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Yield Optimization of Reducing Sugars from Acid Hydrolysis of *Chlorella vulgaris* Waste Biomass

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Abstract

Microalgal biomass is a suitable feedstock for biofuel production. This study aimed to evaluate the potential of *Chlorella vulgaris,* a widely-cultured microalgae species, for the production of reducing sugars via acid hydrolysis. Reducing sugars yield from acid hydrolysis was optimized using Response Surface Methodology (RSM). Acid concentration and reaction time were found to be the most significant process parameters affecting reducing sugars yield. Optimum parameter conditions were found to be 3.71 % (v/v) and 73.98 min for sulfuric acid concentration and reaction time, respectively, which correspond to 44.96% conversion of waste biomass carbohydrates to reducing sugars. The findings suggest that *C. vulgaris* biomass can be harnessed as a renewable source of biofeul production.

Keywords: Microalgae waste biomass, acid hydrolysis, reducing sugars

Introduction

The potential of microalgae as feedstock for biofuel production continues to be a focus of high concern and contention. Different oil extraction techniques have been extensively investigated for certain microalgae species due to their ability to accumulate large quantities of lipid inside their cells (Lam and Lee, 2012) and rapid biomass production capacity (Mussatto et al., 2010). As reported, the biomass yield of microalgae is approximately 200 ton/ha/year (Dismukes et al., 2008), producing an ethanol yield at the range of 50,000-140,000 L/ha/year (Mussatto et al., 2010).

Microalgae can fix CO_2 from the atmosphere, flue gases, or soluble carbonate while simultaneously capturing solar energy (Lam and Lee, 2012). It has been estimated that one kilogram of dry algal biomass utilizes about 1.8 kg of CO_2 (Brennan and Owende, 2010). One of the widely-cultured species of microalgae is *Chlorella vulgaris*. This species has been studied widely because of its efficient CO_2 fixation and its copious production of lipids that may be converted to biodiesel. Ho et al. (2013) and Hirano et al. (1997) demonstrated the potential use of *C. vulgaris* biomass solely for bioethanol production. While studies on biodiesel production from *C.*

vulgaris abound, reports on the utilization of waste biomass for bioethanol production remain non-existent.

The biomass left after the extraction of lipid through solvent extraction comprises mostly carbohydrates and protein, which usually becomes part of the waste stream of biodiesel production. Since carbohydrates can be broken down to sugars which are important precursors to bioethanol production, it may be possible to produce bioethanol from the waste biomass (Brennan and Owende, 2010). To produce reducing sugars from biomass, two of the widely-used technologies are enzyme hydrolysis and acid hydrolysis (Harun & Danquah, 2011a; Ho et al., 2013; Hernandez et al., 2015). Although the former can produce higher reducing sugar yield compared to the latter, acid hydrolysis has faster reaction rates (Ho et al., 2013). In addition, acid hydrolysis does not require specialized sets of equipment and methods for the reaction to proceed, which reduces the cost of production. Being cost effective, acid hydrolysis is utilized in industries rather than enzyme hydrolysis.

C. vulgaris production in the country is currently at its infancy stage. However, realizing its huge potential over terrestrial land-based biomass for both biofuel and CO_2 mitigation might help solve our fuel-import dependency as well as decrease the greenhouse gas emission of the country (Dismukes et al., 2008). In this study, the production of reducing sugars from waste *C. vulgaris* biomass using acid hydrolysis was explored. Hydrolysis parameters were identified, screened, and optimized using established techniques to come up with the most economical process.

Methodology

Cultivation of C. vulgaris

C. vulgaris was grown in batches in improvised 6L bioreactors with an initial inoculum size of 0.10-0.20 g/L. The growth medium was composed of 0.2 g/L and 0.02 g/L of urea and ammonium phosphate, respectively. Ambient air supplied by aquarium aeration pumps served as CO_2 source. The bioreactors were illuminated with fluorescent lamps (GE F220W Super Daylight 6500K) at a room temperature maintained between 25-30 °C.

Preparation and characterization of waste C. vulgaris biomass

C. vulgaris biomass harvested after 14 days of cultivation were dried at 65 °C for four hours in an air-circulating oven then ground to small particles using mortar and pestle. The lipid content of *C. vulgaris* was extracted using hexane and isopropanol as modified by Ryckebosch et al. (2012). For 0.1 g of dried microalgal cells, 30 mL hexane and 20 mL isopropanol was added and the reaction mixture was kept at room temperature for 1 hour with occasional mixing. After extraction, the microalgae were centrifuged at 6000 rpm for five minutes to separate the aqueous layer and the residual biomass. The residual biomass was collected and washed twice with 50 mL distilled water prior to hydrolysis.

The lipid extracted biomass was sent to the National Institute of Molecular Biology and Biotech (BIOTECH, UP Los Baños, Laguna) – Central Analytical Services Laboratory (CASL) for moisture, ash, crude fat, crude protein, crude fiber, and total carbohydrate determination. Characterization of the biomass was done following the National Renewable Energy Laboratory Procedures (NREL LAP) and the Association of Official Analytical Chemists (AOAC) method. Briefly, the moisture content of the residual biomass was determined by drying the sample in a 105°C drying oven until constant weight (NREL LAP 001). The ash content was analyzed by placing the biomass in a 575°C furnace for 24 hours (NREL LAP 005).

In determining the crude fat, microalgal fat content of the residual biomass was extracted using petroleum ether as extracting solvent and Soxhlet apparatus (AOAC 920.39). The defatted biomass was then washed with boiling sulfuric acid to boiling sodium hydroxide. Afterwards it was washed with boiling water and the residue was placed in a crucible then into a 550°C furnace for 3 hours for the fiber determination (AOAC 978.10). Appropriate nitrogen factor was used to estimate the protein content of the biomass sample (NREL/TP 510-42625; AOAC 976.06). The total carbohydrate of the lipid extracted biomass was hydrolyzed using sulfuric acid and

the neutralized hydrolysate was analyzed by high performance liquid chromatography using the sugar assay (NREL LAP 002).

Experimental Design

A 2^k factorial experimental design was initially employed to determine whether the process parameters (temperature, acid concentration, reaction time) have significant effects on the response (reducing sugar yield), and to check whether the set range of low and high parameter values captures the optimum response. Response Surface Methodology (RSM) – Central Composite Design (CCD) was used to generate a mathematical model of the response variable in terms of the significant parameters. The mathematical model was then numerically optimized to determine the optimum process parameters.

2^k factorial analysis with midpoints

The factors considered in the 2^k full factorial experimental design include temperature, acid concentration and reaction time. Reducing sugar yield (% w/w) which served as the response variable was calculated using the following equation:

$$RSY = \frac{(C_{RS}V_{RS})}{\%C \cdot (C_B V_B)}$$

where RSY is the percent reducing sugar yield, C_{RS} the reducing sugar concentration in mg/mL, V_{RS} the initial volume in mL of the hydrolysate containing the reducing sugars, the percent total carbohydrate obtained from the characterization of waste *C. vulgaris* biomass, C_B the initial biomass concentration in mg/mL, and V_B the initial volume of the reaction mixture in mL.

Analysis of variance (ANOVA) was used to determine whether each factor significantly affects the reducing sugar yield. The values set for the different factors are summarized in Table 1. Each combination of factorial points was done in duplicates, while the midpoint was done in four trials. The Design Expert® package program (Trial version 7.0.0, Stat-Ease Inc., USA) was used to generate the experimental design for the 2^k factorial with midpoints. The standard runs generated are summarized in Table 2.

Table 1. Process parameter values for determining the significant factors in the acid hydrolysis of waste *C. vulgaris* biomass.

PARAMETERS	UNIT	LEVELS		
		LOW	MID-POINT	HIGH
Temperature	°C	130	140	150
Acid concentration	% v/v	1	2	3
Reaction time	min	20	40	60

Table 2. Standard runs with midpoints for the 2^k factorial experiments.

STANDARD NO.	TEMPER-ATURE (°C)	ACID CONCEN-TRATION v/v	REAC-TION TIME (min)
1	130	1	20
2	130	1	20
3	150	1	20
4	150	1	20
5	130	3	20
6	130	3	20
7	150	3	20
8	150	3	20
9	130	1	60
10	130	1	60
11	150	1	60
12	150	1	60
13	130	3	60
14	130	3	60
15	150	3	60
16	150	3	60
17	140	2	40
18	140	2	40
19	140	2	40
20	140	2	40

Response Surface Methodology

The optimum parameters for the acid hydrolysis of *C. vulgaris* biomass waste were determined using RSM-CCD which is the most commonly used second-order response surface design. Being a second-order design, CCD is able to capture "interaction and quadratic effects within the factors tested" (Gardiner & Gettinby, 1998). CCD includes all full two-level factorial designs with a center point and axial point(s), making it an efficient tool for optimization.

Results of the 2^k factorial analysis showed that reaction temperature does not significantly affect reducing sugar yield. As such, only acid concentration and reaction time were considered in optimizing the reducing sugar yield. In addition, the low and high values of the two parameters were adjusted because the surface plot from the 2^k factorial analysis did not produce a curvature. The modified low and high values of acid concentration and reaction time are summarized in Table 3. The corresponding standard optimization runs generated using Design Expert® are tabulated in Table 4. Midpoints were replicated five times, and the value of alpha was set to 1.6. The reaction temperature was fixed at 130 °C.

Table 3. Process parameter values of acid concentration and reaction time for generating the standard optimization runs.

PARAMETERS	UNIT	LEVELS	
		LOW	HIGH
Acid concentration	% v/v	2	4
Reaction time	Min	30	90

Table 4. Standard runs for optimizing reducing sugar yield by acid hydrolysis of waste microalgae biomass.

STANDARD NO.	ACID CONCENTRATION (% v/v)	REACTION TIME (min)
1	2.00	30
2	4.00	30
3	2.00	90
4	4.00	90
5	1.59	60
6	4.41	60
7	3.00	18
8	3.00	102
9	3.00	60
10	3.00	60
11	3.00	60
12	3.00	60
13	3.00	60

Acid hydrolysis of waste C. vulgaris biomass

The batch acid hydrolysis of lipid extracted microalgal biomass was done thermochemically using dilute sulfuric acid solution. Specifically, 0.2 g of microalgal waste biomass was mixed with varying concentrations of sulfuric acid in 90 mL test tubes. Using a fume hood, the resulting mixture was placed in a preheated oil bath at varying temperatures and reacted at different time durations. After hydrolysis, the samples were cooled to room temperature using running water. The hydrolysate was obtained by centrifugation at 6000 rpm for five minutes prior to reducing sugar determination. The hydrolysis set-up is illustrated in Figure 1.

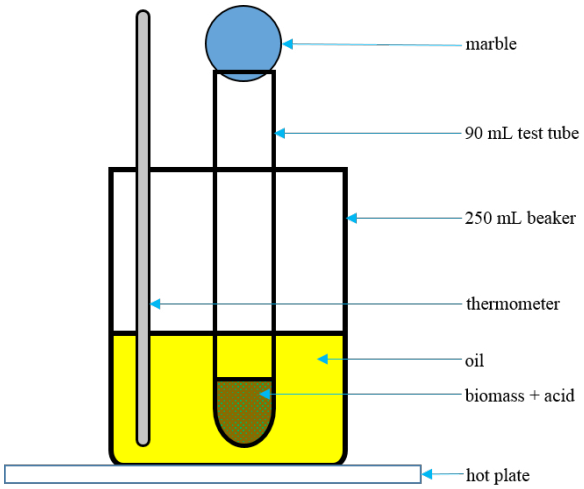


Figure 1. Acid hydrolysis set up with labels.

After hydrolysis, the samples were cooled to room temperature using running water. Lost volume due to evaporation was replaced with distilled water. The supernatant containing the reducing sugars was separated from the residue containing unreacted components by centrifugation at 6000 rpm for five minutes. The concentration of the reducing sugar was analyzed using the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952). A 0.5 mL hydrolysate was mixed with alkaline copper tartrate solution and was brought to boiling water bath for 10 minutes. After cooling to room temperature, 0.5 mL of the arsenomolybdate reagent and 3 mL of water was added. The resulting solution was stabilized for 15 minutes to produce the blue solution. The absorbance of the sample was read using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) at an absorbance of 620 nm. The absorbance reading was converted into a corresponding reducing sugar concentration using glucose as standard.

Experimental verification

The maximum reducing sugar yield (% w/w) predicted by RSM-CCD was validated by performing actual acid hydrolysis experiments operated at the optimum conditions. Validation was performed in two trials, and the average of the outcomes was compared to the predicted response.

Results and Discussion

Characterization of waste C. vulgaris biomass

The composition of *C. vulgaris* biomass waste was determined following NREL procedures, summarized in Table 5. Results show that about 20% of the waste biomass is carbohydrate and about 25% is protein. Due to prior extraction of the microalgal oil, lipid content was already very minimal.

Table 5. Composition of waste biomass from lipid extraction of *C. vulgaris*.

PROPERTY	PERCENTAGE
Moisture	22.37 ± 0.60
Ash	31.16 ± 0.89
Crude fat	0.40 ± 0.09
Crude protein	24.43 ± 0.36
Crude fiber	1.23 ± 0.02
Total carbohydrate	20.41

Screening of process parameters using 2^k factorial analysis

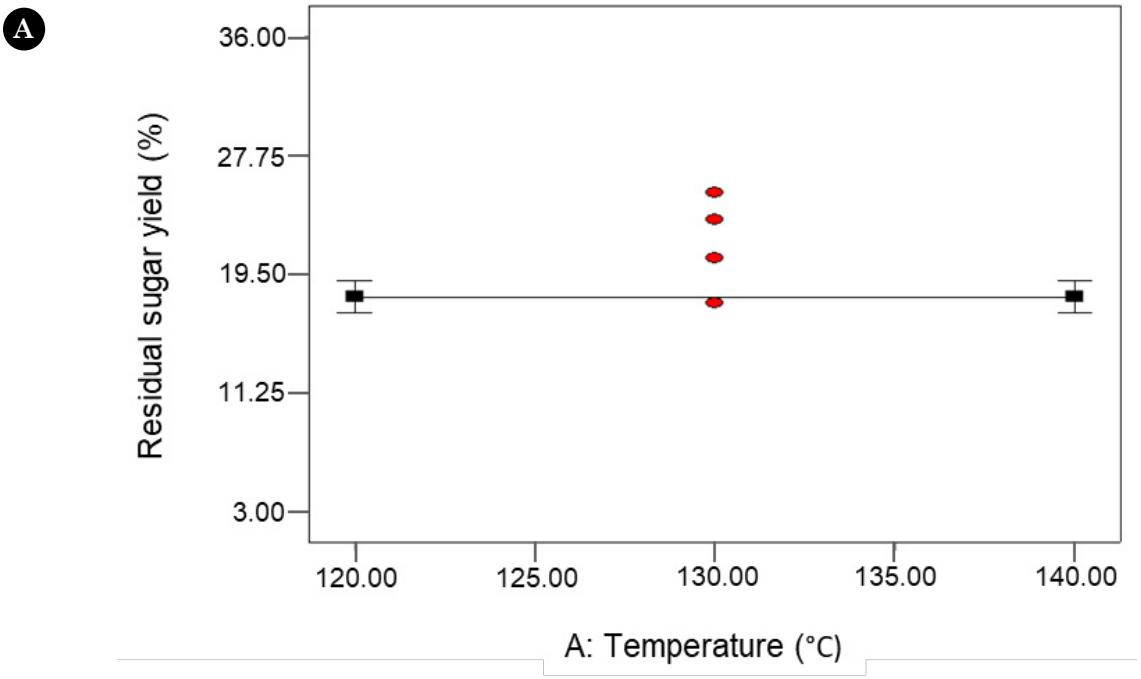
Figures 2 show the individual effects of temperature, acid concentration and reaction time, respectively, to reducing sugar yield. In the figure, the error bars indicate the standard error bar of a mean value. The red dots in the one-factor diagram represent raw data points of the four experimental runs of the midpoint value of the parameter. On the other hand, the highest and lowest points of the experiment runs are presented with square symbol. The one-factor representation is to show the relationship of the response (amount of reducing sugar) to the parameters selected. Skewness of the line represents significant interaction of the parameter to the response variable.

Results show that for the range of values employed, reaction temperature had minimal effect on reducing sugar yield. Meanwhile, previous studies on dilute acid hydrolysis of biomass from other microalgae species or strains report that the best temperature is in the range of 140 to 160 °C (Harun & Danquah, 2011b; Grohman et al., 1986). Our results somehow confirm these reports, but improve upon them by pointing to the possibility of a substantially lower optimal temperature at 130 °C, which is advantageous on a process economics perspective.

Reducing sugar yield was found to increase with increasing acid concentration. This may be explained by the greater abundance of hydronium ions available to initiate glycosidic cleavage at higher acid concentrations. Prolonged reaction periods essentially had a similar effect on reducing sugar yield. Longer reaction periods allow more glycosidic bonds to break, liberating more sugars in the process.

For a more complete picture of the factors affecting reducing sugar yield, statistical results using ANOVA of the 2^k factorial model are presented in Table 6. The *p*-value in the table shows the relation of the obtained experimental data and model predictions. If the *p*-value of the parameter is less than 0.05, the parameters are considered statistically significant. Hence, "lack of fit" with a *p*-value of 0.5612 means that the model predictions statistically fits well with the experimental data. The parameters found to have significant effects on the reducing sugar yield using $\alpha = 0.05$ were acid concentration (B) and reaction time (C). The effect of temperature (A) and all parameter combinations (AB, BC, AC) were found to be not significant.

To better visualize the model from the 2^k factorial analysis, the contour and three-dimensional surface plots of reducing sugar



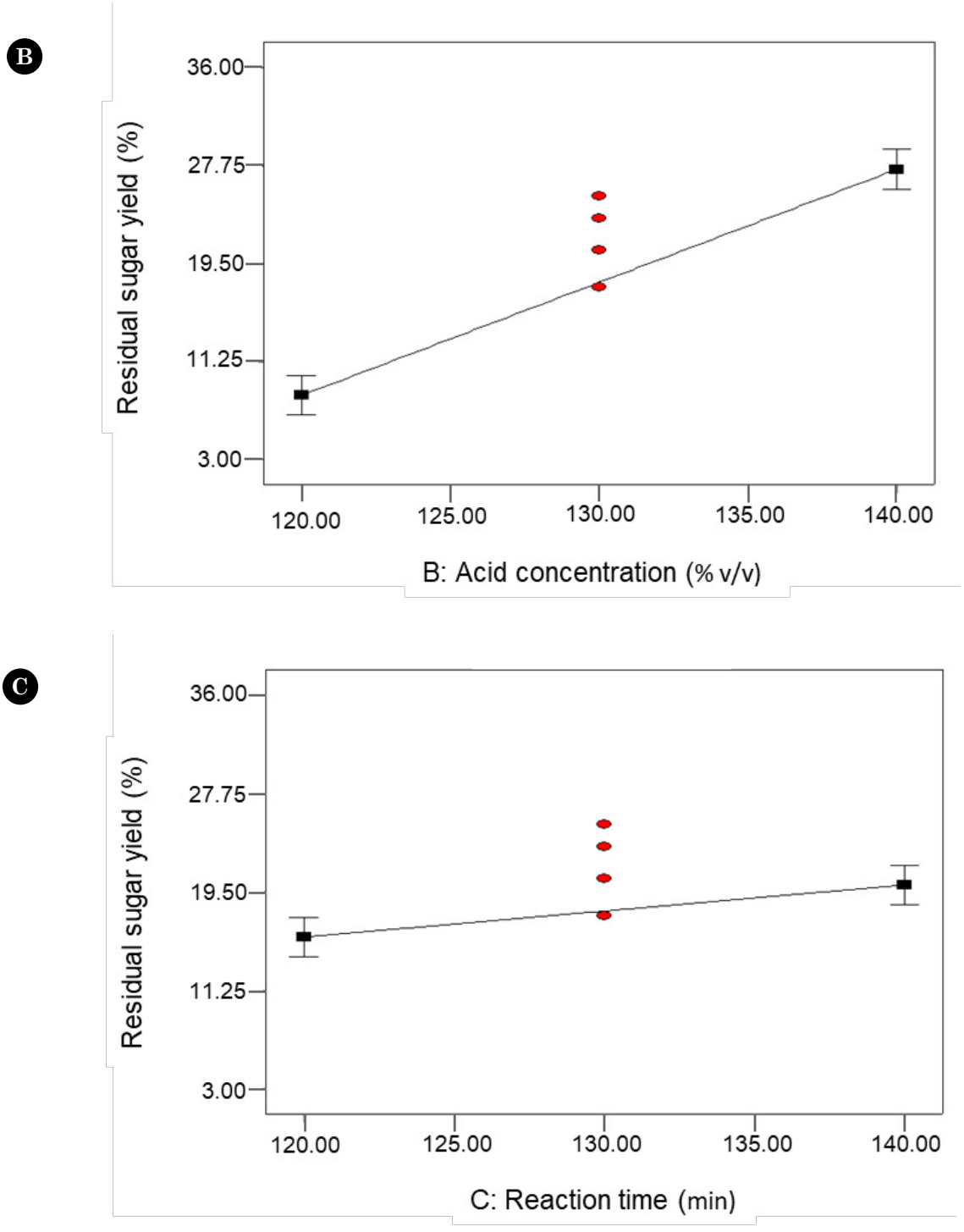


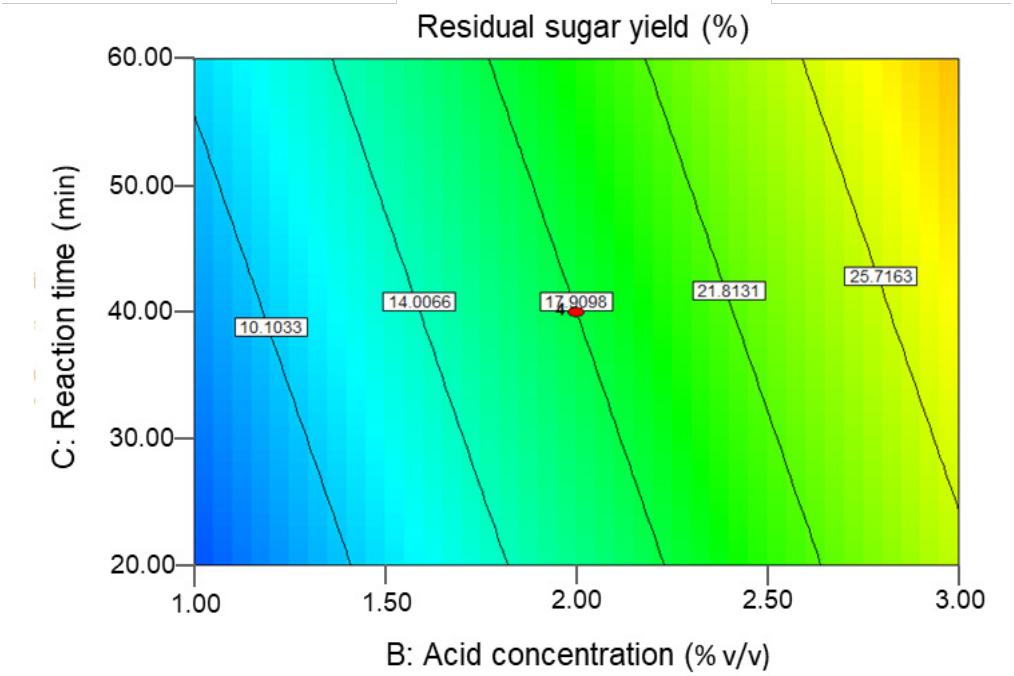
Figure 2. 2^kfactorial analysis of temperature, acid concentration and reaction time (**A**: Reducing sugar yield (% w/w) vs. temperature (°C), **B**: Reducing sugar yield (% w/w) vs. acid concentration (v/v), **C**: Reducing sugar yield (% w/w) vs. reaction time (min)).

Table 6. ANOVA of the 2^k factorial model.

SOURCE	SUM OF SQUARES	DF	MEAN SQUARE	F VALUE	P-VALUE PROB > F
Model	1562.1229	6	260.3538	21.7068	< 0.0001
A-Temp	0.2517	1	0.2517	0.0210	0.8872
B-Acid Concn	1451.4928	1	1451.4928	121.0171	< 0.0001
C-Time	76.3962	1	76.3962	6.3695	0.0267
AB	32.7160	1	32.7160	2.7277	0.1245
AC	0.0019	1	0.0019	0.0002	0.9901
BC	1.2643	1	1.2643	0.1054	0.7510
Curvature	44.8340	1	44.8340	3.7380	0.0771
Residual	143.9294	12	11.9941	-	-
Lack of Fit	4.5498	1	4.5498	0.3591	0.5612
Pure Error	139.3796	11	12.6709	-	-
Cor Total	1750.8863	19	-	-	-

Note: Values of "Prob>F" less than 0.05 indicate model terms are significant; not significant otherwise.

A



B

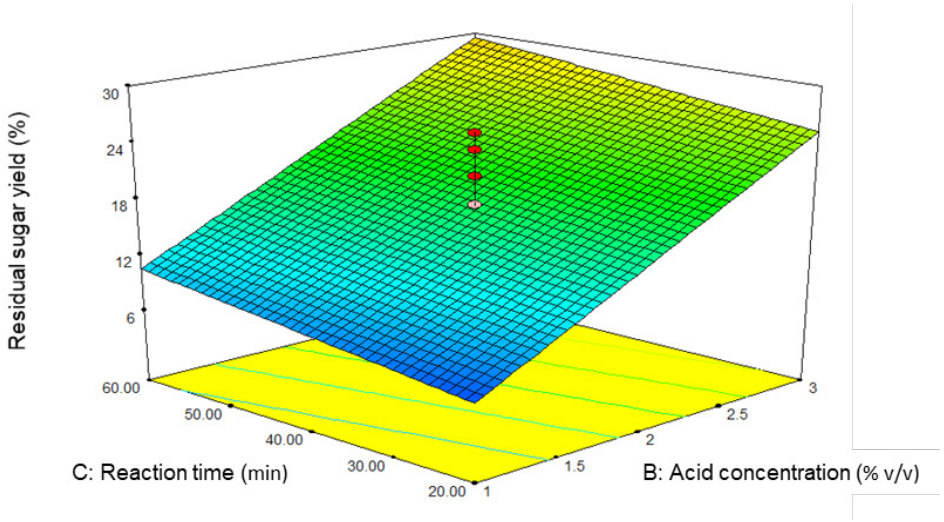


Figure 3. 2^k Factorial analysis model plot of reducing sugar yield at different acid concentration and reaction time values (A: Contour plot, B: Three-dimensional surface plot)

yield versus acid concentration versus reaction time were constructed. Blue indicates low values while red means high values on the surface plot. As seen in Figure 3, the peak response or the maximum reducing sugar yield appears at the high values of both acid concentration and reaction time. However, since no curvature was observed, the equation that describes this model cannot be optimized numerically within the range of process parameters.

Optimization by RSM

Based on the 2^k factorial analysis, acid concentration and reaction time register significant effects on reducing sugar yield. As such, these parameters were considered for the optimization of acid hydrolysis. However, the low and high values of each parameter from 2^k factorial analysis had to be adjusted since no curvature was observed in the model generated, i.e. no optimum point can be obtained. To capture a curvature in the model, the levels of each parameter were adjusted near the high values of each parameter. The new low and high values for acid concentration and reaction time were now 2 and 4 % (v/v) and 30 to 90 min, respectively.

To visualize the model generated and to show the interactions between acid concentration and reaction time, contour and response surface plots were generated. As seen in Figures 4, the reducing sugar yield began declining at the highest values of both factors (4 % v/v sulfuric acid at 90 min) and a curvature indicative of the existence of optimum parameters has materialized. This means that the process parameters that could provide maximum reducing sugar yield can be obtained from the new range of values of acid concentration and reaction time. The mathematical model that embodies these optimum parameters is given below:

$$RSY = -62.0892 + 30.5420[A] + 1.3624[B] - 0.1073[AB] - 3.0463[A^2] - 0.0065[B^2]$$

where:

RSY is the reducing sugar yield (% w/w)
[A] is the acid concentration (% v/v)
[B] is the reaction time (min)

This equation may be used to determine the actual reducing sugar yield as long as the values of acid concentration and reaction time are within 2-4 % (v/v) and 30-90 min, respectively. By numerically optimizing this equation the optimum conditions for the acid hydrolysis of waste *C. vulgaris* biomass was determined to be 3.71 % v/v sulfuric acid and 73.98 min reaction

time. At these conditions, reducing sugar yield will be approximately 44.96 %.

ANOVA was performed to confirm if the model can satisfactorily predict response values. Based on statistical results summarized in Table 7, the mathematical model developed is significant having a p-value of 0.0027. Among the factors considered, acid concentration (A), reaction time (B), and the quadratic term B^2 have significant effects to the response. In addition,

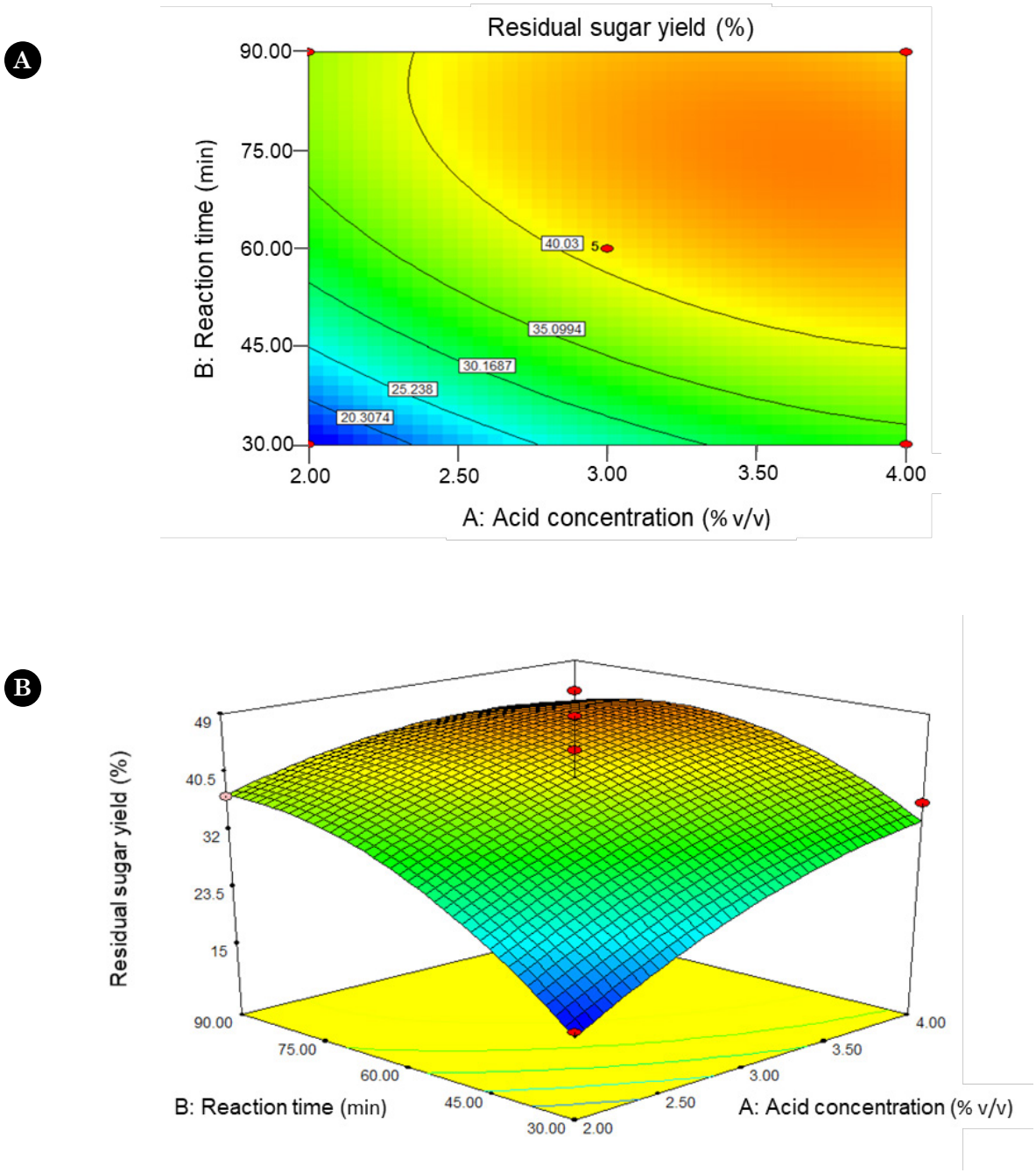


Figure 4. Model plot interaction of reducing sugar yield at different acid concentrations and reaction time (A: Contour plot, B: Three-dimensional response surface plot)

Table 7. ANOVA of the model generated for optimization.

SOURCE	SUM OF SQUARES	DF	MEAN SQUARE	F VALUE	P-VALUE PROB > F
Model	1070.209	5	214.0417	11.6804	0.0027
A-H2SO4 Concentration	271.3297	1	271.3297	14.8067	0.0063
B-Time	481.4253	1	481.4253	26.2718	0.0014
AB	41.48149	1	41.4815	2.2637	0.1762
A^2	64.55504	1	64.5550	3.5228	0.1026
B^2	239.1342	1	239.1342	13.0497	0.0086
Residual	128.2737	7	18.3248	-	-
Lack of Fit	20.5436	3	6.8479	0.2543	0.8552
Pure Error	107.7301	4	26.9325	-	-
Cor Total	1198.482	12	-	-	-

Note: Values of "Prob>F" less than 0.0500 indicate model terms are significant; not significant otherwise.

lack of fit was not significant, which means that the mathematical model generated fits the data well.

The effects of acid concentration (A) and reaction time (B) on RSY may further be explained and predicted using the mathematical model. Factor B having a higher coefficient than factor A means that reaction time has a greater effect on reducing sugar yield. On the other hand, the generated second order model concaving upward as shown in Figure 8 can be explained by the negative coefficients of A^2 and B^2. This indicates that further increasing the values of acid concentration and reaction time will decrease the reducing sugar yield. With respect to the chemistry of the reaction, such decrease may be attributed to the degradation of reducing sugars into simpler byproducts such as propionic acid, acetic acid, formic acid or lactic acid (Hernandez et al., 2015).

Experimental verification

The predicted optimum process parameters for the acid hydrolysis of waste *C. vulgaris* were confirmed through experimental verification. Using the optimum acid concentration (3.71 % v/v) and reaction time (73.98 min), acid hydrolysis of waste *C. vulgaris* biomass was again performed at 130 °C. Results are summarized in Table 8. Results show that the mathematical model generated has good predictive ability, registering an error of only about 2.5%.

Table 8. Comparison of experimental and theoretical reducing sugar yield for the verification of the optimum conditions of the acid hydrolysis.

REDUCING SUGAR YIELD	VALUE (%)
Theoretical	44.96
Experimental	46.09
Error	2.5

The potential of producing reducing sugars from waste *C. vulgaris* by acid hydrolysis was explored in this study. Acid hydrolysis was found to be influenced mainly by acid concentration and reaction time, and minimally by reaction temperature in the range of temperatures studied. Numerical optimization of the generated mathematical model of the reducing sugar yield in terms of the significant factors shows that the acid concentration and reaction time that maximizes reducing sugar yield were 3.71 % (v/v) and 73.98 min, respectively. The numerically optimized mathematical model predicts a maximum reducing sugar yield of 44.96% which agrees well with actual results from verification experiments.

The high lipid and carbohydrate content of *C. vulgaris* shows potential in producing two types of biofuels – biodiesel and bioethanol. Simultaneous optimization of the lipid and carbohydrate composition of *C. vulgaris* during the cultivation stage must therefore be explored to balance the production of these two biofuels by varying media supplied during growth.

Actual production of biodiesel and bioethanol from *C. vulgaris* is suggested to determine the actual energy recovery. Life cycle analysis of the overall process could also be performed for scale up studies.

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