Research Article

Acid Hydrolysis of Pretreated Sugarcane Bagasse, Macroalgae *Sargassum* sp. and Its Mixture in Bioethanol Production

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Abstract
Sustainable biofuel feedstock could become a critical issue in the light of the recent fuel crisis. The use of mixed biomass could reinforce to overcome this issue. The present work examined the parallel use of agricultural residue sugarcane bagasse (SCB) and natural invasive marine seaweed Sargassum sp. (SSP) as a single feedstock and its mixture in two-step concentrated acid hydrolysis followed by yeast fermentation in order to produce reducing sugar with minimal formation of furfural, and bioethanol. In this work, alkali pretreated SCB and SSP were used as feedstock in acid hydrolysis. To investigate the influenced parameters of acid hydrolysis, biomass type (SCB, mixed biomass MB (SCB and SSP in 1:1 ratio by weight) and SSP), initial acid concentration (64–80 wt%), reaction time (30–90 min) and solid loading (10–20%w/w) were optimized by using Taguchi method. The optimized conditions were obtained with mixed biomass type, the initial acid concentration of 64 wt%, reaction time of 60 min and solid loading of 10%w/v. Under these conditions, 0.51 g/g of reducing sugar was achieved without furfural formation although ethanol yield was relatively low compared to that of Taguchi experimental runs. The result indicated that biomass type highly influenced the acid hydrolysis on sugar yield and furfural formation. This study provides the potential route for converting pretreated cellulosic biomass to value-added products, such as sugar and ethanol via the biorefinery process.

Keywords: Sugarcane bagasse, *Sargassum* sp., Acid hydrolysis, Reducing sugar, Furfural, Bioethanol

1 Introduction

Reduction in fossil energy sources, increase in the demand for energy, recent fuel crisis and related global environmental impacts, such as climate changes currently become major issues worldwide [1], [2]. Thus, searching of alternative, inexpensive, and eco-friendly energy sources has notably expanded [3]. Subsequently, the high demand for bioethanol extremely rises a few decades ago to use in transportation sector by blending with gasoline [1], [2]. So, it necessitates for a sustainable increase in bioethanol production in order to meet the high demand [4], making a need for sustainable feedstock. Sugar and starch are well-known feedstock for bioethanol. However, sugars and starch-based bioethanol has limitation upon feedstock and food vs.

fuel crisis [5]. Therefore, massive attention has been paid on the advanced bioethanol using non-food lignocellulosic biomass. It is a cheap renewable source for biofuel and other bioproducts.

Lignocellulosic biomass, accounting for 50% of the world biomass, contains cellulose, hemicellulose, from which bioethanol can be produced by a biochemical pathway through several steps, and lignin [6]. Despite different lignocellulosic feedstocks are used in ethanol production, it has been well known that there are three major steps in bioethanol production: 1) pretreatment and hydrolysis, 2) fermentation, and 3) separation of pure ethanol [7].

Effective hydrolysis of lignocellulosic biomass to value-added products, sugars and bioethanol requires the noticeable enhancement of fermentable sugar along with efficient reduction of inhibitors. Concentrated acid hydrolysis of lignocellulose, such as a two-step acid hydrolysis, has higher efficiency than enzymatic acid hydrolysis and provides complete and fast conversion of cellulose to sugar with low degradation [8], [9]. Sulfuric acid is more widely used in the acid hydrolysis of lignocellulose and it is much cheaper than commercial enzymes. Thus, this is more economically feasible for commercial ethanol production from lignocellulosic materials than enzymatic hydrolysis [9], [10].

However, in specific conditions like high temperature and high solid loading, acid hydrolysis of lignocellulosic material can produce sugar degradation products, such as furfural, 5HMF, acetic acid and phenolic compound, which can decrease sugar yield as well as ethanol yield [11], [12]. The major challenges of concentrated acid hydrolysis are to minimize unavoidable key inhibitors and to recover the spent acid [2]. The latter will not be considered in this study. It has been well known that these inhibitors could be reduced by different detoxification processes [13]. However, these processes cannot definitely remove all inhibitors when the concentration of inhibitors is too high [14]. Sugar degradation that can form inhibitors in acid hydrolysis process usually depends on hydrolysis conditions, such as biomass species, acid concentration, reaction time, temperature and solid loading [15]. Therefore, it is necessary to evaluate the optimal conditions for acid hydrolysis to obtain the high reducing sugar with little degradation. Some studies reported that a high concentration of sugar was achieved with little or large formation of inhibitors under mild operating conditions using a two-step concentrated sulfuric acid hydrolysis [9], [10], [12], [15]–[18].

However, previous studies have almost exclusively focused on single biomass for bioethanol production. A sustainable feedstock supply from various sources in the biorefinery process is necessarily important for the development of the biofuel goal [11]. This can be certainly overcome by the use of mixed biomass. It is of interest to know whether mixed biomass affects sugar yield and sugar degradation for sustainable biomass feedstock because little is known about mixing the two different natural resources such as agricultural waste sugarcane bagasse (SCB) and marine seaweed SSP.

Sugarcane is the world's largest production of crops over the period of 2000–2019, accounting for 21 percent with 1.9 billion tons of the global crop production in 2019 [19]. After juice extraction of sugarcane, approximately 240 kg of SCB with a moisture content of 50% per ton of sugarcane is produced in 2021 [20] and this waste cannot be fully utilized in order to reduce environmental impact. Likewise, marine seaweed SSP is widely and rapidly distributed in the sea of tropical regions, and can also adversely impact the environment. Therefore, it is necessary to effectively utilize it in the biorefinery process due to the enrichment of different carbohydrates in it [21], [22].

The present study aims to investigate the influence of parameters, such as biomass type, acid concentration, reaction time and solid loading on reducing sugar yield and furfural formation in two-step concentrated acid hydrolysis through Taguchi design method. In this study, alkali-pretreated SCB, SSP and its mixture were used as acid hydrolysis feedstock. After neutralization and detoxification, fermentation of all acid hydrolysates was also performed using *Saccharomyces cerevisiae* IAM 4178 under the same conditions to evaluate their fermentation efficiency.

2 Materials and Methods

2.1 *Materials used and sample preparation*

SCB was obtained from vendor shops in Yangon, Myanmar. SSP were collected in Talibon, Bohol, Philippines. Pretreatment is necessary to disrupt the complex structure of lignocellulosic biomass and remove lignin for enhancing hydrolysis efficiency using

enzyme or acid. Therefore, SCB and SSP were individually pretreated by sodium hydroxide before acid hydrolysis as described in our previous works [23], [24]. Optimized alkali pretreated SCB and SSP were used in this study. Pretreated SCB and SSP contained the following compositions: glucan, 46.6 and 21.34 wt%; xylan, 38.9 and 1.24 wt%.; ash, 0.023 and 3.66 wt%, respectively, which were measured by NREL/TP-510-42618 standard method [25]. Chemicals including sulfuric acid, sodium hydroxide and calcium carbonate used in this study were analytical grade and purchased from local suppliers in Manila, Philippines.

2.2 *Two-step concentrated acid hydrolysis*

A two-step acid hydrolysis was performed by subsequent concentrated and diluted sulfuric acid in order to subject to saccharification of cellulose and hemicellulose [12]. For the first step of acid hydrolysis, each of the pretreated samples was mixed with different initial concentrations (64–80 wt%) of sulfuric acid in a 1 L glass container at a weight ratio of 1:1.25 [12]. The mixture was kept at 30 °C in a waterbath for 1 h and constantly stirred to obtain a thorough mixing. Subsequently, for the second step, the mixture was diluted by adding different volume of distilled water in order to achieve a different solid loading (10–20%w/v) and to reduce acid loading in the second step. The diluted mixture was then autoclaved at 121 °C for a different time (30–90 min). After autoclaving, the acid hydrolysate was maintained at room temperature for 1 h and neutralized with calcium carbonate to obtain a pH of 5.6. Thereafter, the neutralized mixture was filtered through vacuum filtration using a 0.45 µm membrane filter to remove the solid residues. The filtrate was then detoxified with 10%w/v of activated carbon and continuously stirred at room temperature using a magnetic stirrer for 1 h and filtered again as mentioned above. The mixed feedstock was prepared using pretreated SCB and SSP in an equal ratio (1:1) w/w of dry matter. The acid hydrolysis of mixed feedstock was conducted in the same procedure as indicated above.

2.3 *Fermentation of acid hydrolysates*

Prior to fermentation, all hydrolysates were supplemented with 5 g/L of peptone and 3 g/L of yeast extract and sterilized in an autoclave at 121 °C for 20 min. Yeast strain *Saccharomyces cerevisiae* IAM 4178 was used in the fermentation of hydrolysates and maintained on yeast extract agar medium containing glucose, 10 g/L; peptone, 5 g/L; yeast extract, 3 g/L; and agar, 20 g/L at 4 °C as described in previous studies [12], [26]. For the preparation of inoculum, *S. cerevisiae* IAM 4178 was precultured in 50 mL of yeast extract liquid medium prepared as described above at 30 °C for 48 h. Then, the precultured liquid medium was centrifuged at 6000 rpm for 15 min and the supernatant was discarded. Yeast pellet collected was washed with sterilized distilled water and centrifuged again. Yeast pellet was then added into each hydrolysate medium previously supplemented with nutrients and inoculated at 30 °C, continuously stirring at 120 rpm for 72 h.

2.4 *Sugar, furfural and ethanol analysis of the hydrolysate*

Reducing sugar content in acid hydrolysates was measured by dinitrosalicyclic acid (DNS) method [27]. The furfurals in acid hydrolysate and ethanol concentration in the fermentation medium were measured using HPLC (Agilent, 1200 series) equipped with an RI detector. Sugar 1011 column (Shodex, Japan) was used with degassed 5 mM sulfuric acid as mobile solvent and a flow rate of 1 mL/min and the column temperature was operated at 60 °C. Before injection, the sample was filtered through 0.45 µm syringe membrane filter. Reducing sugar yield (Y_1) (g/g biomass), furfural (g/g biomass) (Y_2) and ethanol yield (g/g reducing sugar consumed) were calculated as described in the Equations (1)–(3), respectively below. Duplicate analyses for each sample were performed.

$$Y_1 = \frac{C_{RS} \times V}{W \times 1.1} \quad (1)$$

$$Y_2 = \frac{C_F \times V}{W} \quad (2)$$

$$Ethanol = \frac{C_E}{C_{RSC}} \quad (3)$$

where C_{RS} represents concentration of reducing sugar

(g/L); V represents volume of acid hydrolysate (L); W represents weight of pretreated biomass (g); 1.1 is cellulose conversion factor; C_F represent concentration of furfural (g/L); C_E represents concentration of ethanol (g/L); and C_{RSC} represents concentration of reducing sugar consumed (g/L) in fermentation process.

2.5 *Optimization of concentrated acid hydrolysis process using Taguchi robust design method*

For the effective acid hydrolysis of lignocellulosic biomass, acid concentration, reaction time, hydrolysis temperature and solid loading are key factors to be optimized [13]. In this study, only sulfuric acid was used and the temperature was constantly set at 121 °C. Thus, biomass type, initial acid concentration, reaction time and solid loading were optimized using Taguchi robust design. With the Taguchi methods, the independent parameters are arranged in an L9 orthogonal array (OA). This method can achieve the rapid and accurate technical information to design and produce low-cost, highly reliable products and processes. This approach is widely used for the optimization of various processes and also in product development [28]. In general, this method comprises of a set of minimum experimental runs providing the complete and accurate information about the influence of all independent variables on the performance parameters and reduces the experiment runs which can save time and cost compared with other full factorial designs [29]. The major advantage of this design is the simplicity with the ability to adapt easily to more complex experiments comprising several factors with various numbers of levels. The optimum condition is identified by evaluating the main effects of each of the factors to conduct the complete analysis suggested by Taguchi. This approach is the combination of design technique and analysis method, which yield high consistent results of predicted performance [30].

In this study, the $L_9 3^4$ orthogonal array was performed with four independent factors and three levels of each factor for experimental design including a totally nine experimental runs. Factors and levels used in this study are shown in Table 1 and the design matrix with response parameters (Y_1 and Y_2; as denoted in section 2.4) is shown in Table 2. In Taguchi method, the signal-to-noise (S/N) ratio is used as the desired function to determine the optimal parameters of the acid hydrolysis. The S/N ratio is the log transformation of the results of desired performance [31], [32]. There are three quality characteristics of the S/N ratio to be considered viz. 1) "the larger the better" criterion when the target value is to be maximized and high S/N value is preferred; 2) "the smaller the better" when the target value is to be minimized and low S/N value is preferred; and 3) "nominal is better" when the fixed value is targeted [29]. The objective function of this study is to maximize the concentration of reducing sugar and to minimize furfural formation. The S/N ratios of objective functions used were calculated as indicated in the Equations (4) and (5) below.
For reducing sugar,

$$S/N = -10\log_{10}\left(\frac{1}{n_j}\sum_{j=1}^{n}\frac{1}{Y_i^2}\right) \quad (4)$$

For furfural formation,

$$S/N = -10\log_{10}\left(\frac{1}{n_j}\sum_{j=1}^{n}Y_i^2\right) \quad (5)$$

where 'i' is the number of experiment, 'n' is the replication number 'i', 'j' is the replicates number and Y is the response parameter.

Table 1: Assigned factors and levels of Taguchi design for two-stage concentrated acid hydrolysis

Level	Factor			
	A	B	C	D
	Type of Biomass	Acid Concentration (wt%)	Reaction Time (min)	Solid Loading (%w/v)
1	SCB	64	30	10
2	MB*	72	60	15
3	SSP	80	90	20

*Mixed biomass combining pretreated SCB and SSP in 1:1 ratio of dry matter

3 Results and Discussion

Concentrated acid hydrolysis of cellulose biomass generally includes two steps so that high sugar yields can be obtained. In the first step, decrystallization of cellulose occurs while decrystallized cellulose is transformed into sugars in the second step [16]. In this study, a two-step concentrated acid hydrolysis

of single feedstock and mixed feedstock using SCB and SSP was optimized in order to fully understand the effectiveness of feedstock variety in terms of the fermentable sugar, sugar degradation product, furfural formation, and ethanol yield. While maintaining the constant temperature during hydrolysis, it necessitates finding the appropriate time because the long duration of hydrolysis can result in the formation of inhibitors, such as furfural and 5HMF, which can interfere the fermentation process. Furthermore, the concentration of acid influences the yield of sugar extracted from various feedstocks. Therefore, in the two-step concentrated acid hydrolysis process, the factors that affect the yield of the process were considered as follows: 1) type of biomass, 2) acid concentration, 3) reaction time and 4) solid loading along with three different levels for each factor. The influence of process parameters was evaluated using Taguchi design method with response parameters including reducing sugar (g/g pretreated biomass) to be maximized and furfural (g/g pretreated biomass) to be minimized, revealing the effectiveness of acid hydrolysis process. The ethanol yield is commonly described as g ethanol/g dry matter of the raw material. However, this expression was not applicable for this work because different species of biomass were used. Hence, the ethanol yield is calculated based on consumed sugar in the acid hydrolysate. The design matrix with response parameters is presented in Table 2. The mean effect analysis and mean effect plot of each factor corresponding to reducing sugar and furfural are shown in Table 3 and Figure 1, respectively.

Table 2: Taguchi design matrix with responses of two-step concentrated acid hydrolysis

Run	Factor				Response		Ethanol (g/g RS)
	A	B	C	D	Y_1 (g/g biomass)	Y_2 (g/g biomass)	
1	1	1	1	1	0.30	<0.0001	0.07
2	1	2	2	2	0.25	0.006	0.28
3	1	3	3	3	0.20	0.008	0.47
4	2	1	2	3	0.25	0.005	0.34
5	2	2	3	1	0.23	<0.0001	0.21
6	2	3	1	2	0.28	0.004	0.61
7	3	1	3	2	0.12	0.0002	0.17
8	3	2	1	3	0.09	<0.0001	0.03
9	3	3	2	1	0.14	<0.0001	0.51

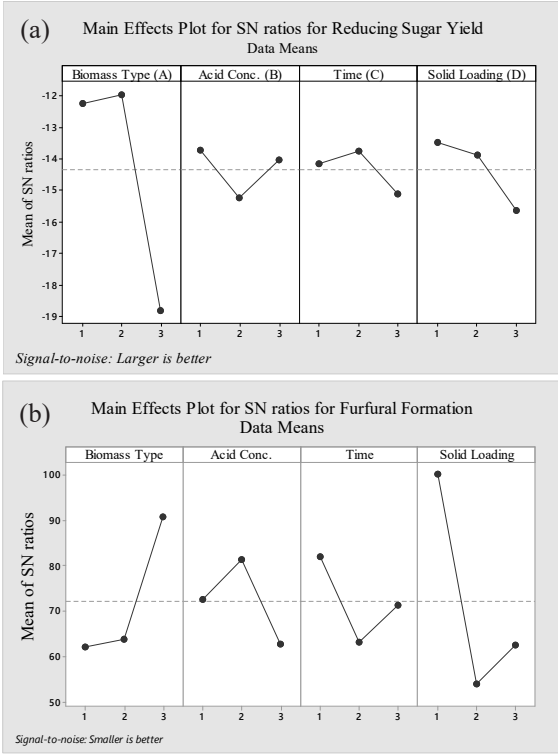

Figure 1: Mean effect plot of (a) reducing sugar yield (Y_1) and (b) furfural formation (Y_2).

Table 3: Response table for signal to noise (S/N) ratio

For Y_1 – Reducing Sugar Yield (g/g pretreated biomass)				
Level	Factor			
	A	B	C	D
1	–12.23	–13.71	–14.13	–13.47
2	–11.94	–15.24	–13.74	–13.86
3	–18.81	–14.03	–15.10	–15.64
Delta	6.87	1.25	1.36	2.17
Rank	1	3	4	2
For Y_2 – Furfural (g/g pretreated biomass)				
Level	Factor			
	A	B	C	D
1	62.08	72.47	82.04	100.00
2	63.90	81.35	63.21	53.99
3	90.60	62.77	71.34	62.60
Delta	28.53	18.57	18.83	46.01
Rank	2	4	3	1

3.1 *Effect of hydrolysis factors on reducing sugar and furfural formation*

The sugar and furfural formation, which are the response

values of Taguchi design in 9 runs for four factors assigned for the concentrated acid hydrolysis process, showed the effectiveness of sugar and furfural formation in producing 0.09–0.30 g/g pretreated biomass (15.41–54.87 g/L) and <0.0001–0.008 g/g pretreated biomass (0.001–1.6 g/L), respectively (Table 2). From the primary result, it is apparent that the lowest yield of sugar was observed in run 8 with a combination of factors of SSP of biomass type, initial acid concentration of 72 wt%, reaction time of 30 min and solid loading of 20%w/v, whereas, the lowest yield of furfural was generated in run 1, 5, 7, 8, and 9. This indicates that a high formation of furfural preferably occurred with high sugar yield under extreme conditions. However, the highest yield of sugar (32.57 g/L, equivalent to 0.3 g/g) was produced in run 1 with the combination of the lowest levels of each factor, whereas the highest yield of furfural (1.6 g/L equivalents to 0.008 g/g) was observed in run 3 using SCB biomass with the highest levels of the factors. This suggests that different operating conditions affected the different species of biomass regarding to reducing sugar yield and inhibitor formation

For the most part, there was a significant proliferation in sugar yield of MB compared to the single SSP and a substantial decrease in furfural formation from MB compared to SCB. For these cases, a similar trend was reported by Vera *et al.* [33], explaining that synergistic effects were observed when using hybrid poplar and wheat straw as a mixture in steam pretreatment followed by enzymatic hydrolysis obtaining more monomeric sugar recovery and less sugar degradation by the interaction of ash and acetic acid formed. As mentioned in section 2.1, the presence of ash in SSP is higher than in SCB. Megawati *et al.* [11] also revealed that sugar yield differed from different feedstocks and the mixture and sugar yield obtained from mixed biomass including twig, corncob, leaf, and sawdust was quite high compared to single biomass except from leaf. Nevertheless, the hydrolysis of single SCB and single SSP in this report is in the comparable result range with the results of other works [34]–[37].

Moreover, the difference in chemical compositions, such as carbohydrates could be observed in different biomass or even in the same biomass type depending on the geographical location, plant variety and processing conditions [38]. For example, brown seaweed contains different carbohydrates including alginate, mannitol and laminarin. Therefore, different yields of sugar were observed when specific biomass was used. Acid hydrolysis of SCB gave a high yield of sugar (Run 1, 2, and 3) while acid hydrolysis with SSP produced a low concentration of sugar (Run 7, 8, and 9). The possible reason was due to the low content of cellulose in brown macroalgae compared to SCB as described in section 2.1, which had been considered a less potential biomass source in bioconversion than land-based biomass [32]. A contrary observation was reported by Nguyen *et al.* [39] conveying that differences in the compositions of pretreated mixed biomass did not show the negative effects on the hydrolysis process. However, a comparable yield of sugar was apparently perceived in hydrolysis of mixed biomass (Run 4, 5, and 6). This result highlights that mixing the biomass positively affects the sugar yield as well as furfural reduction in hydrolysis process compared to the use of single SCB and SSP. Similarly, Imamoglu and Sukan [40] also reported that combining rice husk and corn stalk had a positive effect on ethanol production with a maximum yield of ethanol. Contrarily, Brodeur-Campbell *et al.* [31] reported that any synergistic or antagonistic effect on sugar yield was not observed for any of feedstock mixtures including aspen, balsam and switchgrass when diluting acid pretreatment and enzymatic hydrolysis were subsequently performed.

Generally, there is a limit of acid concentration that sugar formation can still be observed and over which acid hydrolysis becomes a harsh condition transforming sugar to degradation product such as furfural [15]. It was clearly seen that increase in acid concentration with increased time resulted in the reduction of sugar yield and consequently a high yield of furfural, especially for SCB (Table 2). Furfural is commonly generated from degradation of xylose at high acidic and temperature conditions. As mentioned in section 2.1, pretreated SCB has more xylan content than pretreated SSP. In addition, variations of acid concentration in the second step of hydrolysis would affect the sugar yield and sugar degradation. Acid loadings of run 1–9 in second step hydrolysis became 7.7, 12.6, 18.1, 14.67, 8.6, 13.9, 11.3, 16.4, and 9.5 wt%, respectively.

The effect of reaction time in the presence of biomass and acid with different solid loadings, strongly affected the sugar yield of acid hydrolysis resulting decrease in sugar yield with increased time. This can be explained that sugar from the acid treatment of biomass at a long time degraded into furfural as depicted in

Table 2 and other degradation products such as 5HMF and acetic acid [32].

For the effect of solid loading, sugar yield decreased with an increase in solid loading since a decrease in hydrolysis efficiency at high solid loading was associated with less penetration of acid into solid [41] or accumulated inhibitors that also result in low fermentation yield [42]. This is strongly supported by Figure 1(b), indicating that high furfural formation occurred with high solid loading. In addition, furfural is more likely to be found in SCB than in SSP. However, as presented in Table 2, it decreased when the mixture was used as feedstock. It can be concluded that mixed biomass can reduce the formation of furfural in bioethanol production compared to the use of single biomass SCB. This is a promising finding in the use of mixed biomass for acid hydrolysis in order to produce reducing sugar. However, the reason remains unclear to which degree of biomass mixture is attribute to the yield. In the previous literature on acid hydrolysis, Kumar *et al.*, [10] reported that sugar recovery of up to 88% of theoretical sugars with negligible furfural/HMF formation was achieved in two stages acid hydrolysis of sugarcane bagasse. Wijiya *et al.*, [15] stated that a sugar yield of 52 wt% was obtained from a two-step concentrated acid hydrolysis of pine wood. Tanaka *et al.*, [12] revealed that high-concentration sugar was produced with no furfural formation after detoxification in acid hydrolysis of cassava stem. Huang *et al.*, [17] demonstrated that a high concentration of xylose (101.1 g/L) with furfural removal of 92% was produced in acid hydrolysis of wood pulping after polystyrene divinylbenzene resin detoxification.

Our primary results indicate that the yields of reducing sugar and formation of furfural highly depend on the biomass species and hydrolysis conditions, including acid concentration, reaction time or solid loading. In turn, this finding also suggests that SCB can be taken into consideration for the production of value-added products, such as furfural from agricultural waste rather than bioethanol.

3.2 *Statistical analysis of Taguchi Method for optimization of acid hydrolysis*

3.2.1 Mean effect analysis

The factors for two-step concentrated acid hydrolysis and their levels exhibited the significance of optimization for achieving maximum sugar yield and minimum formation of furfural as two responses. The influence of factors on response parameters was determined by analysis of rank and delta calculated from differences between the largest S/N values and lowest S/N values of each factor. Then, the factors are arranged in order of rank from the largest effect to the lowest effect on the performance of each response as depicted in Table 3 and the main effect of the S/N ratio were plotted in Figure 1. Since the objective functions used in this study were to maximize the response Y_1, the larger the S/N value, the better the influenced level; and to minimize response Y_2, the smaller the S/N value, the better the influenced level. The highest S/N ratio in each factor is preferable to achieve the maximum sugar and the lowest S/N ratio is preferable to obtain less formation of furfural. From Table 3 and Figure 1(a), it can be clearly observed that biomass type of level 2 (mixed biomass), initial acid concentration of level 1 (64 wt%), reaction time of level 2 (60 min) and solid loading of level 1 (10%w/v) had the largest S/N values for a response of reducing sugar (i.e. –11.94, –13.71, –13.74, and –13.47, respectively). According to the rank, type of biomass achieved the first rank as the best significant factor on reducing sugar yield in the a two-step concentrated acid hydrolysis of pretreated biomass, followed by solid loading, acid concentration and reaction time. Whereas, significant change was observed in biomass type affected the sugar yield as depicted in Figure 1(a). Moreover, high solid loading did not produce a high sugar yield. The contrary observation was reported in the literature [12] and high solid loading (20%w/v) of cassava stem produced high sugar yield in repeated acid hydrolysis.

Meanwhile, biomass type of level 3 (SSP), initial acid concentration of level 2 (72 wt%), reaction time of level 1 (30 min) and solid loading of level 1 (10%w/v) ensured the S/N values for the response of furfural (i.e. 90.6, 81.35, 82.04, and 100, respectively) which were desirable to achieve the minimum formation of furfural. Contrasting to the rank of sugar yield, the rank values corresponding to furfural formation suggest that solid loading had the utmost significant effect on the furfural formation next to biomass type, reaction time, and acid concentration. Dramatic changes were found in biomass type and solid loading when using different

biomass and lowering the level of solid loading as conveyed in Figure 1(b), in which minimum formation of furfural was achieved in SSP biomass and lowest solid loading. This may be due to the fact that SSP has low xylan content, which is the main source of furfural formation and less accumulation of inhibitor on low solid loading [9]. According to the mean effect analysis based on reducing sugar data, the optimized parameters were type of mixed biomass, initial acid concentration of 64 wt%, reaction time of 60 min and solid loading of 10%w/v.

3.2.2 Analysis of variance (ANOVA)

Contributions (the mean response magnitudes) of factors affecting the acid hydrolysis on reducing sugar yield and furfural formation were calculated through analysis of variance (ANOVA) tool. Initial acid concentration showed the least sum of squares on reducing sugar yield and reaction time had the least sum of squares in furfural formation according to initial analysis of variance. Therefore, in analysis of variance (ANOVA), these factors were necessary to be pooled up to improve the significance of other factors [30] and it is shown in Table 4. From Table 4, an evaluation of the effect of each factors on reducing sugar formation in acid hydrolysis by ANOVA confirmed that the type of biomass highly represented as the most dominant factor comprising 83.07% of the total contribution followed by solid loading (7.29%), reaction time (6.14%) with pooled error (3.50%). As $F_{0.05,2,2}$ at a 5% significant level is 19.0, the factor of biomass type with F-value of 21.88 is statistically significant. The results obtained from ANOVA analysis were consistence with the mean effects analysis. It can be concluded that the impact of biomass type used was highly significant in a two-step acid hydrolysis of lignocellulosic material.

ANOVA data corresponding to furfural formation showed the influence of the principal factor on the acid hydrolysis process (Table 4). Biomass type and solid loading were equally distributed with each 40 % contribution, granting the most influential factors for furfural formation in acid hydrolysis. From F-value test, none of the factors showed a statistically significant effect on furfural formation. However, contribution results are also in agreement with the mean effects analysis as shown in Table 3.

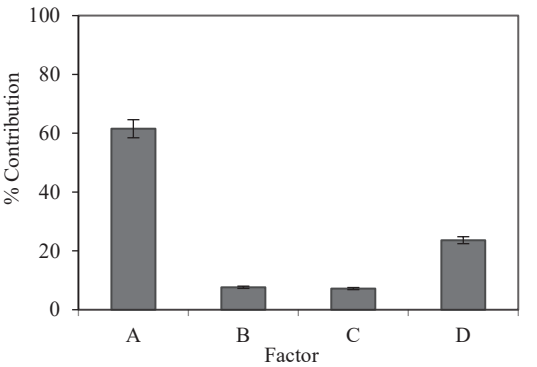

Figure 2: Bar chart showing overall % contribution of reducing sugar yield and furfural formation.

Table 4: ANOVA analysis corresponding to Y_1 and Y_2

For Y_1 – Reducing sugar yield					
Source of Variations	**DF**	**Sum of Squares**	**Mean Square**	**F-value**	**% Contribution**
Types of Biomass	2	0.0361	0.0181	21.88	83.07
Reaction Time	2	0.0027	0.0013	1.56	6.14
Solid loading	2	0.0032	0.0016	1.96	7.29
Error	2	0.0015	0.0008		3.5
Total	8	0.0435	0.022		100
For Y_2 – Furfural formation					
Source of Variations	**DF**	**Sum of Squares**	**Mean Square**	**F-value**	**% Contribution**
Type of Biomass	2	0.000034	0.000017	4.9	40
Acid Conc.	2	0.00001	0.000005	1.43	11.76
Solid Loading	2	0.000034	0.000017	14.9	40
Error	2	0.000007	0.000004		8.24
Total	8	0.000085	0.000043		100

Figure 2 shows the overall contribution for reducing sugar and furfural formation. The overall chart clearly shows that biomass type is the main determinant with contribution of 51.56%, followed by solid loading at 23.64% and the rests are less effect.

Regression analysis was also performed based on observed data of reducing sugar. The regression equation for reducing sugar yield is shown in the Equation (6) below.

$$Y_1 = 0.434 - 0.066A - 0.007B - 0.02C - 0.021D \quad (6)$$

Variation of the regression equation (R^2 = 0.73) is

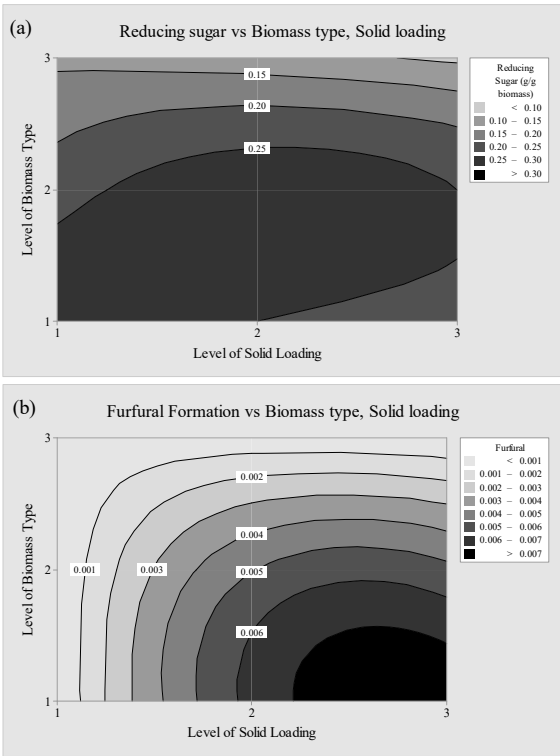

Figure 3: Contour plots of interaction between biomass type and solid loading corresponding to (a) reducing sugar and (b) furfural formation.

73% indicating that the equation fairly fitted with experimental data. The interaction between biomass type and solid loading on reducing sugar yield and furfural formation was evaluated using the observed data.

The contour plots, as depicted in Figure 3, illustrate the relationship between the biomass type and solid loading used in the a two-step concentrated acid hydrolysis to produce maximum reducing sugar and minimum formation of furfural. The darker regions represent the higher yield. As shown in Figure 3(a), the higher yield of reducing sugar appeared to make a shape from lower left to middle right, indicating that both SCB (level 1) and MB (level 2) produced the higher yield of sugar with low level to a high level (10–20%w/v) of solid loading and only MB (level 2) released maximum yield of sugar in a moderate level of solid loading. The maximum sugar yield by this combination effect is expected to appear within these ranges. As mentioned in section 3.1, comparing the sugar yield of experimental runs (1–3), the highest sugar yield (0.30 g/g) was obtained with SCB at a low level of solid loading and, subsequently, sugar yield increased with decreased solid loading using mixed biomass. The upper part of the plot represents the biomass type and solid loading combination that SSP produces the low sugar yield at any level of solid loading. On the contrary, from Figure 3(b), the highest formation of furfural occurred at the lower right corner, indicating that furfural formation increased with increasing level of solid loading and decreasing level of biomass type (i.e., SSP<MB<SCB). Therefore, the highest formation of furfural would be generated within biomass type of level 1 and solid loading of level 3.

The predicted value of sugar yield at the optimized level of conditions (the type of mixed biomass, initial acid concentration of 64 wt%, reaction time of 60 min and solid loading of level 10%w/v) was 0.29 g/g pretreated biomass. To confirm the Taguchi optimized data, the confirmation experiment was conducted with duplicate runs using optimized conditions of a two-step concentrated acid and performed as same procedures mentioned in section 2.2 and 2.3. It was observed that a reducing sugar yield of 0.51 g/g of pretreated biomass was obtained in the confirmation run and it was a 1.56-fold increase compared to the predicted value. This was probably due to the fact that acid concentration of hydrolysis and low solid loading with a high volume of solution would make more penetration into an inner matrix of biomass disrupting the crystalline structure and producing high sugar concentration [43]. Furthermore, furfural was not detected in the acid hydrolysate. This can be explained by the fact that furfural formation would reach below the lower limit of quantification of the HPLC as a result of detoxifying action. From the economic point of view, the low acid concentration is the better choice and low solid loading is good for mixing. Therefore, it reveals that the Taguchi method of acid hydrolysis process can be applied for the desired product in biorefinery process. Likewise, it is important to note that a two-step acid hydrolysis process effectively provides the enhanced reducing sugar yield with minimum furfural formation, which is one of the key inhibitors in bioconversion process.

3.3 *Fermentation of resulted acid hydrolysates*

All acid hydrolysates from acid hydrolysis process were subjected to a fermentation process using *S. cerevisiae*

IAM 4178 to produce ethanol as described in section 2.3. The results of ethanol yield are presented in Table 2. It can be seen that the ethanol yield of acid hydrolysates ranges from 0.03 to 0.61 g/g consumed sugar (0.40 to 20.37 g/L). The theoretical yields of bioethanol from run (1–9), calculated based on their initial reducing sugar, were 7.1, 30.3, 91.3, 18.4, 21.1, 70.9, 14.9, 3.9, and 43.1 %, respectively. The maximum ethanol yields of 0.47, 0.61 and 0.51 g/g sugar were found in run 3, 6 and 9, respectively, even in the presence of high furfural (i.e., 1.5 and 0.7 g/L) except of run 9, which used SCB and MB in acid hydrolysis. This indicates that furfural did not show the adverse effects on the fermentation process. The minimum ethanol yield of 0.03 g/g was obtained in run 8 using SSP. This is possible that ethanol yield from yeast fermentation practically depends on a concentration of sugar obtained from acid hydrolysis rather than the type of biomass and another reason is probably that the yeast (*S. cerevisiae* IAM 4178) can assimilate the proper amount of furfural during fermentation [12]. However, it was also found that high sugar yield did not produce high ethanol yield as seen in run 1. Megawati *et al.* [11] claimed that the presence of different sugar types and sugar degradation products impeded the fermentation process. Moreover, it was possible that presence of salt (generated during neutralization of excess acid) and fermentation conditions (such as pH, temperature, nutrient supplement and initial concentration of yeast) would limit the fermentation process [44]. Therefore, fermentation conditions probably reflect the extent of fermentation efficiency regardless of sugar concentration. The present study utilized the established fermentation parameters based on the previous reports [12], [26].

Nevertheless, our result of ethanol yield was higher than the previous report [36]. Moreover, hydrolysis of MB is reportedly a better result than mixed algae [43] and a similar result with the whole sugarcane residues (mixture of bagasse, straw and top) [45].

Ethanol yield obtained from acid hydrolysate of confirmation run was 0.16 g/g sugar (3.9 g/L) produced from initial reducing sugar concentration of 56.1 ± 0.17 g/L (0.51 g/g pretreated biomass). It was lower than that from run 4 operated by similar conditions except for solid loading, producing ethanol yield of 0.34 g/g sugar with an initial reducing sugar concentration of 54.9 g/L. Moreover, theoretical yield and volumetric productivity of ethanol were 13.64% and 0.05 g/L/h, respectively. Lowering ethanol yield and fermentation rate can be explained by the fact that possible formation of inconsumable sugars by yeast, insufficient nutrient supplement and use of unoptimized conditions in fermentation, such as inoculum type, pH, temperature, inoculum concentration would limit the growth and productivity in the bioconversion process. On the other hand, the certain existing technologies would provide the diverse results, positive and negative effects on the entire production process [46].

Mass balance of acid hydrolysis and fermentation was also evaluated based on the data of confirmation run operating at optimized conditions. It was found that total reducing sugar of 18.6 g in 40 g of pretreated mixed biomass (equal ratio of SCB and SSP) produced a reducing sugar of 15.98 g in the a two-step acid hydrolysis process. Subsequently, an ethanol concentration of 1.1 g with a theoretical yield of 13.64 % was obtained in the fermentation process. This reveals that mixed biomass can be considered a potential feedstock in biorefinery process for bio-based product.

4 Conclusions

The present work investigated the influence of operating parameters including biomass type on reducing sugar yield and furfural formation through Taguchi robust design. Biomass type and solid loading relatively influenced the two-step acid hydrolysis on maximizing the reducing sugar yield and minimizing the furfural formation. The use of mixed biomass could effectively reduce the furfural formation with a high sugar yield. High solid loading significantly impacted the furfural formation. Reducing sugar yield of 0.51 g/g pretreated biomass without furfural formation was obtained at optimized conditions – mixed biomass, initial acid concentration of 64 wt%, reaction time of 60 min and solid loading of 10%w/v. However, ethanol yield was comparatively lower than those using acid hydrolysates from Taguchi experimental runs. This may be considered as further optimization process of fermentation to improve ethanol yield. Overall, our results demonstrate the efficient use of agricultural waste SCB and invasive marine seaweed SSP in acid hydrolysis for bioethanol production and provide the potential process for converting pretreated mixed biomass to reducing sugar with low furfural

and ethanol. Additionally, further research is needed to verify the complexities of mixed biomass that can be drawn from this study.

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Author Contributions

K.W.: research idea, methodology, investigation, data analysis, writing the original draft, reviewing and editing; J.A.: conceptualization, supervision, research idea, data analysis, project administration, funding acquisition and reviewing; L.A.: conceptualization, supervision and reviewing; P.G.: conceptualization, supervision and reviewing; K.N.: conceptualization, supervision and reviewing. The authors have read and agreed to publish this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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