JEAS

JOURNAL OF ENGINEERING AND APPLIED SCIENCES

Journal of Engineering and Applied Sciences, Volume 21, Number 1, Dec. 2022, 890 - 906

The Effect of Furan Extraction on the Ethanol Fermentability of *Canarium schweinfurthii* (African Elemi) Seed

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Abstract
The potential of cellulosic ethanol as a panacea to environmental degradations associated with fossil fuel has recently spurred research on the technicalities to improve product yield amidst the myriad of inhibitors domicile in a lignocellulosic feedstock. The effect of the extraction of furan products on the fermentability of *Canarium schweinfurthii* (African Elemi) seeds was the focus of this research. Proximate characterization (PC), Fourier Infrared Spectroscopy (FTIR), and Scanning Electron Microscopy (SEM) were applied to ascertain the level of the hydrolyzable components and the need for pretreatments on the substrate. The PC indicated a 55% hydrolyzable component, recalcitrating lignin at 29% and hemicelluloses above 15%. The PC, the FTIR, and the SEM results underscored the need for chemical pretreatments before the enzymatic hydrolysis and fermentation. One of the pretreatments was furan extraction in an atmospheric refluxing setup, targeted at getting rid of furan inhibition of fermentation and obtaining furfural and hydroxyl methyl furfural (HMF) as complementary products. The pretreatment extract was 72% HMF; the residue, along with the non-pretreated, the acid, and the alkaline pretreated, were subsequently hydrolyzed with three different crude enzymes from cellulosic fungal strains and a commercial enzyme from Trichoderma reesei. The GC/MS analysis revealed that the hydrolyzate from the furan extracted substrate was about 90% glucose, that of the non-pretreated was about 70% xylose. So, the furan extraction enriched the hexose compositions over the pentoses, and the hexose enrichment translated into more ethanol fermentability. The maximum ethanol conversion from the non-pretreated and the furan extracted substrates was 8.8 and 63%, corresponding to ethanol yield of 0.045 and 0.332 g ethanol/ g sugar, respectively. Moreover, the statistical analysis and the kinetics parameters of the fermentation affirmed that the furan-extracted substrates suffered less inhibition to cell growth and ethanol yield.

Keywords: Fermentation, hydrolysis, inhibition, furan, ethanol

Nomenclature:

Com	A commercial enzyme from *Trichoderma reesei*
$CF1, CF2, CF3$	The crude enzymes from cellulosic fungi
C	The non-pretreated African Elemi seed
CA	The acid pretreated African Elemi seed
CB	The alkaline pretreated African Elemi seed
CAF	The furan-extracted African Elemi seed
K_S	The Monod's constant for growth on substrates (g/l)
K_{SP}	The Monod's constant for product formation on substrates (g/l)
K_i	The inhibition constant for growths on substrates (g/l)
K_{ip}	The inhibition constant for product formation (g/l)
m	The maintenance coefficient (h^{-1})
P	The product concentration (g/l)
P_m	The product concentration above which cells do not grow(g/l)
P_{mS}	The product concentration above which cells do not yield products (g/l)
S	The substrate concentration (g/l)
X	The cell dry weight (g/l)

$Y_{X/S}$	The cell yield constant (g-cells/ g-substrate)
$Y_{P/S}$	The product yield constant (g-product/ g-substrate)
μ	The specific growth rate (h^{-1})
μ_{max}	The maximum specific growth rate (h^{-1})
v	The specific rate of product formation (h^{-1})
v_{max}	The maximum specific rate of production (h^{-1})
β, γ	The constants with the inhibitions due to product formation

1. Introduction

Yeast cells, especially *Saccharomyces cerevisiae* and some industrial native strains, are famous for generating energy by converting sugars into ethanol and CO_2 in the absence of oxygen in a process known as ethanol fermentation (Yuan, Rao, Relue & Vernas, 2011). Inhibition of cell growth has been the major obstacle facing the development of cellulosic ethanol as the fermentation, an intracellular metabolic activity in fungal and bacterial cells, is susceptible to over thirty-five known inhibitors, especially when the hydrolysate is prepared by chemical means, of which furan products have been the most daunting (Luo, Brink, & Blanch, 2002). Many fermentation applications prefer commercial *Saccharomyces cerevisiae* for fermentation of C6 and C5 sugars to ethanol due to the high productivity and its relative toughness in the fermentation environment, so researchers continued to search for possible solutions to combat the inhibitors and enhance the productivity of bioethanol.

Secondary metabolites such as acetic acid, lactic acid, propionic acid, citric acid, succinic acid, and glycerol are a group of chemicals that have a detrimental presence in ethanol fermentation by S. cerevisiae. The inhibitory effects of the secondary metabolites could be relieved by operating at a high pH level (Fan, *et al.*, 2014). The co-existence of pentoses, typically xylose, and hexoses, such as glucose, in a fermentation broth, the natural case in the fermentation of lignocellulosic hydrolyzates, is equally inimical to ethanol fermentation because the microorganisms can only ferment xylose when the glucose concentration has fallen to a certain level. Wang, York, Ingram & Shanmugam (2019) reported that a co-culture of a mutant E-coli constructed to ferment xylose at a high rate and S. cerevisiae could virtually ferment the entire xylose and glucose molecules at a high rate. Reactive oxygen species (ROS) such as peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha oxygen, as defined by Herb, Gluschko & Schramm (2021), are highly reactive chemical molecules formed due to the electron receptivity of oxygen, and this species of chemicals can damage the fermentation cells and lead to reduced ethanol yield and productivity. Qi, Xia & Zhong (2015) reported that with cells prepared to have a high intracellular biotin pool, the effects of the ROS on the fermentation could be combated. The presence of water-insoluble phenolic compounds such as 2-furoic acid, ferulic acid, *p*-coumaric acid, guaiacol, and *p*-hydroxybenzoic acid in lignocellulosic hydrolysates has also been tagged detrimental to the metabolic activities of S. cerevisiae, but Gu, Zhang & Bao (2014) reported that their effects could be circumvented to a reasonable extent by an evolutionary adaptative approach. Another phenolic compound with notable inhibitory effects is vanillin. Zheng *et al*. (2017) reported that S. cerevisiae cells could grow to be more vanillin tolerant through a breeding strategy based on genome duplication and large-scale chromosomal variations. Even the product, ethanol, hurts the activities of the yeast cells and should be separated from the cells for optimum performance of the cells; one of the ways of dealing with this is using cell immobilization and the integration of fermentation and pervaporation processes (Cai *et al*. 2016).

Overall, there are lots of inhibitors and challenges facing high ethanol yield from lignocellulosic biomass, and studies had proffered solutions to douse the obstacles; however, much has not been reported as the panacea to furfural and other furan products, which were the main inhibitors to the metabolic activities of saccharomyces cerevisiae. It is this gap that this study intended to address by incorporating furan extraction into the dilute acid pretreatment of *Canarium schweinfurthii* (African Elemi) seed, getting rid of the furan-related inhibitors and simultaneously generating a complementary product. This research modeled the kinetics of cell growth, ethanol production, and substrate consumption and analyzed the effects of furan inhibition on the parameters.

Moreover**,** African Elemi is a tropical tree that is native to some African countries, and the fruit is commonly eaten mainly due to its vast medicinal and nutritional values, hence it is an economic tree, but the inner seed is often discarded as it's not generally used for anything (Opkala, 2016; Maduelosi & Angaye, 2015); as a result, the non-edible inner seed of African Elemi is one of the major solid wastes in dumpsites around the markets and homes, both in the metropolis and the rural areas in Africa. This research intended to harness the lignocellulosic inner seed of

African Elemi as a feedstock for bioethanol and furan productions, thereby sanitizing the environment of the solid wastes and generating products from waste, which will, in turn, encourage more planting of the tree.

2.0 Material and methods

2.1 Preparation and characterization of the hydrolysates

The non-edible inner seeds of African Elemi were sourced from a dumpsite at New Market in Enugu, Nigeria, in March 2021. The seeds were washed, sundried, crushed with a commercial blender and packed in an air-tight plastic container. Proximate analysis was later carried out following the National Renewable Energy Laboratory protocol (Sluiter, & Sluiter, 2011), with some modifications, mainly by using GC/MS analysis to differentiate the hexoses from the pentoses (Senila, Gog, Senila, Roman & Silaghi-dumitrescu, 2011). As part of the characterization of the biomass, FTIR (Agilent Technologies, Cary 630) analysis was carried out using the Attenuated Total Reflection (ATR) mode (Zajsek & Gorsek, 2010; Sindhu, Binod & Pandey, 2015) and an SEM (Thermo Scientific, Prisma E) image of the original biomass was obtained to compare with the internal structure of crystalline cellulose.

The substrate was divided into four: one part (C) was left non-pretreated. A part of the substrate was pretreated with 1% NaOH solution to obtain the alkaline pretreated biomass (CB) which was meant to selectively degrade the lignin content (Kim, Lee & Kim, 2015), and a part was applied for furan extraction to obtain the furan-extracted biomass (CAF) according to Ambalkar & Tablib (2012), although with some modifications. One of the modifications was to use a direct refluxing mechanism to provide a better contact between the solid and the liquid phases. Besides, the extraction temperature was reduced to 80°C to avoid decomposing the substrates for ethanol production. Furan extraction involved the use of 1% H_2SO_4 as a catalyst and butanol as the solvent, so a part of the substrate was treated with only 1% H_2SO_4 to obtain the acid pretreated biomass (CA).

Then, the differently pretreated substrates were enzymatically hydrolyzed using crude enzymes produced from soil-isolated fungal strains. The fungal strains were sourced from the Biotechnology Laboratory, School of the Environment, Florida Agric and Mechanical University, USA, in July 2021. The isolates were identified by 18S rDNA gene sequencing as *Talaromyces pinophilus, Talaromyces funiculus, and Penicillium sp*. and labeled as CF1, CF2, and CF3, respectively. Freshly grown spores from the three cellulosic isolates were used to produce crude cellulase enzymes following standard laboratory procedures (Sridevi *et al.*, 2015). Enzymes from the three fungal isolates and a commercial cellulase from *Trichoderma reesei (*C2730-500Ml, from Fisher Chemical)-labeled as COM, were applied to study the effects of the source of enzymes on the fermentation of the hydrolysates. The hydrolysis was carried out in an Erlenmeyer flask at 50°C for two days in a rotary incubator, while the hydrolysates characterized with a GC/MS (Agilent Technologies, 7890B). The hydrolysates were derivatized into trimethylsilyl oximes (TMSO) using the two-step derivatization method. The GC/MS eluted the *Syn (Z)* and the *Anti(E)* glucose forms in the hydrolysates at 30.2 and 30.3 minutes, respectively (Senila, Gog, Senila, Roman & Silaghi-dumitrescu, 2011). Four substrates (C, CB, CA, and CAF) were hydrolyzed with four cellulase enzymes, CF1, CF2, CF3, and COM, making a total of sixteen different hydrolysates applied for this study.

2.2 The fermentation of canarium hydrolysates

Ethanol was produced through the fermentation of the sixteen hydrolysates that were obtained from the enzymatic hydrolysis of the differently pre-treated biomass samples. The hydrolysates were concentrated with the target of getting an equal concentration of simple sugars, and this was achieved by monitoring the absorbance of simple sugars in 3,5-dinitrosalicylic acid (DNS) while the solution was being heated in a hot plate (Biocyclopedia. 2022). The initial simple sugar concentration was approximately 200 mg/ml. *Saccharomyces cerevisiae* (from Fisher Chemicals) was aerobically activated and propagated at 30°C for 2 h in an enrichment medium comprising of (g/l): yeast extract, 5; peptone, 15; $MgSO_4.7H_2O$, 1; K_2HPO_4, 1, which was autoclaved at 121°C for 20 min before inoculation (Cao & Liu, 2013). For the inoculation, 5mL of the concentrated hydrolysate was mixed with 4 ml of the nutrient media and 100 µl of the yeast inoculum, making the total volume 10 mL and the initial sugar concentration in the fermenter 100 mg/ml. The yeast inoculum was prepared by mixing 6 g of dry *Saccharomyces cerevisiae* with 60 mL of water, then the initial cell concentration in the fermenter was 1.0 mg/ml. The mixture was stirred for 1 minute, then about 200 µl was pitted out for the analyses at zero hours. After the aerobic activation, the broth was sealed in anaerobic bottles for the fermentation at varying times, pH, and yeast dosage. The anaerobic bottles were sealed with rubber stoppers to ensure a completely anaerobic environment and 21-gauge needles were inserted to vent the CO2 that was generated as a by-product (Yuan et al., 2011). Every 3h, about 200 µl of the fermentation product was centrifuged at 8000 rpm for 10 min, and the supernatant was characterized for ethanol, simple sugars, and cell dry mass using FTIR, the DNS, and the dry mass spectrophotometric methods, respectively.

2.3 Measuring the ethanol concentration

The ethanol concentration was accurately measured using the Attenuated Total Reflection (ATR) mode FTIR (Zajsek & Gorsek (2010). The FTIR (Agilent Technologies, Cary 630) spectra of ethanol in water showed a peak at a wavenumber of 1043 cm^{-1}, which corresponded to the peak for an alcoholic C-O group (Yadav, Verma, Patgiri, & Prajapati, 2016). There was no peak at the wavenumber with zero percent ethanol, but the peak height increased with the increase in ethanol concentration. The correlation coefficient, R^2, was 0.998, so the standard graph was applied to estimate the ethanol concentration after the fermentation. The calibration standard is shown in Fig. 7a.

2.4 Measuring the cell growth and the sugar concentration

The cell growth was measured using the method described by Biorenewable Education Laboratory (2011) with little modifications. A solution of the nutrient media (yeast extract, 5; peptone, 15; $MgSO_4.7H_2O$, 1; K_2HPO_4, 1) and the concentrated hydrolysate in the ratio of 4:5 was prepared for each of the hydrolysates and covered with aluminum foil. The solution was applied to dilute the fermentation broth each time the cell concentration was measured, and the solution was equally blank for the spectrophotometer readings. Initially, a calibration standard was prepared by dissolving known weights of the yeast in the blank solution and taking the absorbance at 620 nm. The concentration of the yeast ranged from 2 to 10 mg/ml and diluting by a factor of 20, the absorbances were between 0 and 0.2, so the calibration standard was obtained with an R^2 value of 0.9889 (Fig. 7c)

The total sugar concentration was measured with time in the fermentation broth, and to prepare the standard graph, different glucose standards ranging from 2 to 10 mg/mL were prepared using distilled water, and 3 mL of the standards were mixed with 3 ml of the DNS solution in a glass test tube. DNS is a solution of 3,5-dinitrosalicylic acid (10 g/l), sodium potassium tartrate (30 g/L), and NaOH (16 g/l) (Wood *et al.*, 2012). While the tubes were stopped, they were heated in a water bath at 100°C for 10 minutes, and after cooling, the absorbances were recorded at 540 nm. The glucose DNS calibration standard was obtained with an R^2 of 0.9932 (Fig. 7b).

2.5 Statistical analysis

The statistical significance of the differences in means of the ethanol conversions obtained from the various pre-treatments of the lignocellulosic biomass was determined using the One-Way ANOVA of the Excel Analysis Tool Pack. Following the One-Way-ANOVA was the Tukey Kramer post hoc test that was carried out to estimate the pairwise Honest Significant Difference (HSD) among the pre-treatment groups (Glen, 2022). The HSD statistic, T, was calculated using Eq. 2.1.

$$T = q \times \sqrt{\frac{MSE}{n}} \qquad (2.1)$$

Where:

q= The critical value of the studentized range, obtained from Studentized Range q Table (Zaiontx, 2020).

MSE= The Mean Square Error, or the average variance of the pretreatment groups

n= The number of items in each group

To read out the values of q, the numerator degree of freedom is the number of groups, and the denominator degree of freedom is the difference between the total number of observations and the number of items in each group. The absolute difference between the means of the pairwise comparison was compared with the estimated T. A pairwise comparison is significant if the absolute difference is greater than the estimated T.

2.6 The fermentation kinetics models

The modified Gompertz model is commonly applied (Zajsek & Gorsek, 2010, Fan et al., 2014), however, the model does not account for the effect of the substrate and product inhibition. In this study, a modified Monod Equation that incorporated both the substrate and the product inhibitions was proposed (Krishna, Ho & Tsao, 1999). The model equations describing the cell growth, the product formation, and the substrate consumption are given in Eq. 2.2 to 2.4, respectively. The unknown parameters were estimated using Solver programming of the Analysis Tool Pack of MS Excel (2021 version). The Error Sum of the Squares (SSE) between the experimental observation and the model was minimized with a constraint that the parameters were non-negative. The Excel Solver layout for the kinetics of ethanol production from the acid pretreated substrate hydrolysed with CF1 enzyme (CA-CF1), for example, is shown in Table 4.

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_m S}{K_s + S + {S^2}/{K_i}}\left\{1 - \left(\frac{P}{P_m}\right)^{\beta}\right\} \quad (2.2)$$

$$v = \frac{1}{X}\frac{dP}{dt} = \frac{v_m S}{K_{SP} + S + {S^2}/{K_{ip}}}\left\{1 - \left(\frac{P}{P_{ms}}\right)^{\gamma}\right\} \quad (2.3)$$

$$-\frac{dS}{dt} = \frac{1}{Y_{P/S}}\frac{dP}{dt} + \frac{1}{Y_{X/S}}\frac{dX}{dt} + mX \quad (2.4)$$

3.0 Results and Discussions

3.1 The Proximate Composition of Canarium Seeds.

The result of the proximate analysis, Fig.1 shows that the canarium seed was rich in hydrolyzable carbohydrates, but its lignin content was equally high., meaning that the hydrolyzable components were wrapped up in lignin, and they would require pretreatments to distort the structure and expose the cellulose for enzymatic attacks (Phitsuwan, Sakka & Ratanakhanokchai, 2013).

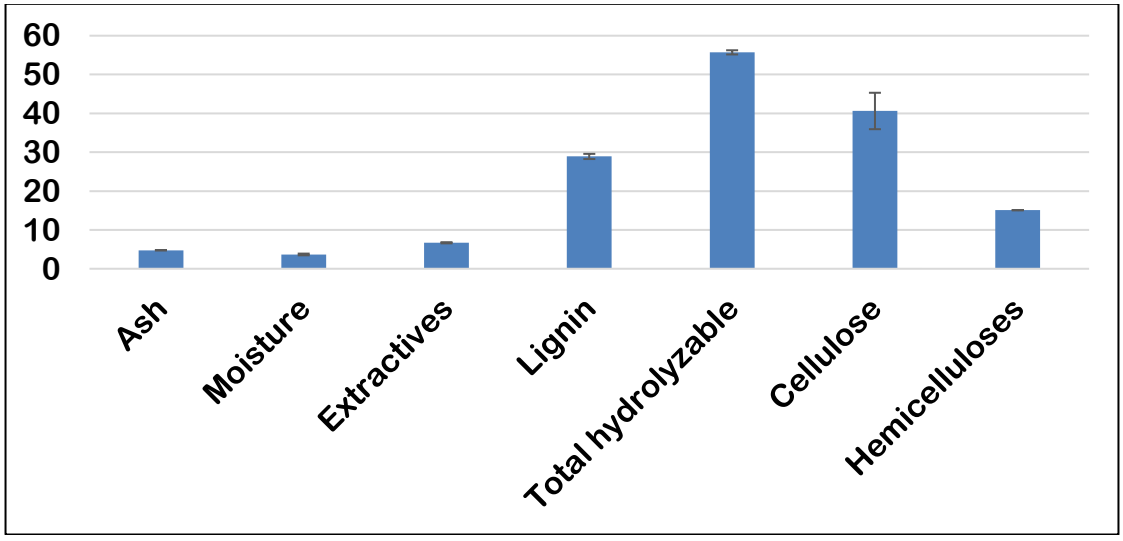


Figure 1: The percentage proximate composition of canarium seeds

3.2 The FTIR spectra and the SEM images of the substrates

Figure 2(a & b) are the Scanning Electron Microscopy (SEM) images of the canarium seed and crystalline cellulose, respectively. Though the morphologies of the structure were almost the same, the arrangement of the canarium seeds was not totally uniform, indicating heterogeneous components, but the pattern of crystalline cellulose were uniform. So, chemical pretreatment was necessary for exposing the cellulose in the canarium seeds to enzymatic hydrolysis. As explained by Bajpai (2016), polymers in a lignocellulosic biomass associate with one another in a hetero-matrix format at varying compositions, depending on the source of the material. The FTIR spectra are equally shown in Fig. 2 (c). The absorbance heights were plotted against the wavenumber. Meanwhile, the spectra for the differently pretreated canarium seeds were stacked, and the absorbance heights normalized within a vertical distance of 0.0 to 0.1 for each spectrum. The carbonyl C-O group appeared between wavenumber 1650 and 1780 cm^{-1}, the carboxylic OH-group between wavenumber of 2500 to 3000 cm^{-1}, the alcoholic C-O group between the wavenumber of 1025 and 1200 cm^{-1}, and the alcoholic OH-group between the wavenumber of 3000 and 3600 cm^{-1} (Zajsek & Gorsek, 2010). Looking at the spectra heights, in Fig. 2 (c), it was observed that the pretreatments reduced the carbonyl C=O group at the wavenumber of 1650-1780 cm^{-1} and the carboxylic OH group at the wavenumber of 2500-3000 cm^{-1}.

3.3 The compositions of the hydrolysates

The hydrolysates were characterized based on the percentage values of *Syn* (*Z*), *Anti*(*E*) glucose, and the xylose in the total hydrolysates. The pentoses are represented by the percentage of xylose while the hexoses are represented by the percentage of glucose either in Syn or Anti-form (Senila *et al*., 2011). The result showed that the hydrolysates

were almost of the same composition irrespective of the source of the enzyme. Figure 2(d) represented the values hydrolyzed with the CF1 enzymes. The differences were mainly due to the pretreatment method. This observation was confirmed from the results of the post hoc analysis on ethanol conversions from the different hydrolysates shown in Section 3.6 where the pairwise honest significant differences due to the enzyme sources were statistically insignificant. On the other hand, the composition of the hydrolysates varied with the type of pre-treatment given to the canarium seed. The non-pretreated seed, C, showed higher content of pentoses than hexoses, and the pretreated substrates (CA, CB, and CAF) had higher content of hexoses than pentoses. It can be observed that the acid pretreated, CA, and the alkaline pretreated, CB, had almost the same pentoses composition. However, the acid pretreated had a higher *Anti*-form of glucose than the alkaline pretreated. In the same way, the furan extracted, CAF, had the lowest pentoses than the others, and the glucose composition was equally high. Therefore, the pretreatment reduced the pentoses composition and enriched the hexoses, while the furan extraction further enhanced the removal of the pentoses. According to Ebert (2018), the composition of pentoses is directly linked to the formation of furan products, which would inhibit the ethanol formation with *saccharomyces cerevisiae*, so the furan extraction has been demonstrated to be effective in eliminating furan-forming components of the hydrolysates.

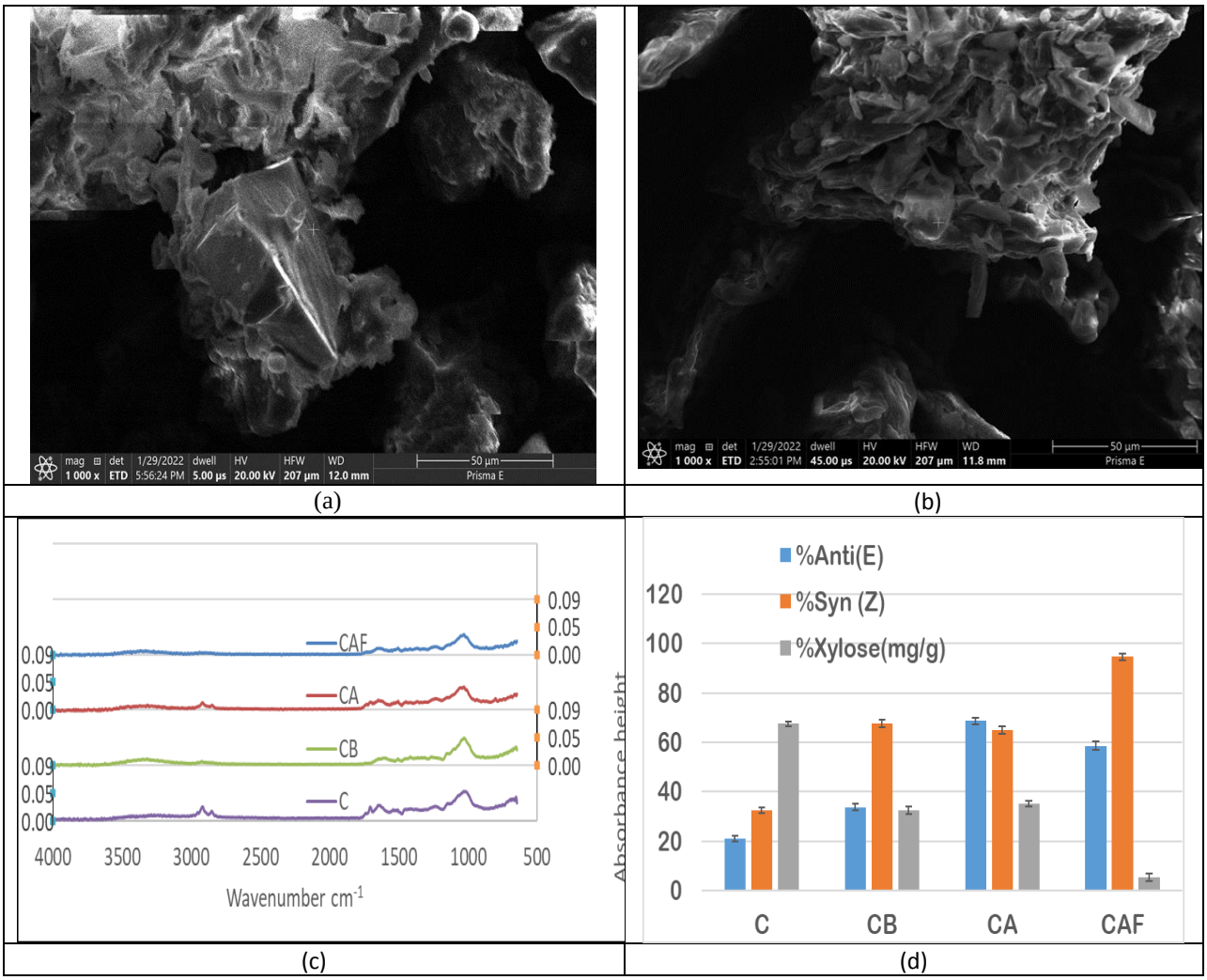
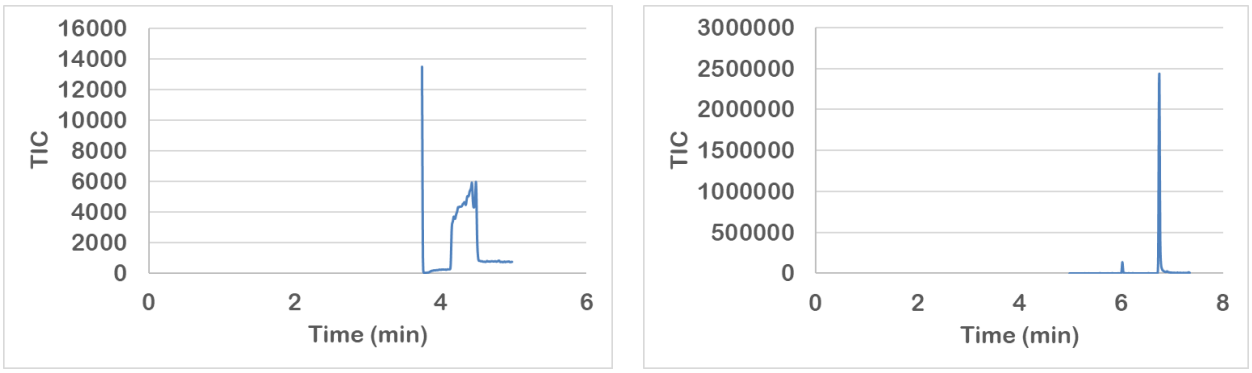


Figure 2: Characterization of the substrates: (a)SEM image of the canarium seed, (b)SEM image of crystalline cellulose, (c)FTIR spectrum of differently pretreated canarium seeds(d) sugar compositions of the enzymatic hydrolysate of the differently pretreated canarium seeds

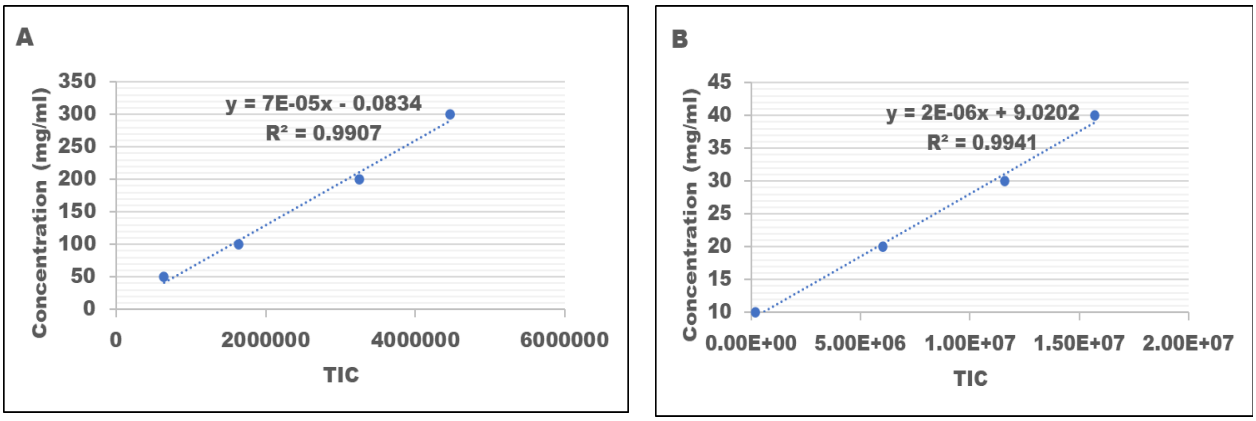
3.4 Characterization of the furan product

The furan product was characterized by the GC/MS, and the chromatograms of the furfural and the hydroxyl methyl furfural (HMF) fractions are in Fig. 3. The furfural and the HMF eluted at 4.3, and 6.8 min, respectively; the standard calibration curves, of coefficient of determination, R^2, above 0.99, were prepared, and the furfural was 2%, while the HMF was 72%. The standard calibration curves are shown in Fig.4 (a &b) for the furfural and HMF, respectively. The results and calculations for the percentage compostions of the furan components are shown in Table 1.



(a) (b)

Figure 3: The GC/MS chromatogram of the furan product: (a)the furfural fraction (b)the HMF fraction



(a) (b)

Figure 4: The GC/MS standards for furfural (a) and HMF (b) in butanol. The primary product ions for furfural and HMF were 95 and 69, eluting at 4.43 and 6.87 minutes, respectively

Table 1: Calculations for the percentage compositions of furfural and HMF in the furan product

	Furfural		**HMF**	
Parameter	Formula	Value	Formula	Value
Total ionic charge	X	6000	X	2431757
Concentration (mg/ml)	$\mathrm{C} = 7\mathrm{E} - 05 * \mathrm{X} - 0.08$	0.420	$\mathrm{C} = 2\mathrm{E} - 06 * \mathrm{X} + 9.02$	13.88
Total Volume (ml)	V	0.481	V	0.481
Dilution factor	D	60	D	60
Total Weight(g)	W	0.557	W	0.557
Component weight (g)	$M = (V * D * C)/1000$	0.010	$M = (V * D * C)/1000$	0.400
Weight Fraction (%)	$\frac{M}{W} \times 100$	2.180	$\frac{M}{W} \times 100$	71.94

3.5 The ethanol fermentation yield

The initial simple sugar composition of the hydrolysates was approximately 100 mg/ml, which would yield theoretical ethanol of 51 mg/ml. However, due to the presence of the inhibitors from the substrates and the products, the ethanol yield was impaired, and the actual yield from each hydrolysate was a function of the substrate preparation. Figure 5 shows the effects of the type of pretreatment on the percentage conversion of ethanol. The non-pretreated substrates, C-CF1, C-CF2, C-CF3, and C-COM had less than 10% ethanol conversion, meaning that the inhibition suppressed the conversion of about 90% of the simple sugars in the fermenter. The furan extracted substrates had about 63% conversion of the theoretical values, hence the combination of furan extraction and acid pretreatment enhanced the yield of ethanol from the canarium seed. It was equally observed that the type or the source of the cellulase enzyme could not contribute significantly to the differences in ethanol conversion, but a pretreatment, whether alkaline or acidic was necessary to improve the quality of the hydrolysate, and an additional furan extraction further enhanced the hydrolysates and the subsequent ethanol yield. This observation can be seen from the results of the pairwise Honest Significance Difference (HSD) in Section 3.6 (Table 3). Krishnan et al. (1999) used a mixture of pure glucose and xylose with a recombinant *S. cerevisiae* and recorded conversion of over 90% of the theoretical ethanol, so the heterogeneous nature of the hydrolysates in this work could have accounted for the relative lower conversion of ethanol. The maximum ethanol conversion in Fig. 5 was 63%, from the CAF-CF1. This value was equivalent to ethanol concentration of 3.32 g/100ml, corresponding to 0.332g ethanol for a 10 ml fermentation broth. With 1.0 g initial sugar in the fermenter, the maximum ethanol yield was 0.332 g ethanol/ g sugar. The maximum ethanol conversion from the unpretreated C-CF2 was 8.8%, corresponding to an ethanol yield of 0.045 g ethanol/g sugar. The ethanol yield from the furan-extracted substrate can be compared with the report by Ozmihci & Kargi (2007), which indicated that the maximum ethanol yield per sugar were 0.54 g ethanol/ g sugar, and Ahmad, *et al.* (2011) fermented glucose using S. cerevisiae and reported an ethanol yield per sugar of 0.668 g/g.

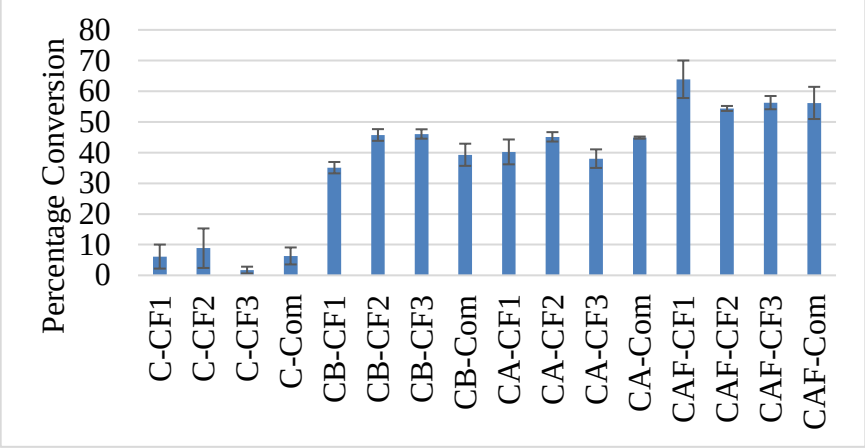


Figure 5: The ethanol percentage conversion from differently pretreated canarium seed

3.6 The statistical analysis of the ethanol conversion

The result presented in section 3.5 showed the differences in ethanol conversion from the differently pretreated canarium seed, but the statistical analysis was necessary to ascertain the statistical significance of the differences, hence a One-Way Analysis of Variance (ANOVA) was carried out using the MS Excel Analysis Tool Pack, and the p-value was much less than 0.01, meaning that the hypothesis that the conversions of ethanol from the differently pretreated canarium seed were the same could not be accepted. In other words, the alternative hypothesis that the ethanol conversions from the differently pretreated canarium seed were different cannot be rejected even at a 0.01 significant level. The ANOVA result is shown in Table 2. However, even though the ANOVA results affirm the significance of the ethanol conversion differences, a post hoc analysis was necessary to ascertain the significance of the pairwise differences among the groups. The results of a pairwise comparison of the ethanol conversion indicated that the conversions from the pretreated were significantly higher than that of the non-pretreated, and the conversions from the furan-extracted substrates were significantly higher than the other substrates, irrespective of the source of the enzyme. The result equally confirms that the ethanol conversion was not a function of the source of cellulase enzyme, but the nature of the hydrolysates.

The pairwise Honest Significance Difference (HSD), following the Turkey Kramer post hoc analysis (Glen, 2022) is in Table 3 for the differently pretreated canarium seeds. The HSD statistic, T, was calculated using the formula in Eq. 2.1 (Section 2.5), and the critical values of the studentized range, q, were obtained from the studentized range q table (Zaiontz, 2020**)**. The absolute difference between the ethanol conversions of the treatment pair was compared with the HSD statistics, and the "stat" was "S" (Significant) when the absolute difference was more than the HSD statistic and "NS" (Not Significant) when the absolute difference was less than the HSD statistic. The "stat" among the non-pretreated, the alkaline pretreated, the acidic pretreated, and the furan-extracted substrates with different enzymes were generally "NS," signifying that the source of the enzyme could not significantly affect the conversion of ethanol from the non-pretreated substrates. The "stat" among the non-pretreated and the alkaline pretreated, the non-pretreated and the acid pretreated, the non-pretreated and the furan-extracted substrates were generally "S," meaning that the ethanol conversions from the pretreated substrates were significantly higher than that from the non-pretreated substrates. The "stat" among the alkaline and the acidic pretreated substrates were more of "NS," indicating that the acidic and the alkaline pretreatment had almost the same effect on the ethanol conversions of the lignocellulosic substrates. The "stat" among the alkaline pretreated, or the acid pretreated, and the furan-extracted substrates were generally "S," showing that the furan-extraction further enhanced the ethanol fermentability of the lignocellulosic substrates.

Table 2: The ANOVA result for ethanol conversion from the differently pretreated canarium seeds.

Biomass	Source of Variation	SS	df	MS	F-value	P-value	F-crit
Canarium seeds	Between Groups	18017.46	15	1201.16	210.38	1.37E-27	1.99
	Within Groups	182.69	32	5.70			
	Total	18200.16	47				

Table 3: The pairwise comparisons of ethanol conversions from differently pretreated canarium seeds

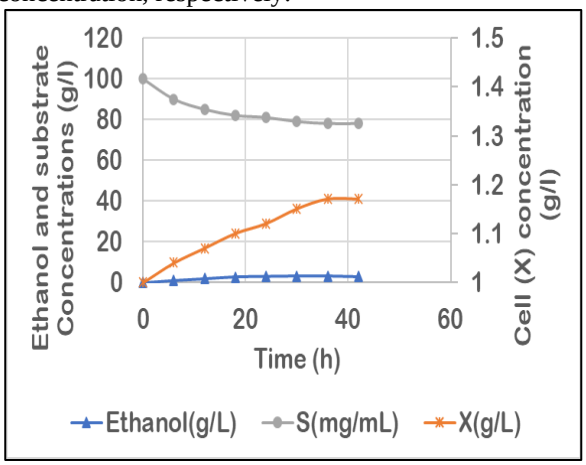
Comparison	AD	Stat	Comparison	AD	Stat	Comparison	AD	Stat
C-CF1 to C-CF2	2.7	NS	C-CF1 to CAF-CF3	50.2	S	CB-CF2 to CAF-CF1	18.2	S
C-CF1 to C-CF3	4.4	NS	C-CF1-to CAF-Com	50.1	S	CB-CF2 to CAF-CF2	8.6	S
C-CF1 to C-Com	0.2	NS	C-CF2 to CAF-CF1	55.1	S	CB-CF2 to CAF-CF3	10.5	S
C-CF2 to C-CF3	7.1	NS	C-CF2 to CAF-CF2	45.5	S	CB-CF2 to CAF-Com	10.4	S
C-CF2 to C-Com	2.5	NS	C-CF2 to CAF-CF3	47.4	S	CB-CF3 to CAF-CF1	17.8	S
C-CF3 to C-Com	4.6	NS	C-CF2 to CAF-Com	47.3	S	CB-CF3 to CAF-CF2	8.3	NS
C-CF1 to CB-CF1	29.0	S	C-CF3 to CAF-CF1	62.2	S	CB-CF3 to CAF-CF3	10.2	S
C-CF1 to CB-CF2	39.6	S	C-CF3 to CAF-CF2	52.7	S	CB-CF3 to CAF-Com	10.1	S
C-CF1 to CB-CF3	40.0	S	C-CF3 to CAF-CF3	54.6	S	CB-Com to CAF-CF1	24.6	S
C-CF1-to CB-Com	33.2	S	C-CF3 to CAF-Com	54.5	S	CB-Com to CAF-CF2	15.1	S
C-CF2 to CB-CF1	26.2	S	C-Com to CAF-CF1	57.6	S	CB-Com to CAF-CF3	17.0	S
C-CF2 to CB-CF2	36.9	S	C-Com to CAF-CF2	48.1	S	CB-Com to CAF-Com	16.9	S
C-CF2 to CB-CF3	37.2	S	C-Com to CAF-CF3	50.0	S	CA-CF1 to CA-CF2	4.9	NS
C-CF2 to CB-Com	30.4	S	C-Com to CAF-Com	49.9	S	CA-CF1 toCA-CF3	2.2	NS
C-CF3 to CB-CF1	33.4	S	CB-CF1 to CB-CF2	10.7	S	CA-CF1 to CA-Com	4.7	NS
C-CF3 to CB-CF2	44.0	S	CB-CF1 toCB-CF3	11.0	S	CA-CF2 to CA-CF3	11.1	S
C-CF3 to CB-CF3	44.4	S	CB-CF1 to CB-Com	4.2	NS	CA-CF2 to CA-Com	0.3	NS
C-CF3 to CB-Com	37.6	S	CB-CF2 to CB-CF3	0.3	NS	CA-CF3 to CA-Com	6.8	NS
C-Com to CB-CF1	28.8	S	CB-CF2 to CB-Com	6.4	NS	CA-CF1 to CAF-CF1	23.7	S
C-Com to CB-CF2	39.4	S	CB-CF3 to CB-Com	6.8	NS	CA-CF1 to CAF-CF2	14.2	S
C-Com to CB-CF3	39.8	S	CB-CF1 to CA-CF1	5.1	NS	CA-CF1 to CAF-CF3	16.1	S
C-Com to CB-Com	33.0	S	CB-CF1 to CA-CF2	10.0	S	CA-CF1-to CAF-Com	16.0	S
C-CF1 to CA-CF1	34.1	S	CB-CF1 to CA-CF3	2.9	NS	CA-CF2 to CAF-CF1	18.8	S
C-CF1 to CA-CF2	39.0	S	CB-CF1-to CA-Com	9.8	S	CA-CF2 to CAF-CF2	9.3	S
C-CF1 to CA-CF3	31.9	S	CB-CF2 to CA-CF1	5.5	NS	CA-CF2 to CAF-CF3	11.1	S
C-CF1-to CA-Com	38.8	S	CB-CF2 to CA-CF2	0.6	NS	CA-CF2 to CAF-Com	11.1	S
C-CF2 to CA-CF1	31.4	S	CB-CF2 to CA-CF3	7.7	NS	CA-CF3 to CAF-CF1	25.9	S
C-CF2 to CA-CF2	36.3	S	CB-CF2 to CA-Com	0.9	NS	CA-CF3 to CAF-CF2	16.4	S
C-CF2 to CA-CF3	29.2	S	CB-CF3 to CA-CF1	5.8	NS	CA-CF3 to CAF-CF3	18.3	S
C-CF2 to CA-Com	36.0	S	CB-CF3 to CA-CF2	0.9	NS	CA-CF3 to CAF-Com	18.2	S
C-CF3 to CA-CF1	38.5	S	CB-CF3 to CA-CF3	8.0	NS	CA-Com to CAF-CF1	19.0	S
C-CF3 to CA-CF2	43.4	S	CB-CF3 to CA-Com	1.2	NS	CA-Com to CAF-CF2	9.5	S
C-CF3 to CA-CF3	36.3	S	CB-Com to CA-CF1	0.9	NS	CA-Com to CAF-CF3	11.4	S
C-CF3 to CA-Com	43.2	S	CB-Com to CA-CF2	5.8	NS	CA-Com to CAF-Com	11.3	S
C-Com to CA-CF1	33.9	S	CB-Com to CA-CF3	1.3	NS	CAF-CF1 to CAF-CF2	9.5	S
C-Com to CA-CF2	38.8	S	CB-Com to CA-Com	5.6	NS	CAF-CF1 toCAF-CF3	7.6	NS
C-Com to CA-CF3	31.7	S	CB-CF1 to CAF-CF1	28.8	S	CAF-CF1 to CAF-Com	7.7	NS
C-Com to CA-Com	38.6	S	CB-CF1 to CAF-CF2	19.3	S	CAF-CF2 to CAF-CF3	1.9	NS
C-CF1 to CAF-CF1	57.8	S	CB-CF1 to CAF-CF3	21.2	S	CAF-CF2 to CAF-Com	1.8	NS
C-CF1 to CAF-CF2	48.3	S	CB-CF1-to CAF-Com	21.1	S	CAF-CF3 to CAF-Com	0.1	NS

Note: C-CF1, C-CF2, C-CF3- Non-pretreated with different crude enzymes; C-Com-Non pretreated with the commercial enzyme; CB-CF1, CB-CF2, CB-CF3, CB-Com-The alkaline pretreated with the enzymes; CA-CF1, CA-CF2, CA-CF3, CA-Com-the acid pretreated with the enzymes; CAF-CF1, CAF-CF2, CAF-CF3, CAF-Com- the furan-extracted with the enzymes

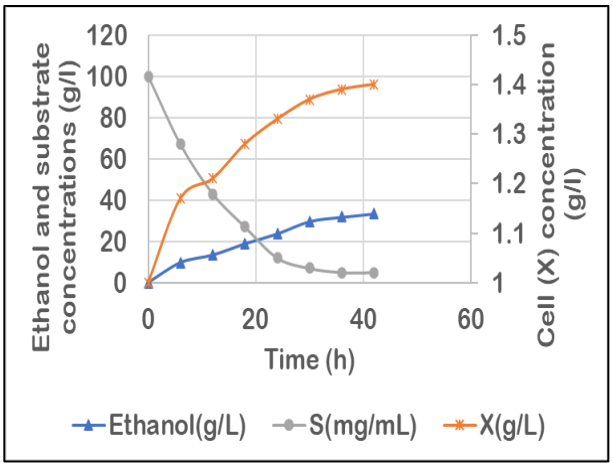
3.7 The kinetics study

The ethanol conversion, the substrate concentration, and the cell growth were measured, and the results from the non-pretreated and the furan extracted substrates hydrolyzed with the crude enzyme, CF1, are in Fig. 6. The results from CF1 were presented because the ethanol conversion was not a function of the source of cellulase enzyme, but the nature of the hydrolysates, as shown in Table 3. The increasing ethanol and the decreasing substrate concentrations were plotted on the primary vertical axis on the left while the growing cell concentration was plotted on the secondary axis on the right. There were hindrances to the availability of the substrate for cell utilization, and this affected both the growth of the cells and the ethanol conversion from the non-pretreated substrate as seen in Fig.6(a). The maximum yield from the non-pretreated canarium seeds was 2.8 g/l (8.8% conversion), corresponding

to an ethanol selectivity of 25%, meaning that the substrate was converted to other unwanted products; however, part of the substrate was consumed for the cell maintenance. As shown in Fig. 6(b), the maximum ethanol yield from the furan-extracted canarium seed was 33 g/l (63% conversion) with corresponding ethanol selectivity of 69%, so the furan extraction further improved the maximum ethanol yield and the selectivity. Figure 7(a, b & c) shows the FTIR calibration standard for ethanol, the UV spectrophotometer calibration standards for simple sugars and cell concentration, respectively.

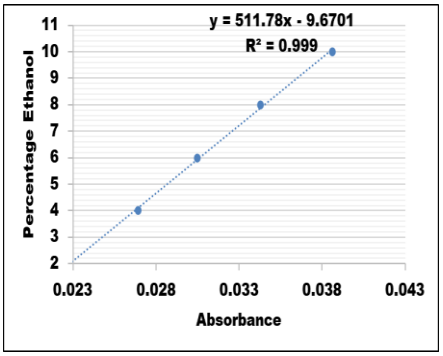


(a)

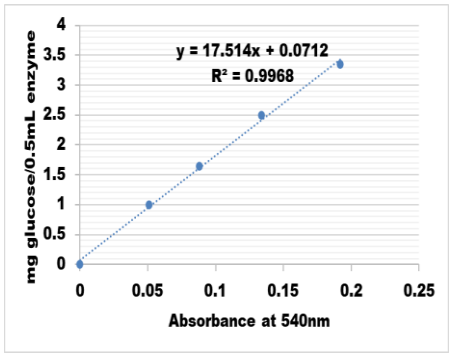


(b)

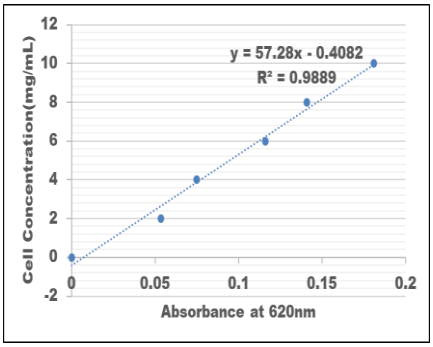
Figure 6: The variation of the ethanol, the cells, and the substrate concentrations with time from (a)the non-pretreated and (b) the furfural-extracted substrates



a



b

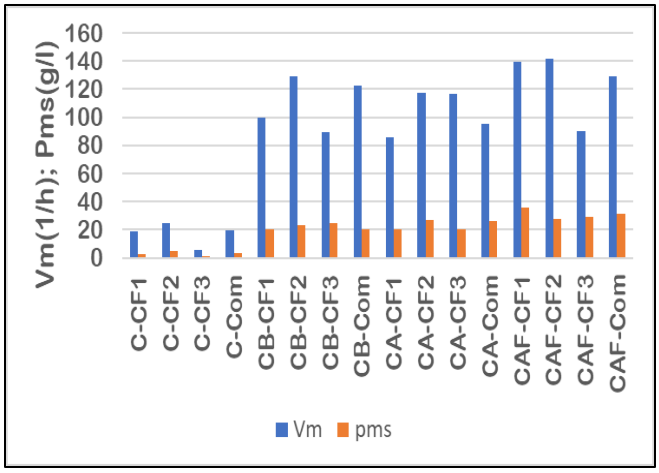


c

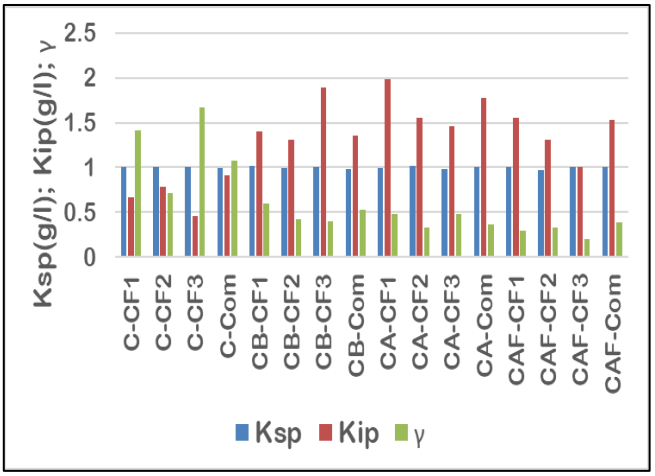
Figure 7: The calibration standards for (a) ethanol with FTIR, (b) simple sugars with UV-spectrophotometer, and (c) cell concentration with spectrophotometer.

3.7.1 The kinetics model of ethanol production

The kinetics of the ethanol production was modeled with Eq. 2.3, and the kinetics parameters were Fig. 8. Both the maximum specific rate of ethanol formation (Vm) and the maximum ethanol concentration (Pm) were higher with the pre-treated substrates than with the un-pretreated substrates, but the lambda values for the un-pretreated were higher than the pretreated. The higher lambda values raised the inhibition due to the product formation, and this suppressed the specific rate of the product formation. While the Monod's constant seemed relatively the same for the groups, the inhibition constant for product formation was higher with the pretreated substrates than with the non-pretreated as in Fig. 8(b), and this means a higher specific rate of product formation.



(a) Vm, Pms

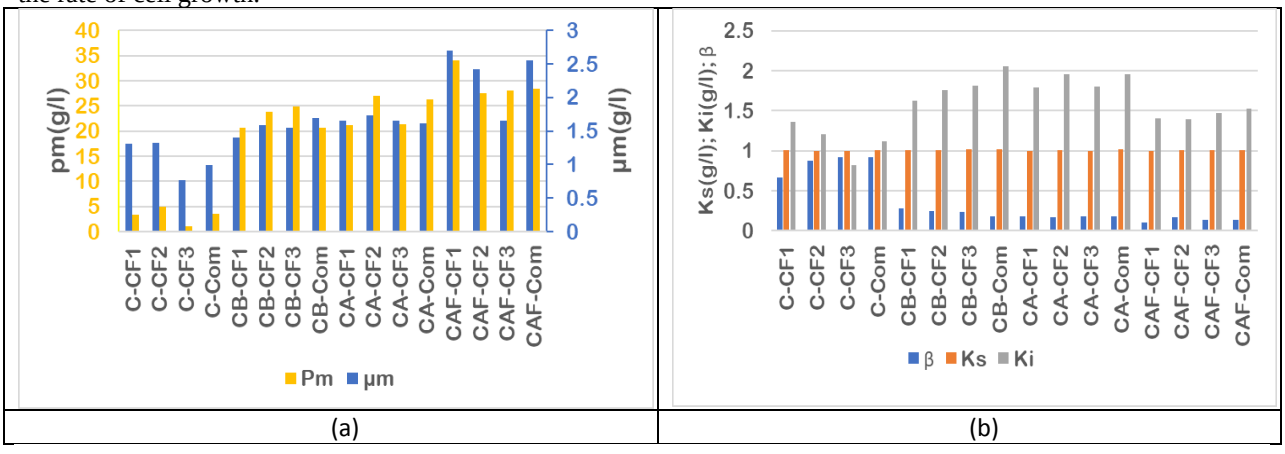


(b) Ksp, Kip, γ

Figure 8: The kinetics parameters for the ethanol production: (a)the maximum specific rate of product formation (Vm), and the product concentration above which product could not form (Pms), (b) the Monod's constant for product formation (Ksp), the inhibition constant for product formation (Kip), and the product formation inhibition coefficient (γ)

3.7.2 The kinetics of the cell's growth

The cell's specific growth rate was modeled as a function of substrates and product concentrations using Equation 2.2 and the maximum specific growth rate and the maximum product formation are plotted in Fig. 9(a). The average maximum specific growth rate of the cells with the furan extracted substrates was 2.0 h^{-1}, but the growth rates were suppressed to values below 1.5 h^{-1} with the non-pretreated substrates. While the Monod's constants in Fig. 9(b) were relatively the same, which could be attributed to the yeast strain, the corresponding inhibition constants were higher than the Monod's constants, especially with the pre-treated substrates, and this means that the pre-treatment eliminated some inhibitions to the growth of the cells. The inhibition of cell growth due to the product formation (β) was lower than the inhibition of product formation due to the product (γ), as can be seen in Figs. 8(b) and 9(b). But the differences in the values of β and γ can be attributed to the fact that the rate of product formation was higher than the rate of cell growth.



(a) (b)

Figure 9: The kinetics parameters of the cell growth: (a) the maximum specific rate of cell growth (μm), and the maximum product concentration above which cells could not grow (Pm), (b) the Monod's constant for cell growth (Ks), the inhibition constant for cell growth (Ki), and formation inhibition coefficient on cell (β)

3.7.3 The kinetics of the substrate consumption

The effects of the furan inhibition can also be seen from the substrate consumption rate, as the substrate consumption would be higher with inhibition-free substrates. The product yield constant, $Y_{p/s}$, is an indication of the amount of the substrate that translated directly to the product, and the results in Fig. 10 affirmed that the furan extracted canarium seeds with the highest $Y_{p/s}$ values were more available for ethanol conversion than the other substrates. Comparing the $Y_{p/s}$ with the composition of the hydrolysates in Figure 2(d), the $Y_{p/s}$ values were proportional to the glucose composition in the biomass, but some of the substrates were involved in the cell growth as indicated by the cell yield constant, $Y_{x/s}$. The Yp/s values for the furan-extracted substrates ranged between 0.36 and 0.45 and these fall within the range reported for similar works. Ozmihci & Kargi (2007) reported a maximum productivity and growth yield coefficients of 0.54 and 1.2, respectively, from the fermentation of cheese-whey powder using lactose utilizing yeast strain, and Chen et al. (2012) reported an ethanol yield coefficient of 0.36 from a closed circulating system with a pervaporation membrane. Germec et al. (2019) investigated different kinetic models and reported that Weibull Model predicted accurately the experimental findings, and the ethanol and cell yield coefficients were 0.48 and 0.11, respectively, and Krishnan, Ho & Tsao (1999) applied the modified Monod's models and reported ethanol and cell yield coefficients of 0.47 and 0.11, respectively, supporting that the ethanol yield coefficients in this work were in close agreement with other reports, irrespective of the kinetics model, but the cell yield coefficient depends on the model, the strain of the yeast, and the fermentation system.

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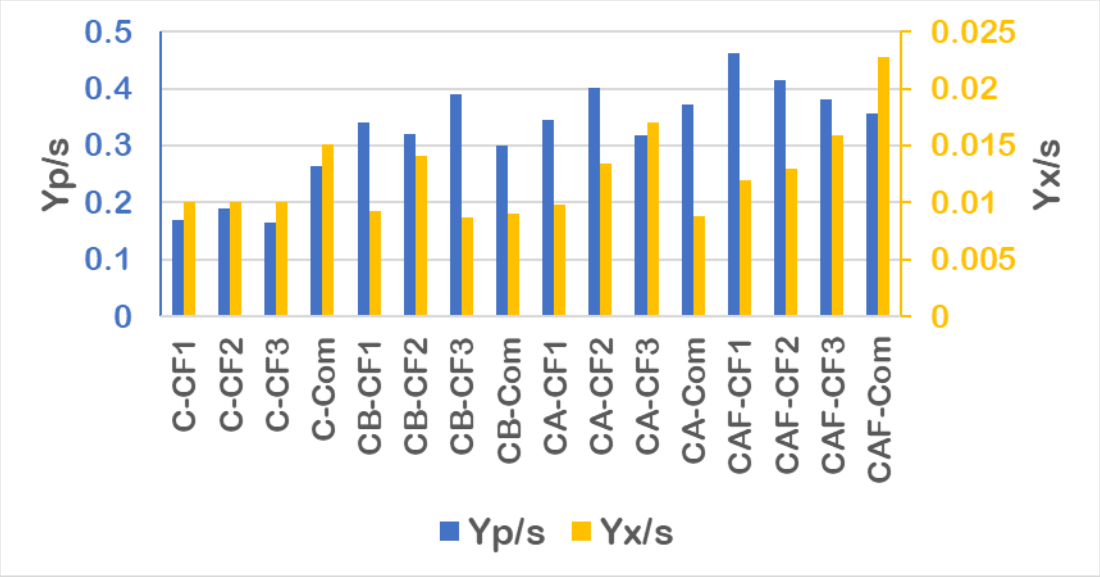


Figure 10: The product yield and the cell growth constants per substrate

Table 4: The excel solver layout for the estimation of the kinetics constants for ethanol production from acid pretreated substrate hydrolyzed with CF1 enzyme

Estimation of the maximum specific cell growth rate μm (Eq. 2.2)

Time	Ethanol (g/L)	S(mg/mL)	X(g/L)	dX/dt	μ= (1/X(dX/dt))	Model	Error Square	μm	1.658
0	0.000	100.000	1.000	0.029	0.029	0.029	2.29E-11	Ks	1.001
6	7.890	72.000	1.120	0.011	0.010	0.006	1.02E-05	Ki	1.790
12	11.430	58.000	1.130	0.002	0.001	0.005	1.36E-05	Pm	21.21
18	15.610	37.000	1.140	0.003	0.003	0.004	1.27E-06	β	0.177
24	18.960	25.000	1.170	0.004	0.004	0.002	1.9E-06	sum	0.000
30	19.340	21.000	1.190	0.003	0.003	0.002	4.7E-07	R^2	0.956
36	19.719	19.000	1.210	0.003	0.002	0.002	5.36E-08		
42	20.027	18.000	1.220	0.001	0.001	0.002	7.02E-07		

Estimation of the maximum specific product formation rate, vm (Eq. 2.3)

Time (hr)	Ethanol (P) (g/L)	dp/dt	X(g/L)	S(g/L)	V= [(1/x)dp/dt]	V (model)	Error Square	Vm	86.11
0	0.00	1.68	1.00	100.00	1.68	1.68	0.00	Ksp	0.995
6	7.89	0.95	1.06	72.00	0.90	0.84	0.00	Kip	1.987
12	11.43	0.64	1.11	58.00	0.58	0.68	0.01	Pms	20.08
18	15.61	0.63	1.14	37.00	0.55	0.50	0.00	Y	0.485
24	18.96	0.31	1.17	25.00	0.27	0.17	0.01	sum	0.032
30	19.34	0.06	1.19	21.00	0.05	0.14	0.01	R^2	0.986
36	19.72	0.06	1.21	19.00	0.05	0.07	0.00		
42	20.03	0.05	1.22	18.00	0.04	0.01	0.00		

Estimation of the product and the cell yield constants Yp/s and Yx/s (Eq. 2.4)

Time	S(mg/mL)	-(dS/dt)	dp/dt	X(g/L)	dX/dt	Model	Error Square	Yp/S	0.345	
0	100.000	5.833	1.678	1.000	0.029	7.857	4.094	YX/S		0.010
6	72.000	3.500	0.953	1.120	0.011	3.884	0.148	m		0.015
12	58.000	2.917	0.643	1.130	0.002	2.052	0.747	Sum		5.379
18	37.000	2.750	0.628	1.140	0.003	2.177	0.329	R^2	0.917	
24	25.000	1.333	0.311	1.170	0.004	1.344	0.000			
30	21.000	0.500	0.063	1.190	0.003	0.541	0.002			
36	19.000	0.250	0.057	1.210	0.003	0.439	0.036			
42	18.000	0.083	0.045	1.220	0.001	0.235	0.023			

4.0. Conclusion

The canarium seed was high in hydrolyzable sugars, but it was equally high in lignin and hemicelluloses, meaning that its usage as a feedstock for ethanol production can only be efficient if a chemical pretreatment module is incorporated. The alkaline and the acidic pretreatments were effective at enriching the glucose content over the pentoses, but the extraction of furan product further improved the quality of the hydrolysate, reducing the cell growth inhibition and improving the ethanol conversion. Moreover, the furan extract was rich in HMF, so the process simultaneously obtained a complementary product of high economic value. The pairwise post hoc analysis revealed that the rate of ethanol production did not depend on the source of the cellulase enzyme, rather it was a function of the pretreatment. Whereas the non-pretreated substrates had the lowest rate of ethanol yield, the differences from the alkaline and acidic pretreated substrates were not statistically significant, but the yields from the furan extracted substrates were significantly higher than the others. The values of the kinetics parameters indicated that the non-pretreated substrates had lower specific cell growth and product yield, and the substrate consumption rate was higher on the pretreated substrates, especially the furan extracted substrates, integrating furan extraction into the dilute acid pretreatment of canarium seed enhanced the ethanol fermentability and selectivity of the substrates in addition to a complimentary product of high economic importance.

5.0 Recommendation

Research to separate and purify the furan products would improve the economic importance of this project

Acknowledgements

We appreciate the Fulbright Foreign Students Scholarship Board, the Institute of International Education, USA for sponsoring this research.

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