from freshwater algae, *Rhizoclonium sp*. of Trans Himalayas. Sharma *et al.* (2020) stated that the simulation software and life cycle assessment show that second-generation bioethanol production will reduce the environmental impact and it is the regeneration of the bioresource. The novelty of this research lies in the use of the mixed crude co-culture of *T. reesei* and *S. cerevisiae* to produce bioethanol from Napier grass and to reduce operational and capital cost.

This research thus investigates the application of a co-culture of *T. reesei* and *S. cerevisiae* in the ethanol fermentation of Napier grass using SSF. Prior to SSF, Napier grass was pretreated with 3% (w/w) NaOH. The fermentation parameters under study were Napier grass content, mixed crude co-culture loading, and incubation time; orthogonal experimental design was used to optimize the parameters for maximum bioethanol yield. Furthermore, fermentation was upscaled 20-fold, and experiments were carried out under unadded and added sugar. This study will improve the potential of bioethanol production by using NaOH pretreatment and SHF processes generally used in industries. It will help to reduce the cost of ethanol production through the shorter production time and easier enzyme production as well as the utilization of waste that can be used to produce ethanol. The emergence of agriculture has created the possibility of zero waste management.

2. Materials and methods

2.1 Preparation of materials

In this research, Napier grass was acquired from a plantation in Thailand's central province of Suphan Buri. The grass was chopped into smaller pieces of 1–3 cm in length and oven-dried at 80°C until the moisture content was below 10%. The dried grass was ground and sieved using sieve No. 100 prior to pretreatment with 3.0% (w/w) NaOH.

2.2 NaOH pretreatment

Alkaline pretreatment was carried out by immersing 10% (w/v) of dried Napier grass powder in 3.0% (w/w) NaOH solution in an Erlenmeyer flask (Pensri, et al., 2016). The slurry was processed in the autoclave at 121C (15 psi) for 60 min and left to cool to room temperature. The solid was then filtrated, washed by tap water to neutralize pH, and oven-dried at 103°C for 60 min. Both pre- and post-treated Napier grass powder were analyzed and compared for cellulose, hemicellulose, and lignin. The compositional analysis was carried using the Technical Association of Pulp and Paper Industry (TAPPI) standard: TAPPI T203 om-88 test method for cellulose and hemicellulose contents and TAPPI T222 om-88 test method for lignin content.

2.3 Co-culture fermentation preparation

Mixed crude co-culture of *T. reesei* and *S. cerevisiae* (1:1) was utilized to enhance ethanol production. According to (Prajankate, 2011), co-culture contributed to enhanced ethanol yields compared with utilization of one strain. The mixed crude co-culture fermentation was cultured by co-culturing microorganisms in the same potato dextrose agar plate at 25°C for 7 days. Afterward, solid-state cultivation was performed at pH 5 in raw dried tapioca chips (5–15 mm) and incubated at 24°C ± 2°C for 5 days for mixed crude enzyme powder. Samples were collected on a daily basis to determine microorganism concentration and reducing sugar.

2.4 DOE

An orthogonal experimental design was utilized to optimize three independent variables (x1, x2, and x3), under seven different levels for an optimal fermentation condition with maximum ethanol yield, where x1, x2, and x3 are respectively Napier grass powder content, mixed crude co-culture loading, and incubation time. Table 1 tabulates L49 (73) orthogonal design with Napier grass powder content (g), mixed crude co-culture loading (g), and incubation time (d) under seven experimental levels. The experiments were performed in duplicate. The ethanol concentration (g/L) was considered as response values to analyze the influential order of the amounts of substrate and mixed crude enzyme and incubation time to optimize the conditions. Statistical analysis was conducted using standard statistical software (SPSS version 22). A value of $p < 0.05$ was regarded as statistically significant. Moreover, the data was reported using mean ± standard deviation.

Statistical analysis of orthogonal experiments for ethanol yields were calculated based on two important parameters of k_{ab} and K_{ab}. K_{ab} equals to the sum amount of ethanol productivity at all levels (a = level 1-7 in Table 1) in each factor (b= A, B, and C in Table 1). k_{ab} is the average of K_{ab} in each level (a) of factor (b).

2.5 Bioethanol fermentation

Laboratory-scale SSF for Napier grass-based ethanol was carried out under the optimal condition from the orthogonal array design. The liquid media (LM) was first prepared from 1 g $CaHPO_4$, 1 g $MgSO_4{\cdot}7H_2O$, 8 g urea (46% $(NH_4)_2SO_4$), 15 g phosphate (NPK-0-52-34), and 1000 mL pure water at pH 5 and stirred for 30 min. Dried Napier grass powder already treated with NaOH solution as mentioned in section 2.2 and LM were autoclaved at 121°C for 15 min. Then, sterilized Napier grass and mixed crude co-culture with various contents indicated in Table 1 were introduced into the 500-mL Erlenmeyer flask with 300-mL sterilized LM mixture. The flasks were capped with cotton wool and shaken at 100 rpm and 30°C until termination following the design incubation time (Table 1). The laboratory-scale schematic diagram of orthogonal experiments used in the study is shown in Figure 1. Samples were collected on a daily basis, and cell mass, reducing sugar, and ethanol yield were determined.

Table 1
Design of orthogonal experiment under study

Level	Factors		
	A	B	C
	Napier grass (g)	Mixed crude co-culture (g)	Incubation time (d)
1	5	2.5	3
2	10	5	4
3	15	7.5	5
4	20	10	6
5	25	12.5	7
6	30	15	8
7	35	17.5	9

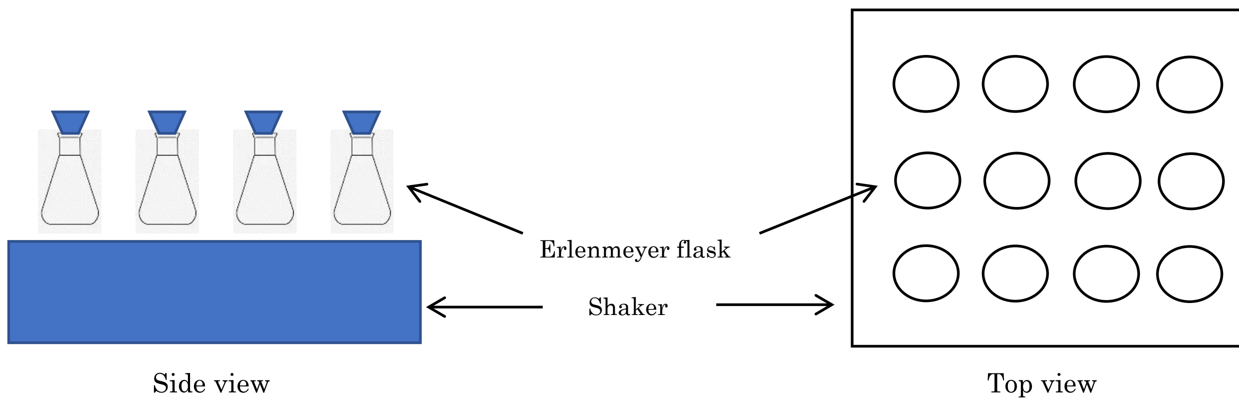

Fig 1 The laboratory-scale schematic diagram of orthogonal experiments for simultaneous saccharification fermentation (SSF) for Napier grass

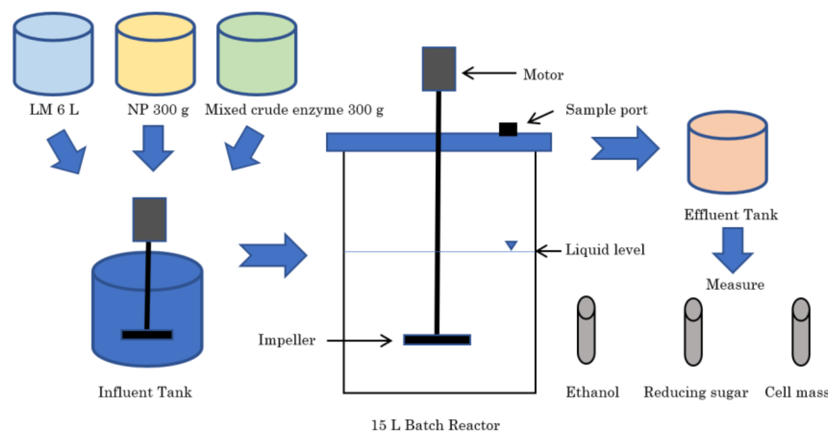

Fig 2 The scale-up schematic diagram of batch reactor for simultaneous saccharification fermentation (SSF) for Napier grass

To scale up, the fermentation was enlarged 20-fold by using the ratio with the highest total concentration of ethanol from the orthogonal fermentation method in Table 1. The highest amount of ethanol when using 15 g of NP and 15 g of mixed crude co-culture when the aforementioned results were enlarged to 20 times. The amount of grass used in fermentation and mixed microbial powder was 300 g. LM (without sugar) to be added to the 20-fold expansion fermentation was 6 L in a 15-L reactor using impellers (Figure 2). Sterilization and then mix all materials with as in laboratory-scale experiments at pH 5. The fermentation was carried out under anaerobic conditions at 100 rpm for 3 h. The batch-scale fermentation was performed under the optimal condition from the orthogonal design experiments (the best condition of the amounts of Napier grass (150 g), mixed crude enzyme (150 g), and incubation time (7 days)) with different experimental settings, with and without added sugar, and the ethanol yields were compared. The theoretical bioethanol yields were calculated using the equation (1) based on the maximum (51%) (%conversion of glucose into ethanol by yeast).

$$\text{The ethanol yield}(\%)=\frac{\text{Ethanol produced(g)}}{\text{Initial sugar(g)}\times 0.511}\times 100 \quad (1)$$

Sugar was added more to provide energy for the microorganisms and to see the effect of adding sugar on increased ethanol yield. This is because sugar is typically added in the ethanol production process to provide microorganisms with a sufficient energy source. Finally, samples were collected on a daily basis to determine cell mass, reducing sugar, and ethanol yield.

2.6 Analytical methods for this study

The microorganism concentration (cell mass) was prepared in pure water suspension and determined using a hemacytometer (Boeco, Germany) with a 40x microscope. Reducing sugar concentrations were estimated with 3,5-dinitrosalicylic acid reagent (Miller, 1959). The ethanol concentrations were determined using the dichromate colorimetric method (Williams & Reese, 1950)

3. Results and Discussion

3.1 The effect of alkaline pretreatment

Napier grass contains essential nutrients, i.e., carbon (48.60%0 ± .80%), hydrogen (6.01 ± 0.14), sulfur (0.32 ± 0.01), and nitrogen (0.99%± 0.33%) for microorganism growth and the structural composition with cellulose (38.8% ± 2.30%), hemicellulose (19.80% ± 1.68%), lignin (27.00% ± 1.29%), and extractives (12.07% ± 0.32%)

(Mohammad *et al.* 2015). Cellulose is dominant in plant cell walls, which is enzymatically converted into monosaccharides for bioethanol production (Rahayu *et al.*, 2017). Hemicellulose and lignin, however, hinder enzymes from breaking down cellulose into glucose during fermentation (Chaturvedi & Verma, 2013). Before Napier grass is used as the carbon substrate in ethanol fermentation, pretreatment is required to remove lignin and hemicellulose, thereby improving digestion and subsequent fermentation efficiency (Eliana *et al.*, 2014).

Figure 3 illustrates the chemical composition of dried Napier grass powder before 3% (w/w) NaOH pretreatment, consistent with the previous report (Pensri *et al.*, 2016; He *et al.*, 2017). Figure 1 also depicts the compositional properties of dried Napier grass powder after NaOH pretreatment, indicating changes in the composition of lignocellulosic biomass as lignin was removed and hemicellulose was transformed into cellulose. The chemical compositions of native and pretreated Napier grass were averaged at 9.2% ±0.56% and3 .6% ± 0.63% for lignin, 52. ±%851.12% and 82.45% ± 0.78% for cellulose, and 18.2% ± 0.32% and 8.6% ± 0.85% for hemicellulose. Native Napier grass has significantly different chemical composition changes after pretreatment ($p < 0.05$). NaOH pretreatment can improve native Napier grass as a proper material for bioethanol production by increasing 29.6% of cellulose and removing 5.6% of lignin and 9.6% of hemicellulose. The compositional properties of native Napier grass include cellulose, hemicellulose, lignin, and others (ash, lipids, sugars, and proteins) (Triantafyllidis *et al.*, 2013). According to (Lui *et al.* 2017), Napier grass consists of cellulose (40%–50%), hemicellulose (25%–35%), and lignin (15%–25%). High cellulose contents of Napier grass render the plant ideal for bioethanol production. According to (Taherzadeh, 2008; Kim *et al.*, 2016; Chen *et al.*, 2017), complex lignin structures hinder chemical and biological degradation, thereby lowering ethanol fermentation efficiency. According to Kamarullah, (2015), hemicellulose is mainly composed of fermentation-resistant pentose. Moreover, pretreatment also improved sugar conversion during hydrolysis (Mafuleka & Kana, 2015). As mentioned in the literature, other components such as proteins, lipids, and ash strongly affect bioethanol yield (Cotana *et al.*, 2015; Burman *et al.*, 2020). The pretreatment step becomes an important step to minimize the composition of the other components. The yield of bioethanol depends on the amount of glucose from raw material.

3.2 Microorganism plate count

Table 2 tabulates the plate counts of microorganisms in the mixed crude co-culture of *T. reesei* and *S. cerevisiae*, consisting of <10 CFU/g total bacteria, <10 CFU/g total yeast, and $2.67×10^8$ CFU/g total fungi. The results revealed that yeast counts (*S. cerevisiae*) were considerably less than that of fungi (*T. reesei*), but the number of microorganisms in the co-culture is enough for fermentation. According to Azhar *et al.* (2017), yeast strains such as *S. cerevisiae* minimally grow on substrate due to their inability to compete with other wild-type yeasts and fungi. The mixed crude co-culture is durable and able to convert sugars into ethanol

3.3 Laboratory-scale ethanol fermentation using mixed crude co-culture

The optimal Napier grass content, mixed crude co-culture loading, and incubation time that maximizes the bioethanol yield (16.90 g/L) were 15 g, 15 g, and 7 days, respectively (Table 3). In Table 4, 15 g of mixed crude co-culture, 15 g of Napier grass, and 7 days incubation time were chosen to be the optimal level for each factor ($B_6A_3C_5$). The major–minor order was considered from a larger R that indicates the effect sequence on the results of ethanol yields. The influence factors from main to secondary were as follows: amount of mixed crude co-culture enzyme > amount of Napier grass > incubation time. In other words, the optimal Napier grass-to-co-culture ratio was 1:1, given the 7-day incubation time. The optimal fermentation condition was further validated in batch-scale experiments using 15-L bioreactor tanks (6-L working volume) in two experimental settings: with and without added sugar. The pilot-scale experiments were performed to collect data from 300 g of mixed crude co-culture and 300 g of Napier grass. Then, the ethanol production of both conditions (with and without added sugar) was collected daily until 7 days was completed (optimum incubation time), and the data collection was extended until the 9th day to determine the trend of ethanol production.

3.4. Batch-scale ethanol fermentation using mixed crude co-culture

The optimal condition to scale up was obtained from the orthogonal experiments of the DOE in Table 4. The optimal ethanol production was obtained under optimal conditions by a 20-fold scale up from the optimization scheme ($B_6A_3C_5$: 15 g mixed crude co-culture, 15 g Napier grass, and 7 days incubation time). To imitate commercial-scale production, the batch-scale experiments using 15-L bioreactor tanks were carried out under the optimal SSF fermentation condition: 15 g Napier grass, 15 g mixed crude co-culture, and 7 days incubation time. The experiments were undertaken under unadded and added sugar conditions to examine the effect of adding sugar on bioethanol production

Table 2
Plate count of microorganisms in mixed crude co-culture

Co-culture	Total bacteria (CFU/g)	Total yeast (CFU/g)	Total fungi (CFU/g)
Co-culture of *T. reesei* and *S. cerevisiae*	<10±0.10	<10±0.2	$2.67×10±^{8}0.5×10^{8}$

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Table 3
Ethanol production of orthogonal experiment for batch fermentation

Experiment	Factors			Ethanol (g/L)
	A	B	C	
1	5	2.5	3	7.03 ±1 .16
2	10	5	3	7.25 ± 1.21
3	15	7.5	3	10.23 ± 1.22
4	20	10	3	7.57 ± 0.02
5	25	12.5	3	7.77±0 .58
6	30	15	3	7.93 ± 0.02
7	35	17.5	3	12.22 ± 4.31
8	5	17.5	4	10.70 ± 0.14
9	10	2.5	4	6.96 ± 0.22
10	15	5	4	9.30 ± 1.30
11	20	7.5	4	5.45 ± 0.18
12	25	10	4	8.52 ± 0.08
13	30	12.5	4	6.53 ± 0.45
14	35	15	4	9.48 ± 0.03
15	5	15	5	11.11 ± 2.12
16	10	17.5	5	10.91 ± 2.49
17	15	2.5	5	7.00 ± 2.53
18	20	5	5	6.88 ± 0.41
19	25	7.5	5	7.95 ± 0.60
20	30	10	5	8.17 ± 0.14
21	35	12.5	5	10.71 ± 0.08
22	5	12.5	6	7.89 ± 0.35
23	10	15	6	10.63 ± 0.31
24	15	17.5	6	12.16 ± 0.02
25	20	2.5	6	7.28 ± 0.58
26	25	5	6	6.55 ± 0.14
27	30	7.5	6	8.70 ± 0.60
28	35	10	6	8.78± 0.44
29	5	10	7	11.63 ± 0.09
30	10	12.5	7	16.17 ± 0.14
31	15	15	7	16.90 ± 0.66
32	20	17.5	7	9.50 ± 0.00
33	25	2.5	7	5.51 ±0 .13
34	30	5	7	8.56 ±0 .14
35	35	7.5	7	12.24 ±0 .64
36	5	7.5	8	11.08 ±0 .25
37	10	10	8	12.06 ±0 .00
38	15	12.5	8	14.62 ±0 .16
39	20	15	8	11.11 ±0 .02
40	25	17.5	8	9.45 ±1 .87
41	30	2.5	8	7.16 ±0 .06
42	35	5	8	9.38 ±0 .48
43	5	5	9	7.48 ±0 .49
44	10	7.5	9	10.13 ±0.12
45	15	10	9	10.40 ±0.52
46	20	12.5	9	9.27 ±0 .53
47	25	15	9	11.07 ±0 .85
48	30	17.5	9	10.27 ±0 .15
49	35	2.5	9	7.50 ±0 .59

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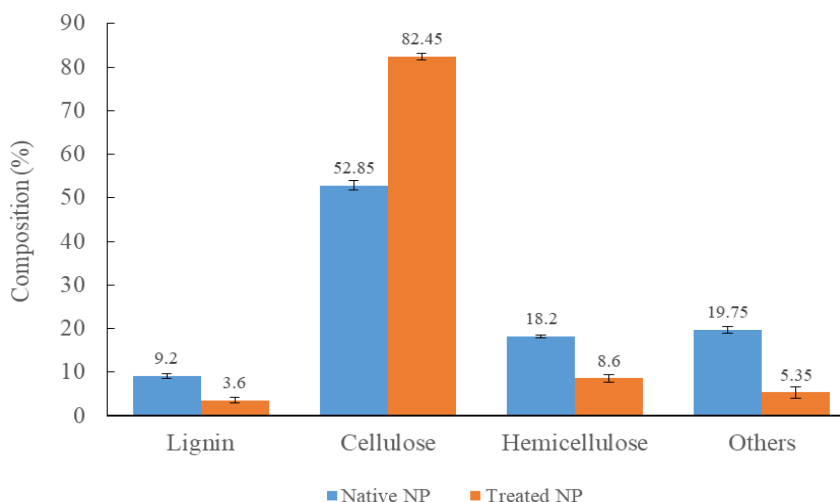

Fig 3 The chemical composition of Napier grass before and after NaOH pretreatment

In Figure 4, the initial reducing sugar (day 0) was as high as 33.0 g as a result of adding sugar. Reducing sugar continually increased as the co-culture fermentation converted cellulose into sugar and peaked to 46.7 g (day 4) and declined. It reversed on day 7 before declining and reversing again at termination (day 9). The phenomenon could be attributed to residual cellulose being converted into sugar. For unadded sugar condition during the initial incubation period, the co-culture fermentation converted cellulose into sugar for growth, causing reducing sugar to rise at an increasing rate at 4.96 g (day 0) and peaked at 12.7 g (day 4) and then was reduced steadily beyond day 4. The added sugar condition had a similar trend with unadded sugar except after day 6, the swing may be due to the microbes' use of the added sugars, causing a slight increase in ethanol production. In Figure 5, the cell mass steadily grew during the first 4 days and peaked in day 5 and declined and reversed on day 8. Since larger proportions of converted sugar were consumed by the microbes, the bioethanol production increased slowly from days 0 to 4 and peaked at day 5 (25.02 g/L) and steadily declined as the reducing sugar decreased. The cell mass minimally grew during the first 4 days of incubation and spiked in day 5 and decreased afterward, consistent with the pattern of reducing sugars. Figure 6 illustrates the bioethanol yields in the unadded and added sugar bioreactor tanks. The bioethanol production increased steadily from days 0 to ,4 peaked at day 5th (33.24 g/L) and declined before reversing on day 8th. The maximum bioethanol yields were 25.02 g/L (5-day incubation time) and 33.24 g/L (5-day incubation time) under unadded and added sugar conditions. The higher bioethanol production under the added sugar condition was attributable to increased amounts of reducing sugar. The trend patterns of the ethanol yield of the unadded and added sugar states were similar and peaked at day 5 as well.

Table 4
Statistical analysis of orthogonal experiments for ethanol yields

Level	Control parameters		
	A	B	C
K1	66.92	48.44	60
K2	74.11	55.4	56.94
K3	80.61	65.78	62.73
K4	57.06	67.13	61.99
K5	56.82	72.96	80.51
K6	57.32	78.23	74.86
K7	70.31	75.21	66.12
k1	22.31	16.15	20.00
k2	24.70	18.47	18.98
k3	26.87	21.93	20.91
k4	19.02	22.38	20.66
k5	18.94	24.32	26.84
k6	19.11	26.08	24.95
k7	23.44	25.07	22.04
R	7.93	9.93	7.86
SD	3.16	3.61	2.83
Major-minor order B>A>C			
The optimization scheme $B_6A_3C_5$			

Table 5
Comparison between batch-scale ethanol yields from Napier grass under different fermentation schemes and co-culture fermentation

Microorganisms	Pretreatment	Process	Maximum ethanol yield		References
			(g/L)	(%)	
Saccharomyces cerevisiae, Accellerase 1500	Alkaline (NaOH)	SSF	27.7	92	Cardona *et al*., (2016)
Aspergillus niger, Saccharomyces cerevisiae	Alkaline (NaOH)	SSF	23.4	78	Aiyejagbara *et al*., (2016)
Aspergillus niger, *Trichoderma reesei, Zymomonas mobilis*	None	SSCF	15	50	Liu *et al*., (2017)
Saccharomyces cerevisiae, β-glucosidase, PEG 6000	Dilute acid (H_2SO_4) and Alkaline (NaOH)	SSF	24	81	Camesasca *et al*., (2015)
Penicillium echinulatum, Saccharomyces cerevisiae	Steam explosion	SHF	4.42	17	Scholl *et al*., (2015)
Trichoderma reesei, *Saccharomyces cerevisiae*	Alkaline (NaOH)	SSF	25.02±1.3	83	This study

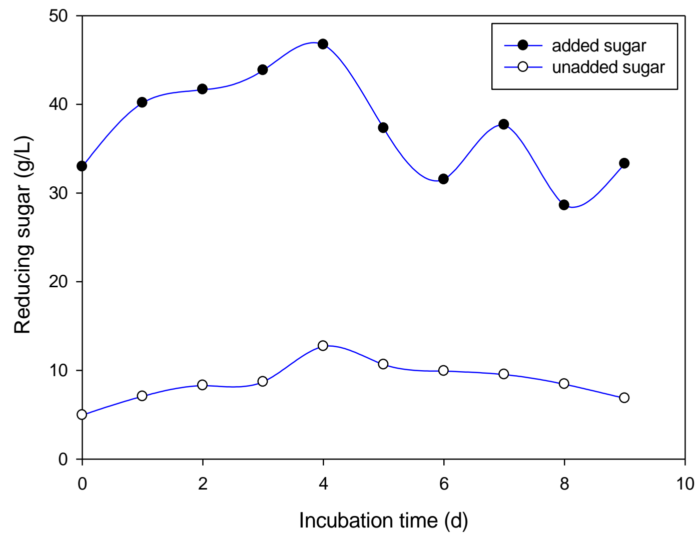

Fig 4 Reducing sugar in batch-scale fermentation of unadded and added sugar conditions

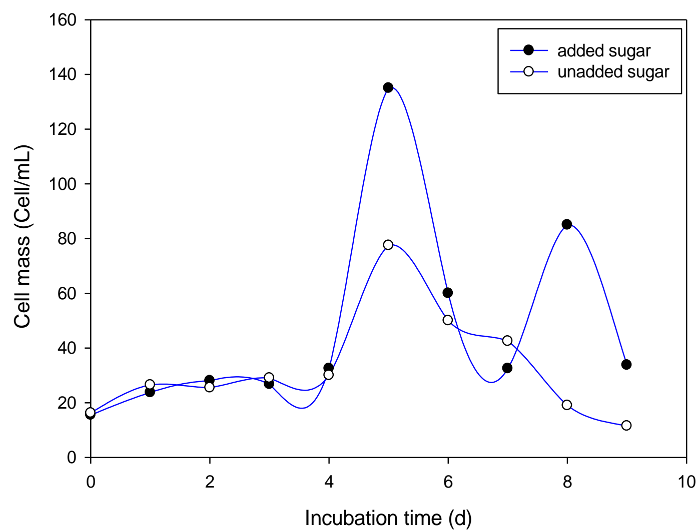

Fig 5 Cell mass in batch-scale fermentation of unadded and added sugar conditions

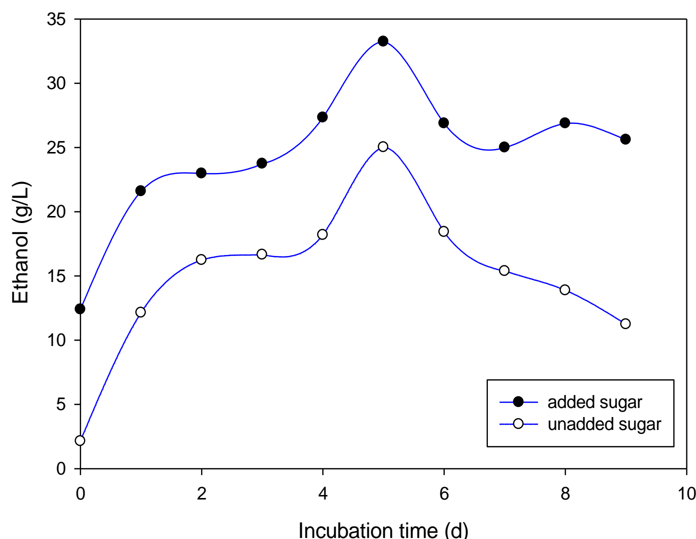

Fig 6 Ethanol yields in batch-scale fermentation of unadded and added sugar conditions

From the orthogonal experiment in Table 4, the effect of each independent variable on the bioethanol yield is based on the biggest average value of k_i. For Napier grass, when the amount was increased, the bioethanol yield was increased. The highest value of the bioethanol yield is at k_3 (15 g). Then, the yield decreased when the amount of Napier grass was further increased. The second variable is the mixed crude co-culture. When the amount of mixed crude co-culture was increased, the bioethanol yield was also increased. The highest value of the bioethanol yield is at k_6 (15 g). The last variable is the incubation time. The change of the bioethanol yield is very small in the first 6 days but it increased to the highest value at k_5 (day 7).

By comparison, reducing sugars under the added sugar condition (33.0 g) was nearly seven times higher than under the unadded sugar condition (4.96 g). However, the maximum bioethanol yield under the added sugar condition (33.24 ± 1.8 g/L) was slightly greater than that under the unadded sugar condition (25.02 ± 0.5 g/L) (merely 8.22 g/L) .The ethanol yield of the two conditions was not significantly different (p-value < 0.05). This result indicated that added sugar minimally enhanced bioethanol production. The small increase in the bioethanol yield, despite increased sugar content, could be attributed to considerably smaller proportions of yeast (<10 CFU/g) to fungi (2.67×10^8 CFU/g) in the co-culture fermentation. Fungi is essential for the conversion of sugar into bioethanol.

Table 5 compares the bioethanol production from Napier grass using different fermentation technologies and co-culture fermentation. In this study, the bioethanol yield under the optimal SSF condition (15 g Napier grass, 15 g mixed crude co-culture, and 5 days incubation) was 25.02 g/L, consistent with Cardona *et al.*, (2016), who used *S. cerevisiae* and Accellerase 1500. Nevertheless, given the relatively lower costs and greater durability, *T. reesei* and *S. cerevisiae* co-culture fermentation is more attractive than that of *S. cerevisiae* and Accellerase 1500 (Cardona *et al.*, 2016).

This work focuses on second-generation feedstock for bioethanol production, which has the limitation of high-cost involvements and energy consumption. The results of this work used the pretreatment method from previous study and scale up only two sizes of the reactors. The authors suggest that a new method of DOE such as the Box–Behnken design based on the response surface methodology should be used in further studies. It combines both mathematical and statistical techniques that are useful in modeling and problem analysis and is effective in reducing the sample count in experiments. Moreover, the economic feasibility should be considered for up-scaling to industrial-scale research in the future.

.4. Conclusion

This research investigated the use of *T. reesei* and *S. cerevisiae* co-culture fermentation in the production of bioethanol from Napier grass using SSF technology. Napier grass was pretreated with 3% (w/w) NaOH, and an orthogonal experimental design was utilized to optimize the Napier grass content, mixed crude co-culture loading, and incubation time for maximum bioethanol production. The pretreatment increased the cellulose content from 52.85% to 82%. The optimal fermentation condition that maximized the bioethanol yield was 15 g Napier grass, 15 g mixed crude co-culture, and 7-day incubation time that gave the maximum bioethanol yield of the laboratory-scale experiments to 16.90 g/L. To scale up, the fermentation was done with 20-fold Napier grass content, mixed crude enzyme, and working volume with and without added sugar. The maximum bioethanol yields of scale up experiments were found at 25.02 and 33.24 g/L for unadded and added sugar, respectively. The results revealed that *T. reesei* and *S. cerevisiae* co-culture fermentation is suitable for bioethanol production of Napier grass.

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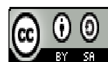

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