Response Surface Optimisation of Enzymatically Hydrolysed and Dilute Acid Pretreated Oil Palm Trunk Bagasse for Succinic Acid Production

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The exploitation of agroindustrial lignocellulose, such as oil palm trunk bagasse (OPTB), as a raw material in the production of succinic acid (SA) may serve as an effective strategy to propel the bio-based industry. This study aimed to optimise the recovery of fermentable sugar, *i.e.*, glucose, from enzymatic hydrolysis of the dilute acid pretreated OPTB (DA-OPTB). The dilute acid pretreatment used in this study was able to remove 59.5% of hemicellulose and 13.3% of lignin. Response surface methodology (RSM) based on central composite design (CCD) was then applied to investigate four independent variables – enzyme loading (10 to 50 U/g), agitation speed (50 to 250 rpm), reaction time (0 to 96 h), and surfactant concentration (0.025 to 0.125%, v/v). The experimental glucose concentration of 21.7 g/L was in good agreement with the RSM-predicted value of 20.5 g/L. Among the parameters investigated, supplementation of a surfactant during enzymatic hydrolysis was significant in influencing glucose recovery, while the extent of the agitation speed was the least influential. The maximum recovered glucose was estimated at 217 g per kg of raw OPTB, with 7.3 g/L of SA attainable from the fermented DA-OPTB hydrolysate using *Actinobacillus succinogenes* 130Z. The results demonstrated that OPTB can be practically utilised in the economical production of high value-added SA.

Keywords: Oil palm biomass; Lignocellulose; Fermentable sugar; Central composite design; Succinic acid

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INTRODUCTION

Recently, utilisation of agro-biomass as sustainable fermentation feedstock has gained considerable attention, given its virtually unlimited and low-cost supply compared to the commercially available refined sugars. One such major lignocellulosic biomass available in Malaysia is oil palm trunk (OPT), which can be found in abundance, particularly during replanting activities at oil palm plantations. It is noteworthy that the economic lifespan of an oil palm tree lies between 20 and 30 years; normally replanting is scheduled at an interval of 25 years. Based on 5.81 million ha of the total oil palm planted area in 2017 (Kushairi *et al.* 2018), approximately 21.64 million tonnes (dry weight) of OPT was generated from 290,500 ha of oil palm trees. Currently, only a small portion of OPT is being utilised for plywood manufacturing, fetching an estimated RM 30 to 40 (equivalent to USD 7 to 10) per trunk. The remaining trunks are largely left in the field as

mulch material or unintentionally serving as a breeding ground for insect pests, *e.g.*, *Ganoderma* sp. and white rot fungi (Ramle *et al*. 2005; Murata *et al.* 2013), which has culminated in rising greenhouse gas (GHG) levels.

The fact that OPT has a high moisture content is indicative of a huge availability of sap amounting to 67% to 82% of the whole trunk weight (Kosugi *et al.* 2010; Nurul Adela and Loh 2015). Regardless of the rich content of simple sugars in the squeezed sap of OPT, the remaining fibre after sap extraction, *i.e.*, OPT bagasse contains a significant amount of structural carbohydrates in the form of cellulose, hemicellulose, and starch that can be further hydrolysed to simple sugars for subsequent microbial fermentation. However, the intrinsic recalcitrant nature of the lignocellulosic bagasse is a bottleneck for efficient sugar recovery due to cross-linkages between polysaccharides (cellulose and hemicellulose) and lignin *via* ester and ether bonds. Therefore, pretreatment is necessary to alter the bagasse's complex structure to enhance cellulose digestibility, hydrolysis rate, and substrate specificity upon enzymatic hydrolysis (Mtui 2009).

Although many biomass pretreatment methods have been developed, only a few can be implemented on an industrial scale concerning both environmental and economic constraints (Akhtar *et al.* 2014). Sequential dilute acid (DA) pretreatment and enzymatic hydrolysis is promising (Loow *et al.* 2016) and has been extensively studied using wood (Zhou *et al.* 2014), corn stover (Avci *et al*. 2013), wheat straw (Satari Baboukani *et al.* 2012), rice straw (Kshirsagar *et al.* 2015), and oil palm empty fruit bunch (EFB) (Nurul Adela *et al.* 2014). DA pretreatment using sulphuric acid is the most commonly used due to low cost and high catalytic performance. The DA-pretreated feedstocks have demonstrated the ability to retain a copious amount of cellulose by expelling phenolic and hemicellulosic components in the resulting liquid portion of the hydrolysate. Despite having specific dissolution characteristics for targeted biomass components, a DA pretreatment is insufficient to remove lignin for subsequent enzymatic hydrolysis (Noparat *et al.* 2015). Therefore, various operational conditions for practical pretreatment of OPTB with DA are required to efficiently use this biomass for the production of many important bioproducts, such as succinic acid (SA).

According to the U.S. Department of Energy (Werpy *et al.* 2004), SA – a four-carbon aliphatic dicarboxylic acid, $C_4H_6O_4$ – has become one of the top 12 building block chemicals. It serves as an important precursor for many industrially manufactured chemical commodities and specialty products (Zeikus *et al.* 1999). Conventionally, SA is synthesized *via* the catalytic hydrogenation of maleic anhydride. However, this process involves an extensive amount of energy and possesses a limited supply of resources. Perhaps, a bio-based production route incorporating sustainable feedstock could augment its market size in the future (Fu *et al.* 2014). Its commercial potential warrants much efforts looking into producing SA from inexpensive and renewable substrates (Tan *et al.* 2014). Among others, sugarcane bagasse (Borges and Pereira 2011), corn stover (Zheng *et al*. 2010), corn fibre (Chen *et al.* 2011), and carob pods (Carvalho *et al.* 2014) have been demonstrated as potential feedstock for SA production with the use of *Actinobacillus succinogenes*. *A. succinogenes* is recognized as one of the most promising microorganisms for industrial SA production due to its great metabolic capability and tolerance to high concentrations of organic acids (Carvalho *et al.* 2016; Jiang *et al.* 2017). However, little is known on the production of SA from oil palm biomasses (Pasma *et al.* 2013; Akhtar and Idris 2017; Luthfi *et al.* 2016, 2017; Tan *et al.* 2016, 2018). To the best of the authors' knowledge, no study yet has elucidated the potential use of OPTB in SA production.

Thus, this study explored and optimised the pretreatment of OPTB *via* sequential DA and enzymatic hydrolysis using response surface methodology (RSM). The investigated variables were enzyme dosage, agitation rate, hydrolysis time and surfactant (Triton X-100) concentration. Initially, optimisation of the pretreatment conditions was conducted to remove hemicellulose to improve cellulose accessibility during enzymatic hydrolysis. The enzymatic hydrolysis was optimised to enhance the production of glucose for subsequent fermentation experiments. Additionally, the fermentability of OPTB hydrolysate for SA production was evaluated. A wild-type bacterium, *viz.*, *Actinobacillus succinogenes* 130Z, was selected as the SA-producing host due to its natural ability to produce SA at high concentrations.

EXPERIMENTAL

Materials

Oil palm trunk bagasse

The oil palm trunk bagasse (OPTB) samples were collected from the Malaysian Palm Oil Board (MPOB) Research Station, Pekan Bangi Lama, Selangor, Malaysia. The OPTB was ground using a laboratory stainless steel grinder to obtain an average particle size of less than 10 mm. The enzyme used was supplied by Universiti Kebangsaan Malaysia-Malaysia Genome Institute (Bangi, Malaysia), namely UKM-enzyme Formulation-3, with a total enzyme activity of 1493 U/mL. A mixture of cellulase and endoglucanase was produced from a submerged fermentation process by a genetically modified *Pichia pastoris* X-33 strain (Invitrogen/Life Technologies, CA, USA). One unit of enzyme activity was defined as the quantity of enzyme needed to digest EFB in achieving 1 µmol of glucose per minute under standard assay conditions at pH 5.0 and 50 ^{o}C.

Methods

Dilute acid pretreatment

The dried OPTB at 10% (w/v) solid loading was immersed in a dilute sulphuric acid (H_2SO_4) solution under different retention times (15, 30, 60, 90, and 120 min), temperatures (110 and 120 ºC), and H_2SO_4 concentrations (0.5, 0.7, 1.0, 1.5, and 2.0%, v/v) to obtain the best pretreatment conditions. The mixture was autoclaved at 121 ºC for 15 min. After that, the DA-pretreated OPTB (DA-OPTB) was washed thoroughly with hot water to a neutral pH and oven-dried at 80 ± 2 ºC to constant weight. The structural carbohydrates of the dried DA-OPTB were calculated gravimetrically, and the optimised DA-OPTB was used for subsequent enzymatic hydrolysis.

Optimisation of enzymatic hydrolysis

The optimised DA-OPTB (5%, w/v) was mixed with 100 mL of 0.05 M citrate buffer solution (pH 5.0) in a 250-mL Erlenmeyer flask. The doses of enzyme and Triton X-100 added to the flask are outlined in Table 1. The resulting mixtures were incubated in an incubator shaker (Innova® 40, New Brunswick, Germany) at 50 ºC and 150 rpm. Samples aliquots were withdrawn at specific periods according to Table 1 and analysed for their released sugars content.

Table 1. Coded Values, Experimental Ranges, and Levels of the Studied Independent Variables

Code	Variable	-α	-1	0	+1	+α
X_1	Enzyme dosage (U/g)	10	20	30	40	50
X_2	Agitation (rpm)	50	100	150	200	250
X_3	Hydrolysis time (h)	0	24	48	72	96
X_4	Triton X-100 concentration (%, v/v)	0.025	0.050	0.075	0.100	0.125

The RSM employed was based on Design-Expert, Version 9.0.5.1 software (Stat-Ease Inc., Minneapolis, MN, USA). Central composite design (CCD) was applied to investigate and obtain combined effects and interactions caused by several independent variables: enzyme dosage (U/g), agitation (rpm), hydrolysis time (h), and Triton X-100 concentration (%, v/v) for optimal glucose production from OPTB. The CCD primarily consisted of a 24 factorial experimental design with six replicated centre points leading to 30 experimental runs covering the four independent variables investigated at five different levels (-α, -1, 0, 1, and +α) (Table 1). Their level of significance was evaluated by a variance analysis (ANOVA).

Three-dimensional (3D) surface plots were drawn to show the effects of the employed independent variables on the response. A quadratic polynomial equation was proposed to describe the mathematical relationship between the response and the variables. The fitness and accuracy of the model were determined by R^2 and adjusted R^2 coefficients, while the F-test was used to ascertain the significance of the model. The effects of the variables and their possible interactions were evaluated. The significance of each variable was determined by its p-value (Prob > F). Coefficients of the full model were analysed to eliminate insignificant variables (p-value > 0.05) from the model. The reduced model was then adjusted. The model-derived optimum value of each selected variable was validated by solving the regression equation using the Design-Expert 9.0.5.1 software. These predicted optimum values were confirmed by running the experiments using each of the derived optimum values for the studied variables.

Fermentation of DA-OPTB Hydrolysate

Actinobacillus succinogenes 130Z used for the fermentation was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany). The inoculum was prepared using Brain Heart Infusion (BHI) medium under aerobic conditions at 37 ºC and 150 rpm for 18 h. The DA-OPTB hydrolysate was supplemented with (per litre of medium): 0.2 g magnesium chloride hexahydrate ($MgCl_2.6H_2O$); 0.2 g calcium chloride dihydrate ($CaCl_2.2H_2O$); 3.0 g potassium dihydrogen phosphate (KH_2PO_4); 1.0 g NaCl; 15.0 g yeast extract, and 40.0 g magnesium carbonate ($MgCO_3$). The OPTB hydrolysate was dispensed into the serum bottles as the main carbon source, capped with a butyl rubber stopper, and then clamped with an aluminum seal. Batch cultivation was carried out in 100-mL serum bottles by adding 10% (v/v) inoculum to a working volume of 50 mL under sterile conditions, and the culture was incubated at 37 ºC and 200 rpm for 48 h.

Chemical analyses

The holocellulose, α-cellulose, and lignin contents of the biomass samples were analysed based on the following standards: ASTM D1104-56 (1978), ASTM D 1103-60

(1978), and TAPPI T222 om-11 (2011), respectively. The hemicellulose content was calculated as the difference in the weights of α-cellulose and holocellulose. The ash and moisture contents were determined by heating the samples at 750 ºC for 120 min using a thermogravimetric analyser (TGA) (TGA710, LECO, St. Joseph, MI, USA). The quantification of the chemical compositions of the DA-OPTB hydrolysate was conducted using a high performance liquid chromatography (HPLC) system as follows: Sugar-pack column (6.5 mm × 300 mm) (Waters, MA, USA); 80 ºC; and a mobile phase of ionized water at 0.5 mL/min (flow rate) for sugars; IC-Pak™ Ion-exclusion 50A 7 μm column (7.8 mm × 150 mm) (Waters, MA, USA); 40 ºC; and a mobile phase of 2.5 mM H_2SO_4 at 0.5 mL/min (flow rate) for succinic, formic, and acetic acids. The apparatus was integrated with an auto sampler (Waters 2707, MA, USA), refractive index detector (Waters 2414, MA, USA), and isocratic HPLC Pump (Waters 1515, MA, USA).

The bacterial growth was estimated by measuring the dry cell weight (DCW) as the cell biomass concentration. The resulting culture broth was centrifuged at 4000 rpm for 10 min to separate the cells. The supernatant was removed and the cells were dried at 80 ºC to a constant weight as cell biomass.

RESULTS AND DISCUSSION

Pretreatment of Oil Palm Trunk Bagasse

To date, DA pretreatment has been successfully exploited to fractionate hemicellulose effectively and improve the accessibility of cellulose in EFB (Nurul Adela *et al.* 2015). In this study, OPTB was pretreated similarly with DA targeting to remove most of the hemicelluloses but retain celluloses to maximise the release of glucose during enzymatic hydrolysis. The efficiency of a biomass pretreatment depends on the rate of hemicellulose hydrolysis into prehydrolysate liquors (Deshavath *et al.* 2017) as indicated by the amount of xylose released. The most influential factors – retention time, temperature, and acid dose – affecting biomass pretreatment (Avci *et al.* 2013) were selected for an OPTB optimisation study. Their effects on sugar recovery are shown in Table 2. Xylose content steadily increased with time at two different temperatures (110 and 120 ºC) using a fixed concentration of 1.0% (v/v) H_2SO_4. The ANOVA and Tukey's tests showed that the xylose removal was significantly different ($p < 0.05$) at 110 ºC for all the tested reaction durations, whereas those at 120 ºC for 90 and 120 min were insignificant ($p = 0.05$). At 120 ºC, although the removal increased initially from 15 to 60 min, it stagnated thereafter, probably due to dehydration of xylose to furfural (Kamireddy *et al*. 2013), which could be prompted at higher temperatures. Based on the findings, 120 ºC and 90 min were regarded as the optimal conditions for DA pretreatment of OPTB. At these conditions, 1.0% (v/v) of H_2SO_4 gave the highest hemicellulose removal, and it was able to get rid of 18.13 ± 0.37 g per 100 g OPTB (Table 2). The acid pretreatment is able to hydrolyse cellulose to an amorphous stage, making glucose more easily accessible during DA pretreatment. The rate of glucose release resulted from an increase of OPTB hydrolysis was due to severe pretreatment employed. In this study such depolymerisation was more pronounced when higher H_2SO_4 concentrations was employed from 1.0% (v/v) to 2.0% (v/v), which led to 71.4% higher glucose yield from 9.08 ± 0.52 g/g to 15.56 ± 1.14 g/g, respectively. Consequently, DA pretreatment of OPTB using 1.0% (v/v) mild acid at 120 ºC for 90 min was used for subsequent optimisation of enzymatic hydrolysis.

The chemical composition of the DA-OPTB differed from that of the raw OPTB (Table 3). The raw OPTB was mainly comprised of 30.86 ± 3.29% cellulose, 25.84 ± 4.61% hemicellulose, and 24.29 ± 5.82% lignin. The cellulose content increased by 64.7% to 50.84 ± 2.41%, while the hemicellulose was reduced substantially (by 59.5%), and the lignin only slightly (by 13.3%), after DA pretreatment. The changes might have been associated with disruption of the ultrastructure of lignocelluloses. Generally, hemicellulose is crosslinked to a lesser extent, relatively amorphous, and more easily hydrolysed than cellulose during pretreatment (Lai and Idris 2013). The more encrusted lignin might require temperature > 160 ºC for better removal (Chen *et al*. 2011). Ash content was reduced by 67.7% from 2.29 ± 0.15% in raw OPTB to 0.7 ± 0.11% after DA pretreatment due to silica removal by acid. The applied DA pretreatment had led to the partial removal of hemicellulose and reduction of crystallinity, thus may also affect loss of the moisture content. The pretreated biomass, DA-OPTB, containing mainly cellulose and lignin became more porous and could easily be hydrolysed by cellulase in the next step.

Table 2. Total Yields of Xylose and Glucose from Oil Palm Trunk Bagasse During Acid (H_2SO_4) Pretreatment at Varying Temperature, Retention Time, and Acid Concentration

Temperature (ºC)	Time (min)	Acid Dose (v/v)	Yield (g 100 g^{-1} raw OPTB)		Conversion (%)[a]	
			Xylose	Glucose	Xylose[b]	Glucose[c]
110	15	1.0	2.16 ^{E} ± 0.21	2.56 ± 0.13	8.35	8.29
110	30	1.0	5.23 ^{D} ± 0.34	4.38 ± 0.28	20.25	14.20
110	60	1.0	9.09 ^{C} ± 0.54	6.98 ± 0.75	35.18	22.61
110	90	1.0	13.49 ^{B} ± 0.74	8.27 ± 0.99	52.22	26.80
110	120	1.0	14.94 ^{A} ± 0.23	9.70 ± 0.83	57.82	31.43
120	15	1.0	10.88 ^{D} ± 0.56	2.25 ± 1.30	42.09	7.30
120	30	1.0	12.09 ^{C} ± 0.48	11.32 ± 1.45	46.79	36.68
120	60	1.0	15.28 ^{B} ± 0.26	11.87 ± 0.42	59.12	38.47
120	90	1.0	17.53 ^{A} ± 0.86	9.78 ± 1.37	67.84	31.69
120	120	1.0	17.22 ^{A} ± 0.20	11.80 ± 0.72	66.63	38.23
120	90	0.5	13.02 ^{B} ± 0.46	5.92 ± 0.06	50.39	19.18
120	90	0.7	15.82 ^{AB} ± 0.69	8.55 ± 0.85	61.22	27.71
120	90	1.0	18.13 ^{A} ± 0.37	9.08 ± 0.52	70.16	29.42
120	90	1.5	16.92 ^{AB} ± 1.63	7.70 ± 0.86	65.48	24.95
120	90	2.0	16.66 ^{A} ± 0.77	15.56 ± 1.14	64.47	50.42

[a] OPTB consists of 30.86 ± 3.29% cellulose and 25.84 ± 4.61% hemicellulose;
[b] % conversion = g xylose (produced) / g xylose (theoretical);
[c] % conversion = g glucose (produced) / g glucose (theoretical);
Note: A, B, C, D, and E represent a grouping information using the Tukey Method at 95% confidence. Means that do not share a same letter are significantly different; data presented are calculated means ± standard deviations of triplicate runs

Table 3. Chemical Composition of Raw and DA-OPTB

Sample	Chemical Composition (g 100 g^{-1} biomass)				
	Cellulose	Hemicellulose	Lignin	Ash	Moisture
Raw OPTB	30.86 ± 3.29	25.84 ± 4.61	24.29 ± 5.82	2.29 ± 0.15	9.46 ± 0.19
DA-OPTB	50.84 ± 2.41	10.47 ± 1.98	21.07 ± 3.72	0.74 ± 0.11	6.65 ± 0.30
Improvement (%)	64.7	59.5	13.3	67.7	-

Optimisation of Enzymatic Hydrolysis of DA-OPTB

The optimisation of the enzymatic hydrolysis of DA-OPTB for glucose production was performed using RSM based on CCD. The response, *i.e.*, glucose concentration (g/L) as a function of enzyme dosage, agitation, hydrolysis time, and Triton X-100 concentration was evaluated (Table 4). The predicted glucose concentrations obtained from the model fitting techniques were sufficiently correlated with the experimental values. In particular, those values at the centre points (Run: 7, 11, 18, 24, 26, and 29) were close to each other. This together with Fig. 1 demonstrated a high reproducibility of the experiments.

Table 4. Total Yields of Xylose and Glucose from OPTB During Acid (H_2SO_4) Pretreatment at Varying Temperature, Retention Time, and Acid Concentration CCD Matrix and Glucose Yield from Enzymatic Hydrolysis of DA-OPTB

Run	Enzyme (X_1, U/g)	Agitation (X_2, rpm)	Time (X_3, h)	Triton X-100 (X_4, %, v/v)	Glucose (g/L)		Yield (% g/g DA-OPTB)[a]	Conversion (%)[b]
					Actual	Predicted		
1	20	100	72	0.100	12.02	12.95	24.04	47.29
2	30	150	48	0.125	16.61	16.34	33.22	65.34
3	40	200	24	0.100	12.34	12.66	24.68	48.54
4	30	50	48	0.075	7.74	8.94	15.48	30.45
5	30	150	0	0.075	0.00	3.50	0.00	0.00
6	40	200	24	0.050	11.59	10.28	23.18	45.59
7	30	150	48	0.075	14.24	13.96	28.48	56.02
8	40	200	72	0.100	20.35	18.00	40.70	80.06
9	20	200	72	0.050	11.97	11.32	23.94	47.09
10	40	100	72	0.050	14.80	14.87	29.60	58.22
11	30	150	48	0.075	12.91	13.96	25.82	50.79
12	40	200	72	0.050	13.13	15.62	26.26	51.65
13	20	200	24	0.100	10.20	8.36	20.40	40.13
14	50	150	48	0.075	18.11	18.26	36.22	71.24
15	30	250	48	0.075	8.17	10.45	16.34	32.14
16	20	200	72	0.100	15.82	13.70	31.64	62.23
17	20	100	24	0.050	6.90	5.22	13.80	27.14
18	30	150	48	0.075	12.91	13.96	25.82	50.79
19	20	100	24	0.100	8.46	7.60	16.92	33.28
20	30	150	48	0.075	5.06	9.66	10.12	19.91
21	30	150	48	0.025	11.19	11.58	22.38	44.02
22	40	100	24	0.050	10.87	9.52	21.74	42.76
23	20	100	72	0.050	13.17	10.57	26.34	51.81
24	30	150	48	0.075	13.00	13.96	26.00	51.14
25	20	200	24	0.050	8.61	5.98	17.22	33.87
26	30	150	48	0.075	12.77	13.96	25.54	50.24
27	40	100	72	0.100	16.53	17.25	33.06	65.03
28	40	100	24	0.100	13.06	11.90	26.12	51.38
29	30	150	48	0.075	15.79	13.96	31.58	62.12
30	30	150	96	0.075	14.20	14.19	28.40	55.86

[a] DA-OPTB consists of 50.84 ± 2.41% cellulose;
[b] % conversion = g glucose (produced) / 100 g glucose (theoretical)

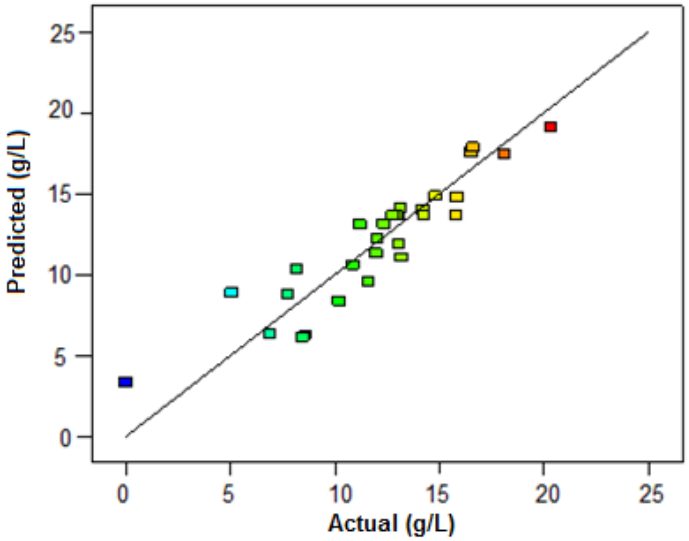


Fig. 1. Actual and predicted plot of glucose response

Two models *i.e.*, linear and quadratic, were proposed based on the statistic summary. The quadratic model was chosen as it has low standard deviation (2.23), high R^2 (0.846), and an insignificant lack-of-fit ($p = 0.0517$). As $R^2 > 0.8$ is indicative of a good fit between the experimental and the predicted responses (Sin *et al.* 2006), the R^2 obtained in this study was acceptable. The model is also coupled with the insignificant lack-of-fit F-value (4.66) implied the validity to predict the variation during enzymatic hydrolysis of DA-OPTB. The ANOVA conducted on the experimental results of the enzymatic hydrolysis of DA-OPTB showed an F-value of 5.89 and Prob > F ($p = 0.0008$), which implied statistical reliability of the model (Table 5). The p-values of enzyme dosage (X_1), hydrolysis time (X_3), Triton X-100 concentration (X_4), X_2^2, and X_3^2 were all < 0.05, demonstrating that they were significant in influencing the response.

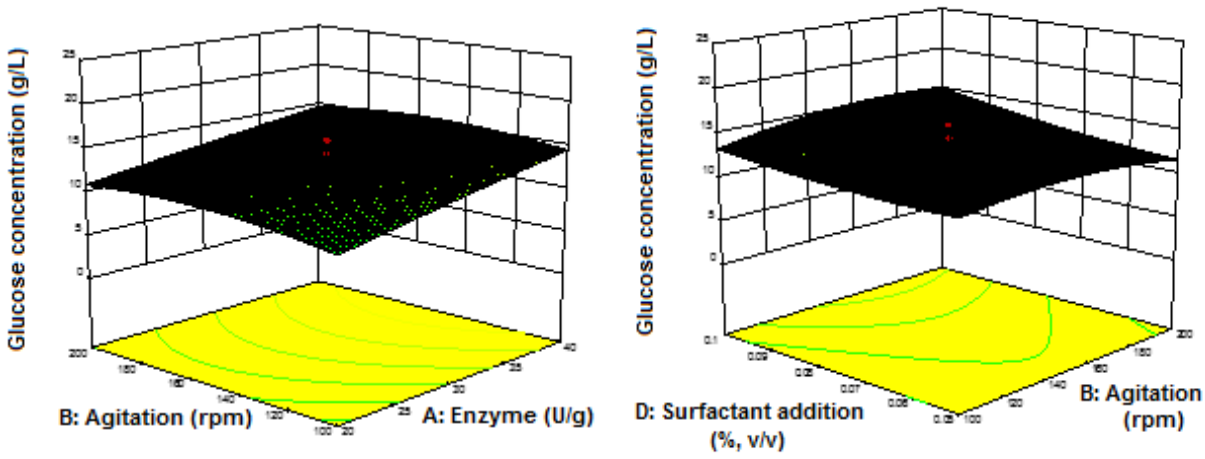
Table 5. Coded Values, Experimental Ranges, and Levels of the Studied Independent Variables ANOVA for Response Surface Reduced Quadratic Model for Glucose Production from Oil Palm Trunk Bagasse

Source	Sum of Squares	Df	Mean Square	F Value	p-value (Prob > F)	Remark
Model	408.32	14	29.17	5.89	0.0008	Significant
X_1-Enzyme dosage	111.00	1	111.00	22.40	0.0003	Significant
X_2-Agitation	3.42	1	3.42	0.69	0.4194	Insignificant
X_3-Hydrolysis time	171.44	1	171.44	34.60	<0.0001	Significant
X_4-Triton X-100 concentration	34.00	1	34.00	6.86	0.0193	Significant
X_2^2	28.59	1	28.59	5.77	0.0297	Significant
X_3^2	41.73	1	41.73	8.42	0.0109	Significant
Residual	74.32	15	4.95			
Lack of fit	67.12	10	6.71	4.66	0.0517	Insignificant
Pure error	7.21	5	1.44			
Cor. total	482.65	29				

The final equation showing the variable coefficient and actual factors is given in Eq. 1. The factors in increasing order of positive effect towards glucose yield from hydrolysed DA-OPTB were: hydrolysis time > enzyme dosage > agitation. The positive value of the variable coefficient indicated favorable response while the negative value showed an inverse relationship between the variable and the response. However, the concentrations of Triton X-100 had inversely affected the enzymatic hydrolysis causing a much reduced glucose yield.

$$\text{Glucose concentration} = -5.66057 + 0.26566 \cdot (\text{enzyme dosage}) + 0.10746 \cdot (\text{agitation}) + 0.27792 \cdot (\text{hydrolysis time}) - 205.35417 \cdot (\text{Triton X-100 concentration}) - 4.08396\text{E-}004 \cdot (\text{agitation})^2 - 2.14147\text{E-}003 \cdot (\text{hydrolysis time})^2 \quad (1)$$

The relationship between the independent variables and the response was visualized by varying two variables within the experimental range while keeping the other two at constant middle level. Figure 2 shows such examples on their effects in producing glucose from DA-OPTB. In Fig. 2(a), changes in the enzyme dosage and agitation rate could positively affect enzymatic hydrolysis and glucose concentration. An increase in enzyme concentration accelerated the cellulose-to-glucose conversion rate as more frequent surface contact occurred between the enzyme and the substrate. Furthermore, a faster agitation speed resulted in more dispersion of the enzyme towards the substrate to promote hydrolysis. The optimum was achieved when the enzyme dose was at 40 U/g and agitation at 150 rpm. Figure 2(b) reveals that an optimal glucose concentration was attainable when Triton X-100 was present at its maximum level but not for agitation. The addition of Triton X-100 in this study changed the nature of the substrate, making the reaction sites more available and increasing enzyme stability (Nurul Adela *et al.* 2015).



(a) **(b)**

Fig. 2. Response surface plots for glucose concentration (g/L): (a) effect of enzyme and agitation (at 48 h hydrolysis time and 0.075% (v/v) Triton X-100, and (b) effect of agitation and Triton X-100 addition (at 30 U/g enzyme loading and 48 h hydrolysis time)

Verification of Optimised Conditions

The quadratic models suggested several solutions for optimum conditions. Using numerical comparison, hydrolysis time of 48 h was selected and the rest of the variables were given in a specific range. From the previously conducted enzymatic hydrolysis of oil

palm EFB with cellulase, prolonged hydrolysis time beyond 48 h was found insignificantly increased the glucose yield (Nurul Adela *et al.* 2014). The solution of highest desirability value of one (1) was selected. Based on the top ranked desirability, the predicted response was 20.5 g/L (Table 6) at conditions as follows: enzyme dosage of 49.9 U/g; agitation of 155 rpm; Triton X-100 concentration of 0.123% (v/v); and hydrolysis time of 48 h. These conditions were then verified to ascertain the prediction. The resulting experimental glucose response of 21.71 ± 1.05 g/L was in agreement with the predicted response (20.49 g/L), thus confirming that the model used was reliable for current study.

Table 6. Experimental Data on Glucose Response Based on Optimum Conditions Suggested by RSM

Enzyme Dosage (U/g)	Agitation (rpm)	Time (h)	Triton X-100 (%, v/v)	Predicted Glucose (g/L)	Actual Glucose (g/L)	Yield (g/g) [a]	Conversion (%)[b]
49.86 (~50)	155	48	0.123	20.49	21.71 ± 1.05	0.43 ± 0.02	85.64 ± 4.10
Note: DA-OPTB - dilute acid pretreated oil palm trunk bagasse; DA-OPTB consisted of 50.84 ± 2.41% cellulose; [a] (g glucose produced per g DA-OPTB); [b] % conversion = g glucose (produced) / g glucose (theoretical)							

Overall, an optimal 21.71 g of glucose was obtained from 100 g raw OPTB employing the RSM-optimised conditions (Table 6). The enzymatic digestibility (based on theoretical maximum yield of glucose) was 85.6% compared to only 8% that of the raw (untreated) OPTB. Only 2.26 ± 0.08 g/L of glucose was released from the untreated OPTB after hydrolysis. The results clearly indicated that the removal of hemicellulose *via* the employed pretreatment was a prerequisite for high enzymatic digestibility of cellulose in OPTB. In fact, using a locally developed recombinant enzyme in this study proved a higher total activity in the hydrolysis of OPTB as a mild acid (1%, v/v) and a lower temperature (120 ºC) was used compared to a stronger acid (3%, v/v) at 180 ºC for 40 min, with 80% enzymatic efficiency (Noparat *et al.* 2015).

Fermentability of DA-OPTB Hydrolysate for SA Production

In fermentation trials, the DA-OPTB hydrolysate for SA production, an equivalent synthetic medium consisting of pure sugars was used as a positive control to imitate the sugars composition of the substrate used. Figures 3(a) and 3(b) show the time course profiles for the respective sugars consumption, SA production, and by-products (formic acid and acetic acid) formation by *A. succinogenes* 130Z at 37 ºC, 200 rpm, and 48 h. The SA production exponentially increased at an early stage of fermentation, reached its maximum production after 18 h, and then remained constant thereafter. At 18 h, approximately 90% of sugars in the DA-OPTB hydrolysate were consumed by *A. succinogenes* 130Z. At 24 h, all of the sugars were consumed yielding 7.3 g/L SA. This concentration was 17% lower compared to that of 8.7 g/L using synthetic medium (Table 7). The SA yields were 0.26 g/g from the DA-OPTB hydrolysate and 0.31 g/g from its counterpart. Although the titer of SA was relatively low in this study, the yield obtained was very close to those by Akhtar and Idris (2017), *i.e.*, 0.23 to 0.29 g/g from autoclave alkali-pretreated EFB fibre, but much further behind the yield (0.47 g/g) achieved using a DA-microwave alkali pretreatment. The organosolv-treated EFB yielded 0.33 g/g SA (Pasma *et al.* 2013) (Table 7).

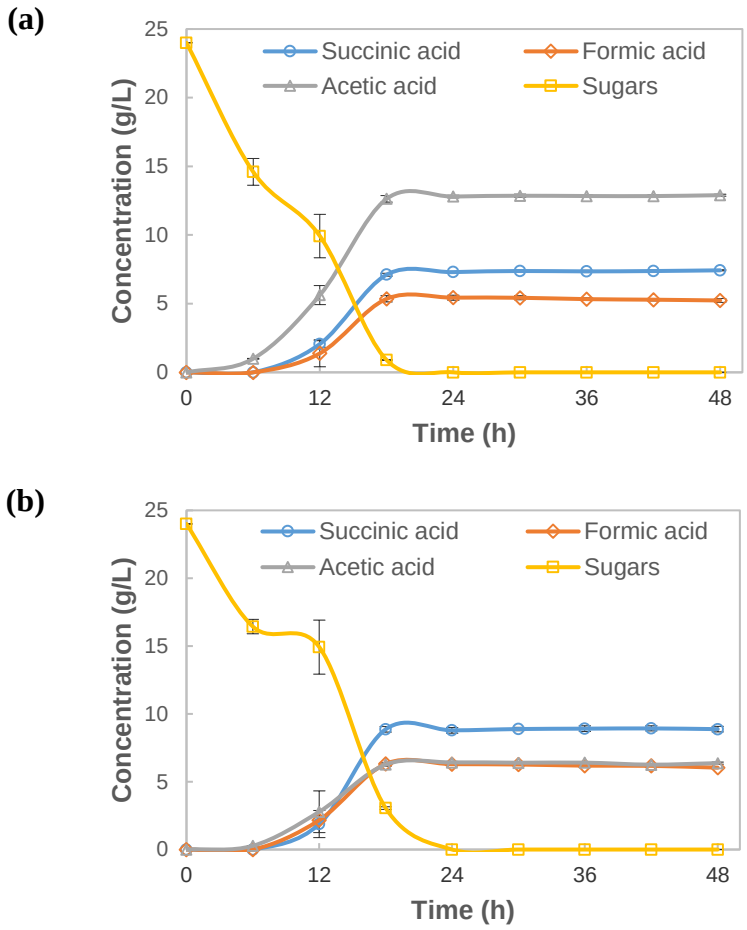


Fig. 3. Time-course profiles of sugars consumption, succinic acid production, and by-products (formic acid and acetic acid) formation by *A. succinogenes* 130Z at 37 ^{o}C, 200 rpm for 48 h in (a) DA-OPTB hydrolysate and (b) reference medium

Table 7. Fermentation Parameters of Succinic Acid (SA) Production from Oil Palm Biomass Hydrolysate in Different Media

Hydrolysate /medium	SA titer (g/L)	SA yield, $Y_{p/s}$ (g/g)	DCW yield, $Y_{x/s}$ (g/g)	Productivity (g/Lh)	Reference
DA-OPTB	7.30 ± 0.04	0.26 ± 0.01	1.15 ± 0.07	0.30 ± 0.00	This study
Reference Medium	8.79 ± 0.20	0.31 ± 0.02	0.66 ± 0.03	0.37 ± 0.02	This study
Autoclave alkali-EFB	20.9	0.29	ND	0.44	Akhtar and Idris (2017)
DA-microwave alkali EFB	33.4	0.47	ND	0.69	Akhtar and Idris (2017)
Organosolv-EFB	23.5	0.33	ND	0.62	Pasma *et al.* (2013)
Note: DCW - dry cell weight; DA - dilute acid pretreated; EFB - empty fruit bunch; ND - not determined					

Although both media consisted of the same amount of initial sugars, the authors found a high acetic acid concentration up to 13 g/L while producing SA from DA-OPTB hydrolysate. Probably it was derived from the acetyl component of the fibre. Its presence together with other minor inhibitor compounds such as furfural and hydroxylmethyfurfural (HMF) in DA-OPTB hydrolysate could likely to suppress the growth of *A. succinogenes* 130Z. However, the results showed the potential use of inexpensive OPTB to partially substitute commercial platform sugars. Further research should focus on maximising SA production by minimising acetic acid formation. This could be done by introducing deacetylation step prior to pretreatment to get rid of the acetyl group. To further enhance SA titer, high initial sugars concentration such as those from xylose-rich prehydrolysates should be used as well. Additionally, it is also important to characterise the nutrient compositions of DA-OPTB hydrolysate to identify any nutrient limiting factors in this kind of fermentation study.

CONCLUSIONS

1. The optimisation of enzymatic hydrolysis of DA-OPTB *via* RSM based on CCD gave 85.6% cellulose-to-glucose conversion and 22% glucose yield (per 100 g raw OPT).
2. The DA pretreatment employed efficiently increased cellulose digestibility for subsequent SA production.
3. The highest SA concentration of 7.3 g/L was produced by *A. succinogenes* 130Z from DA-OPTB hydrolysate incubated at 37 ºC, 200 rpm within 24 h.
4. DA-OPTB hydrolysate could be a fermentative substrate in the biosynthesis of value-added chemicals.

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REFERENCES CITED

Akhtar, J., and Idris, A. (2017). "Oil palm empty fruit bunches a promising substrate for succinic acid production *via* simultaneous saccharification and fermentation," *Renew. Energ*. 114(Part B), 917-923. DOI: 10.1016/j.renene.2017.07.113

Akhtar, J., Idris, A., and Abd Aziz, R. (2014). "Recent advances in production of succinic acid from lignocellulosic biomass," *Appl. Microbiol. Biot.* 98(3), 987-1000. DOI: 10.1007/s00253-013-5319-6

ASTM D 1103-60 (1978). "Standard test method for α-cellulose," ASTM International, West Conshohocken, PA

ASTM D 1104-56 (1978). "Method of test for holocellulose in wood," ASTM International, West Conshohocken, PA

Avci, A., Saha, B. C., Kennedy, G. J., and Cotta, M. A. (2013). "Dilute sulfuric acid pretreatment of corn stover for enzymatic hydrolysis and efficient ethanol production by recombinant *Escherichia coli* FBR5 without detoxification," *Bioresource Technol.* 142, 312-319. DOI: 10.1016/j.biortech.2013.05.002

Borges, E. R., and Pereira, N. (2011). "Succinic acid production from sugarcane bagasse hemicellulose hydrolysate by *Actinobacillus succinogenes*," *J. Ind. Microbiol. Biot.* 38(8), 1001-1011. DOI:10.1007/s10295-010-0874-7

Carvalho, M., Roca, C., and Reis, M. A. (2016). "Improving succinic acid production by *Actinobacillus succinogenes* from raw industrial carob pods," *Bioresource Technol.* 218, 491-497.

Carvalho, M., Roca, C., and Reis, M. A. M. (2014). "Carob pod water extracts as feedstock for succinic acid production by *Actinobacillus succinogenes* 130Z," *Bioresource Technol*. 170, 491-498. DOI: 10.1016/j.biortech.2014.07.117

Chen, K.-Q., Li, J., Ma, J.-F., Jiang, M., Wei, P., Liu, Z.-M., and Ying, H.-J. (2011). "Succinic acid production by *Actinobacillus succinogenes* using hydrolysates of spent yeast cells and corn fiber," *Bioresource Technol.* 102(2), 1704-1708. DOI: 10.1016/j.biortech.2010.08.011

Deshavath, N. N., Dasu, V. V., Goud, V. V., and Rao, P. S. (2017). "Development of dilute sulfuric acid pretreatment method for the enhancement of xylose fermentability," *Biocatal. Agric. Biotechnol.* 11, 224-230.

Fu, L., Gao, X., Yang, Y., Aiyong, F., Hao, H., and Gao, C. (2014). "Preparation of succinic acid using bipolar membrane electrodialysis," *Sep. Purif. Technol*. 127, 212-218. DOI: 10.1016/j.seppur.2014.02.028

Jiang, M., Ma, J., Wu, M., Liu, R., Liang L., Xin, F., Zhang, W, Jia, H, and Dong, W. (2017). "Progress of succinic acid production from renewable resources: Metabolic and fermentative strategies." *Bioresource Technol.* 245, 1710-1717.

Kamireddy, S. R., Li, J., Abbina, S., Berti, M., Tucker, M., and Ji, Y. (2013). "Converting forage sorghum and sunn hemp into biofuels through dilute acid pretreatment," *Ind. Crops Prod.* 49, 598-609.

Kosugi, A., Tanaka, R., Magara, K., Murata, Y., Arai, T., Sulaiman, O., Hashim, R., Hamid, Z. A., Yahya, M. K., Yusof, M. N., *et al.* (2010). "Ethanol and lactic acid production using sap squeezed from old oil palm trunks felled for replanting," *J. Biosci. Bioeng*. 110(3), 322-325. DOI: 10.1016/j.jbiosc.2010.03.001

Kshirsagar, S. D., Waghmare, P. R., Chandrakant Loni, P., Patil, S. A., and Govindwar, S. P. (2015). "Dilute acid pretreatment of rice straw, structural characterization and optimization of enzymatic hydrolysis conditions by response surface methodology," *RSC Adv*. 5(58), 46525-46533. DOI: 10.1039/C5RA04430H

Kushairi, A., Loh, S. K., Azman, I., Hishamuddin, E., Ong-Abdullah, M., Izuddin, Z. B. M. N., Razmah, G., Sundram, S., and Parveez, G. K. A. (2018). "Oil palm economic performance in Malaysia and R&D progress in 2017," *J. Oil Palm Res*. 30(2), 163-195.

Lai, L.-W., and Idris, A. (2013). "Disruption of oil palm trunks and fronds by microwave-alkali pretreatment," *BioResources* 8(2), 2792-2804. DOI: 10.15376/biores.8.2.2792-2804

Loow, Y.-L., Wu, T. Y., Jahim, J. M., Mohammad, A. W., and Teoh, W. H. (2016). "Typical conversion of lignocellulosic biomass into reducing sugars using dilute acid hydrolysis and alkaline pretreatment," *Cellulose* 23(3), 1491-1520.

Luthfi, A. A. I., Jahim, J. M., Harun, S., Tan, J. P., and Mohammad, A. W. (2016). "Biorefinery approach towards greener succinic acid production from oil palm frond bagasse," *Process Biochem*. 51(10), 1527-1537. DOI: 10.1016/j.procbio.2016.08.011

Luthfi, A. A. I., Jahim, J. M., Harun, S., Tan, J. P., and Mohammad, A. W. (2017). "Potential use of coconut shell activated carbon as an immobilisation carrier for high conversion of succinic acid from oil palm frond hydrolysate," *RSC Adv*. 7(78), 49480-49489. DOI: 10.1039/C7RA09413B

Mtui, G. Y. (2009). "Recent advances in pretreatment of lignocellulosic wastes and production of value added products," *Afr. J. Biotechnol*. 8(8), 1398-1415.

Murata, Y., Tanaka, R., Fujimoto, K., Kosugi, A., Arai, T., Togawa, E., Takano, T., Ibrahim, W. A., Elham, P., and Sulaiman, O. (2013). "Development of sap compressing systems from oil palm trunk," *Biomass Bioenerg*. 51, 8-16. DOI: 10.1016/j.biombioe.2012.12.007

Noparat, P., Prasertsan, P., Sompong, O., and Pan, X. (2015). "Dilute acid pretreatment of oil palm trunk biomass at high temperature for enzymatic hydrolysis," *Enrgy. Proced.* 79, 924-929. DOI: 10.1016/j.egypro.2015.11.588

Nurul Adela, B., and Loh, S. (2015). "Optimisation of fermentation conditions for bioethanol production from oil palm trunk sap by *Saccharomyces cerevisiae*," *Malays. J. Microbiol*. 11(2), 163-169.

Nurul Adela, B., Loh, S. K., and Nasrin, A. B. (2015). "The improvement on enzymatic hydrolysis of oil palm (*Elaeis guineensis*) empty fruit bunch lignocellulose," *Malays. Appl. Biol.* 44(1), 95-100.

Nurul Adela, B., Nasrin, A. B., Loh, S. K, and Choo, Y. M. (2014). "Bioethanol production by fermentation of oil palm empty fruit bunches pretreated with combined chemicals," *J. Appl. Environ. Biol. Sci*. 4(10), 234-242.

Pasma, S. A., Daik, R., and Maskat, M. Y. (2013). "Production of succinic acid from oil palm empty fruit bunch cellulose using *Actinobacillus succinogenes*," *AIP Conf. Proc*. 1571, 753-759. DOI: 10.1063/1.4858745

Ramle, M., Wahid, M., Norman, K., Glare, T., and Jackson, T. (2005). "The incidence and use of *Oryctes* virus for control of rhinoceros beetle in oil palm plantations in Malaysia," *J. Invertebr. Pathol*. 89(1), 85-90. DOI: 10.1016/j.jip.2005.02.009

Satari Baboukani, B., Vossoughi, M., and Alemzadeh, I. (2012). "Optimisation of dilute-acid pretreatment conditions for enhancement sugar recovery and enzymatic hydrolysis of wheat straw," *Biosyst. Eng*. 111(2), 166-174. DOI: 10.1016/j.biosystemseng.2011.11.009

Sin, H., Yusof, S., Hamid, N. S. A., and Rahman, R. A. (2006). "Optimization of enzymatic clarification of sapodilla juice using response surface methodology," *J. Food Eng*. 73(4), 313-319. DOI: 10.1016/j.jfoodeng.2005.01.031

Tan, J. P., Jahim, J. M., Harun, S., Wu, T. Y., and Mumtaz, T. (2016). "Utilization of oil palm fronds as a sustainable carbon source in biorefineries," *Int. J. Hydrogen Energ*. 41(8), 4896-4906. DOI: 10.1016/j.ijhydene.2015.08.034

Tan, J. P., Jahim, J. M., Wu, T. Y., Harun, S., Kim, B. H., and Mohammad, A. W. (2014). "Insight into biomass as a renewable carbon source for the production of succinic acid and the factors affecting the metabolic flux towards higher succinate yield," *Ind. Eng. Chem. Res.* 53(42), 16123-16134.

Tan, J. P., Luthfi, A. A. I., Manaf, S. F. A., Wu, T. Y., and Jahim, J. M. (2018). "Incorporation of CO_2 during the production of succinic acid from sustainable oil palm frond juice," *J. CO_2 Util.* 26, 595-601. DOI: 10.1016/j.jcou.2018.06.006

TAPPI T222 om-11 (2011). "Acid-insoluble lignin in wood and pulp," TAPPI Press, Atlanta, GA

Werpy, T., Petersen, G., Aden, A., Bozell, J., Holladay, J., and White, J. (2004). *Top Value Added Chemicals from Biomass. Results of screening for potential candidates from sugars and synthetis gas,* Vol. 1. US Department of Energy, Energy Efficiency and Renewable Energy, Washington, DC, pp. 22-24.

Zeikus, J. G., Jain, M. K., and Elankovan, P. (1999). "Biotechnology of succinic acid production and markets for derived industrial products," *Appl. Microbiol. Biot.* 51(5), 545-552. DOI: 10.1007/s002530051431

Zheng, P., Fang, L., Xu, Y., Dong, J. J., Ni, Y., and Sun, Z. H. (2010). "Succinic acid production from corn stover by simultaneous saccharification and fermentation using *Actinobacillus succinogenes*," *Bioresource Technol.* 101(20), 7889-7894. DOI: 10.1016/j.biortech.2010.05.016

Zhou, X., Ma, J., Ji, Z., Zhang, X., Ramaswamy, S., Xu, F., and Sun, R.-C. (2014). "Dilute acid pretreatment differentially affects the compositional and architectural features of *Pinus bungeana* Zucc. compression and opposite wood tracheid walls," *Ind. Crop. Prod.* 62, 196-203. DOI: 10.1016/j.indcrop.2014.08.035

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