Transformation of Starchy Lignocellulosic Biomass to Ethanol using *Ragi Tapai* Synergized with Microwave Irradiation Pretreatment

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Ethanol production strategy was studied using multiple strain microbes from microwave irradiation (MI) pretreated sago waste. Sago waste (SW) was MI-pretreated for reducing sugars production using 2 heating media (water and sulfuric acid) under pretreatment conditions including MI power, pretreatment duration, and solid loading. When water was used, the pretreatment parameters were optimized using Box-Behnken Design (BBD). However, gelatinized starch and charring of SW led to an insignificant quadratic model. To mitigate the gelatinization problem while determining the best MI pretreatment conditions, water was substituted by sulfuric acid using single factor method. The highest reducing sugar yield of 261.5 mg/g SW was achieved at 7.5% solid loading, 6 min pretreatment duration, and 300 W MI power. The effectiveness of the pretreatment was ascertained by field-emission scanning electron microscopy (FESEM) and chemical-composition analysis. When fermenting MI-pretreated SW using *ragi tapai,* simultaneous saccharification of starch and ethanol production was evidenced from the sugar/ethanol profile. A resulted yield of 7.24 g ethanol/100 g SW confirmed the fermentability of MI-pretreated SW. The ethanol production was well fitted into the modified Gompertz model.

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INTRODUCTION

Sago waste (SW) is a fibrous cellulosic residue generated during the sago starch extraction process (Awg-Adeni *et al.* 2010), and it has no significant industrial or commercial usage. However, by applying pretreatment processes such as acid hydrolysis, alkaline pretreatment, steam explosion, and biological pretreatment (Menon and Rao 2012), the physical and chemical properties of lignocellulosic biomass can be altered to enhance the hydrolysis rate for sugar production.

Like the conventional pretreatments stated above, microwave irradiation (MI) pretreatment imparts structural modification to lignocellulosic biomasses, making them more susceptible to enzymatic attack in facilitating sugar production (Choudhary *et al.* 2012). Despite the reported effectiveness of microwave irradiation pretreatment of lignocellulosic biomasses, thus far the biomasses studied are confined to non-starchy agricultural wastes such as barley husk (Palmarola-Adrados *et al.* 2005), bench wood (Verma *et al.* 2011), rice straw (Ma *et al.* 2009), and switchgrass (Kieshwani and Cheng 2010). Hence, extending MI pretreatment to starchy lignocellulosic waste such as sago waste to produce fermentable sugars is worth investigated.

To convert starchy substrate directly into ethanol, co-culture of microorganisms *i.e. ragi tapai* that are capable of performing saccharification and fermentation *in situ*

simultaneously (Rasmussen *et al.* 2010), had been employed on edible starch. *Ragi tapai* is a co-culture of various strains of fungus, yeast, and bacteria (Merican and Quee-Lan 2004) but its performance on lignocellulosic biomass consisting of starchy component is yet to be confirmed.

In view of the challenges stated above, the study first aimed to evaluate the performance of microwave irradiation pretreatment for sugar production from starchy SW, which has never been attempted. Apart from comparing with the conventional heating method, the efficiency of reducing sugars production from MI pretreatment process was verified *via* surface-structure disruption images from field emission scanning electron microscopy (FESEM) and chemical-composition analysis of the pretreated SW. Subsequently, the fermentability of the reducing sugar from MI pretreated sago waste was investigated by means of sugar and ethanol profiling during the fermentation process using *ragi tapai*. Finally, to consolidate the study, the modified Gompertz model was applied for ethanol production in the fermentation process.

EXPERIMENTAL

Preparation of Sago Waste Sample

Sago waste was collected from Kai Heng Sendirian Berhad, Batu Pahat, Johor, Malaysia. The biomass was dried in an oven (UT6, Thermo Heraeus, USA) at 40 °C for 2 days. The dried SW was ground and sieved into particle size between 250 and 500 µm and stored in a dry cabinet prior to be used.

Microwave Irradiation Pretreatment of Sago Waste

One gram (1 g) of dried SW sample was weighed and put into a 100-mL sample bottle. Various amounts of heating medium (*i.e.*, water or sulfuric acid) were added to the SW sample based on pre-determined solid loading. An 800-W domestic microwave oven (ME711K, Samsung, Korea) was used to pretreat the SW sample at various microwave powers and pretreatment durations. After pretreatment, 20 mL of water was added to the pretreated SW to stop sugar hydrolysis while initiating sugar dissolution. The pretreated SW was then cooled to room temperature in a water bath before it was centrifuged at 3500 rpm for 10 min (Centrifuge 5810 R, Eppendorf, Germany). The supernatant liquid was analyzed for reducing sugar content while the solid residue was subjected to compositional and surface-structure analysis.

Conventional Heating Pretreatment on Sago Waste

In conventional heating pretreatment, water was added into 1 g of SW in a glass test tube to achieve 13% (w/w) solid loading and pretreated at 130 °C for 2 min in an oil bath (MC, Julabo, Germany). Twenty (20) mL of RO water was added into pretreated SW and the mixture was then centrifuged at 3500 rpm for 10 min. The supernatant was analyzed for reducing sugar's concentration.

Optimization of Microwave Irradiation Pretreatment of Sago Waste with Water

In the optimization study using water as heating medium to pretreat SW *via* microwave irradiation, three independent variables, *i.e.*, microwave irradiation power (100 W to 500 W), pretreatment duration (1 to 5 min), and solid loading (13% to 23%) were investigated using Design Expert 7.0.0 software (STAT-EASE Inc., Minneapolis, USA). The design matrix was generated by Box-Behnken Design (BBD), and the selected ranges of the variables determined from the preliminary studies are listed in

Table 1. The performance of the pretreatment process was evaluated based on the yield of reducing sugars, y, which could be fitted into a quadratic model as shown in Eq. 1,

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=1+1}^{3} \beta_{ij} x_i x_j \qquad (1)$$

where x_i and x_j are the independent variables studied, β_0 is the constant coefficient, β_i is the ith linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient. The significance of the model was examined by analysis of variance (ANOVA).

Table 1. Independent Variables with Coded and Actual Levels

Variables	Unit	Level		
		-1	0	1
Microwave Irradiation Power	(W)	100	300	500
Pretreatment Duration	(min)	1	3	5
Solid Loading	(%)	13	18	23

Microwave Irradiation Pretreatment of Sago Waste using Sulfuric Acid

When SW was MI pretreated using water as heating medium, gelatinization of starch and charring of SW under high solid loadings were observed. To accurately determine the range of operating conditions for MI pretreatment, a single-factor approach was adopted when sulfuric acid was used as heating medium at lower solid loadings to overcome the gelatinization and charring phenomena. Various quantities of 0.1 M sulfuric acid were added to SW samples to prepare for solid loadings at 7.5%, 10%, and 12.5%. Each sample bottle containing a specific solid loading was capped, and treated at a moderate microwave power of 300 W for 2 min to avoid charring of SW. After the pretreatment, sugar dissolution and solid-liquid separation process were repeated as previously described.

The solid loading that gave the highest amount of sugar was selected to study the effect of microwave irradiation pretreatment duration between 2 min and 8 min. The mentioned solid loading combining with the best pretreatment duration that produced highest amount of sugar, were then used to study the effect of microwave irradiation pretreatment power at 100, 300, 450, and 700 W. Similar procedures for reducing sugar analysis was performed on the pretreated samples, and all experiments were conducted in duplicate.

Fermentation of MI Pretreated Sago Waste by *Ragi Tapai*

Ragi tapai used in this work was obtained from a local market, Kuala Lumpur, Malaysia. The culture medium for *ragi tapai* consisted of 1% (w/v) yeast extract, 1% (w/v) peptone, and 0.5% NaCl (w/v) and the medium was autoclaved at 121 °C for 20 min. *Ragi tapai* powder at the 4% level was inoculated into the autoclaved medium and incubated at 25 °C and 125 rpm for 60 min prior to fermentation. To initiate fermentation of *ragi tapai*, 5 mL of the inoculum was added to the MI-pretreated SW (adjusted to pH 5.0) and incubated at 25 °C for 120 h. Samples were collected at the fermentation durations of (0, 4, 8, 16, 24, 48, 72, 96, and 120) h. 20 mL RO water was added to fermented SW to dissolve the remaining reducing sugar and the ethanol produced. The mixture was centrifuged at 3500 rpm for 5 min to facilitate solid-liquid separation. The supernatant liquid was analyzed for reducing sugar and ethanol concentration using high-performance liquid chromatography (HPLC; Waters, USA) analysis.

Production Kinetics – Modified Gompertz Model

The ethanol profile obtained from the fermentation process was fitted into the modified Gompertz model by using Matlab software and the modified Gompertz model (Ginkel *et al.* 2001; Lin and Lay 2004) as represented by Eq. 2,

$$P = P_m \cdot \exp\left(-\exp\left[\frac{r_{p,m} \cdot \exp(1)}{P_m}\right] \cdot (t_L - t) + 1\right) \tag{2}$$

where P is product concentration at instantaneous time, P_m is the potential maximum product concentration, $r_{p,m}$ is the maximum rate of production, t and t_L are the fermentation duration and lag phase respectively.

The data were best fitted into the model by reducing the sum of square of residuals, and the fitness of the data can be determined by Eq. 3,

$$R^2 = 1 - \frac{SS_{resid}}{SS_{total}} \tag{3}$$

where R^2 is the coefficient of determination, SSresid and SStotal are the sum square of residuals and the total sum square of residuals, respectively.

Analytical Methods

Reducing sugar analysis

The supernatant solution collected from the pretreated samples was analyzed for reducing sugar using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). Based on the reducing sugar solution standard, the UV absorbance of the samples was measured at 540 nm. The reducing sugar yield was calculated using Eq. 4:

$$\text{Yield}, \ y\,(\%) = \frac{\text{Amount of Reducing Sugar, mg}}{\text{Initial Amount of Biomass, g}} \times 100\% \tag{4}$$

Field emission scanning electron microscopy (FESEM)

To prepare the sample for FESEM images, 1 g of pretreated SW was rinsed thoroughly with water and dried in an oven at 60 °C overnight. The dried samples were then mounted on an aluminum stub with double-sided adhesive carbon tape. The images were acquired at magnifications ranging from 180 to 1600 times and developed using an FEI QUANTA 450 FEG (USA) electron microscope operated at 10 kV under low vacuum.

Analysis of chemical composition

The compositions of both untreated and pretreated SW were analyzed following National Renewable Energy Laboratory (NREL) analytical procedures (Sluiter *et al.* 2008). Two-step sulfuric acid hydrolysis was employed for this purpose. First, 72% sulfuric acid was added to the raw SW and microwave-pretreated SW. The sulfuric acid in the mixture was diluted to 4% with water, and the mixture was then autoclaved to break down the structure of polysaccharides in SW into sugar monomers. The chemical components was quantified using HPLC. A refractive-index detector was used to detect carbohydrate in HPLC. The acid-insoluble lignin (AIL) was determined gravimetrically by subtracting the ash content from the residue obtained from acid hydrolysis. The analytical procedure was performed in duplicate, and the average value was calculated.

Reducing sugar and ethanol concentration analysis

Reducing sugar and ethanol concentration from the fermentation was analyzed by using HPLC equipped with Hi-Plex H-column (300 × 7.7 mm) at 65 °C with ultrapure water as mobile phase at fixed flow rate of 0.6 mL min^{-1}. The sample was filtered by Nylon filter (0.2 μm, Sartorius Stedim Biotech, Germany) before analysis. Ten (10) μL of sample was injected and a refractive-index detector was used to detect the concentrations of reducing sugar and ethanol. Ethanol concentration in the sample was determined from the standard ethanol calibration curve.

RESULTS AND DISCUSSION

Comparison between Reducing Sugar from Conventional Heating and Microwave Irradiation Pretreated Sago Waste

The reducing sugar produced from the conventional heating pretreated SW and the MI-pretreated SW was compared, and the results are tabulated in Table 2.

Table 2. Comparison between the Reducing Sugars Produced from Different Pretreatment Methods at Various Pretreatment Conditions

Pretreatment Method	Pretreatment Conditions	Reducing Sugar Yield (mg/100g)
Without Pretreatment (control)	25 °C 2 min	Not detected
Conventional Heating	130 °C 2 min 13% solid loading	1.4
Microwave Irradiation	300 W 2 min 13% solid loading	4.3

It is no surprise that no reducing sugars were detected from the untreated SW, as the lignocellulosic matrix was intact and the raw starch granules in SW was insoluble in the water without heating. The amount of reducing sugars produced from conventional heating method was 3 times lesser than that obtained from MI-pretreatment under the same pretreatment duration and solid loading.

With better heating efficiency attributable to its heating mechanism, when subjected to MI pretreatment, starch in the SW was hydrolyzed into reducing sugar within 2 min at a moderate microwave power of 300 W. Moreover, MI pretreatment that disrupted the structure of biomass (Hu *et al.* 2008) might have exposed starch granules to hydrolysis and increased the reducing sugar production.

Comparatively, conventional heating method through conduction or convection based heating (Hu and Wen 2008; Liu and Wyman 2005; Mosier *et al.* 2005) requires longer pretreatment duration (> 30 min) and higher pretreatment temperature (> 100 °C).

When pretreatment duration was set at 2 min, limited starch in the SW could be hydrolyzed into reducing sugar. Thus, only 1.4 mg of reducing sugars was achieved for 100 g of SW pretreated. The extent of MI pretreatment on sago waste was studied *via* optimizing operating parameters based on suitable experimental design in the next section.

Optimization of Microwave Irradiation Pretreatment of SW with Water using Box-Behnken Design

Reducing sugar production from microwave irradiation-pretreated SW with water was optimized using Box-Behnken Design. The parameters under investigation and the responses (reducing sugar yield) obtained are listed in Table 3.

Table 3. Experimental Design Matrix with their Respective Reducing Sugar Yields in the Microwave Irradiation Pretreatment of SW with Water

Run	Experimental Variables			Response (y, mg per 100 g SW)
	Microwave Irradiation Power (W)	Pretreatment Duration (min)	Solid loading (%)	
1	500	1	18	6.7
2	300	3	18	5.9
3	300	3	18	6.3
4	100	1	18	6.4
5	100	3	23	6.8
6	100	5	18	7.6
7	100	3	13	7.1
8	300	3	18	6.2
9	500	5	18	7.3
10	500	3	23	6.3
11	500	3	13	7.9
12	300	3	13	6.5
13	300	1	23	7.8
14	300	5	23	6.3
15	300	5	13	7.1
16	300	1	13	6.7
17	300	3	18	6.3

Higher microwave power combined with longer pretreatment duration resulted in lower reducing sugar production at a high solid loading of 23%. At the stated solid loading if low to moderate microwave power, *i.e.* 100 to 300 W, was applied, then the amount of reducing sugar produced decreased from 7.8 to 6.3 mg/g SW even when pretreatment duration was increased (run 5, 13, and 14). At the same solid loading and high microwave irradiation power (500 W; run 10), reducing sugar yield was low at 6.3 mg/100 g SW which could be due to the charring effect of SW as highlighted in Fig. 1.

However, as the solid loading decreased from 23% to 13% while the biomass was treated at 500 W microwave power and for 3 min, the charring phenomenon was diminished with reducing sugar production increased to 7.9 mg/100 g SW (run 11). Under operating conditions of high solid loading (23%) and high microwave irradiation power (500W), water had undesirably evaporated from within the lignocellulosic waste, causing it to burn. A similar charring observation was reported at 850 W and 3 min of pretreatment (Binod *et al.* 2012).

Fig. 1. Charring of pretreated SW (circled) at pretreatment conditions of 500 W microwave power, 3 min pretreatment duration, and 23% solid loading

The results listed in Table 3 were fitted to the quadratic model, and the fitness of the results generated from the ANOVA study are respectively represented in Eq. 5 and Table 4,

$$\text{Reducing Sugar Produced} = 6.38 + 0.034A - 0.17B - 0.038C + 0.57A^2 + 0.94B^2 - 0.040C^2 - 0.60AB + 0.20AC - 0.22BC \tag{5}$$

where A, B, and C are represented by pretreatment duration, solid loading and MI power, respectively.

Table 4. Fitness of Quadratic Model in the Microwave Irradiation Pretreatment of SW

Model to be fitted	Quadratic
p-Value, prob. >F	0.1544 (not significant)
Lack of fit	0.0921(not significant)
R^2 value	0.7395
Adjusted R^2 value	0.4046

The ANOVA study presented in Table 4 clearly denied the significance of the model. This could relate to the gelatinization of starch in the SW that occurred during pretreatment. When SW was heated in the presence of water, the starch granules in the SW swelled because of hydration in the crystalline regions and a viscous solution was formed (Jenkins and Donald 1998; Waigh *et al.* 2000; Vermeylen *et al.* 2006; Prasad *et al.* 2007; Ratnayake and Jackson 2007; Wang and Copeland 2012; Wang *et al.* 2014; Carlstedt *et al.* 2015). This makes the analysis using the DNS method difficult and unreliable, as shown by runs 6 and 9 of Table 3. On the other hand, at 5 min pretreatment duration and 18% solid loading, there was only a slight deviation in reducing sugar production, *i.e.* 0.3 mg reducing sugar/100 g SW, when the microwave power increased from 100 W (Run 6) to 500 W (Run 9). The results obtained had no fixed trend because of the gelatinized starch, and thus they could not be fitted into the quadratic model.

The above findings show the impact of gelatinization and charring on the accuracy if analysis. It also signifies the unsuitability of water as heating medium for MI pretreatment of starchy lignocellulosic biomass. To minimize gelatinization and charring, water was replaced by sulfuric acid in MI pretreatment, and lower solid

loadings were applied to prevent the charring of the sago waste. An elementary optimization based on a single factor approach was applied on the pretreatment. This enabled the study on the effect of each influencing parameter on reducing sugar production and the examination on gelatinization and charring of starchy biomass more thoroughly.

Microwave Irradiation Pretreatment of Sago Waste with Sulfuric Acid

Effect of solid loading

With sulfuric acid as heating medium, MI power and pretreatment duration were kept at 300 W and 2 min, respectively in the MI pretreatment. Under the fixed conditions with varying solid loadings of 7.5%, 10.0%, and 12.5%, reducing sugar yields decreased with increasing solid loadings, as shown in Fig. 2a. When solid loading was increased from 7.5% to 10.0%, the reducing sugar yield decreased from 40.9 to 24.5 mg/g SW (~ 40%). With an increase in the solid loading to 12.5%, reducing sugar yield further dropped to 15.9 mg/g SW.

At high solid loading, energy could not effectively be supplied by microwave to SW through oscillation of water molecules, leading to relatively low reducing sugar production. Conversely, at low solid loading of 7.5%, more sulfuric acid solution was added to the biomass and more energy could be supplied to the biomass from the microwave to disrupt its structure (Hu *et al.* 2008). This clearly shows the importance of moisture content in SW for MI pretreatment.

Effect of pretreatment duration

The effect of the pretreatment duration on reducing sugar production was investigated in a range from 2 to 8 min at microwave irradiation power of 300 W and solid loading of 7.5%. As shown in Fig. 2b, the reducing sugar yield increased as pretreatment was extended from 2 to 6 min. This is attributed to higher energy supplied to SW as the collision frequency between molecules increased at longer pretreatment duration. The heat generated might have penetrated into the inner part of the biomass causing a greater structure disruption, allowing more carbohydrate in SW to be hydrolyzed. When pretreatment duration was lengthened from 4 to 6 min, more energy provided to the pretreatment gave a drastic increase in reducing sugar production, from 90.8 to 261.5 mg/g SW. However, with an increase of the pretreatment duration beyond 6 min to 8 min, charring of SW was observed, resulting in a reduction of 43 mg reducing sugar/g SW reducing sugar. The observed charring effect could be due to high MI power or lengthy pretreatment duration (Binod *et al.* 2012), which might have decomposed reducing sugar such as xylose into furfural (Ma *et al.* 2009).

On the other hand, insufficient pretreatment duration might prevent MI from supplying sufficient energy to excite the dipoles at the surface of the biomass (Gong *et al.* 2010). In this study, 6-min pretreatment seemed to be sufficient to facilitate sulfuric acid hydrolysis of carbohydrates in the SW. The relatively short pretreatment duration is an advantage as compared to the conventional heating method, which required 120 min using sulfuric acid (Kumoro *et al.* 2008) and 30 min using solid acid catalyst (Lee *et al.* 2013) pretreating sago waste.

Effect of microwave irradiation power

To study the effect of microwave irradiation power on reducing sugar production, the conditions were set at 7.5% solid loading and 6 min pretreatment duration with microwave powers ranging from 100 to 700 W. Figure 2c indicates that as microwave power increased from 100 to 300 W, reducing sugar production was

elevated from 22.0 to 261.5 mg/g SW. At 450 W, charring occurred and reducing sugar production decreased to 59.9 mg/g SW.

Apparently, high microwave irradiation power is not conducive as far as acid MI pretreatment of starchy biomass is concerned, as the sulfuric acid solution might have dried up during the pretreatment, and eventually burnt the reducing sugar produced. This explains the fact that high microwave powers such as 700 W had lower reducing sugar yield than that from the low irradiation power of 100 W. This further emphasizes the need to eliminate charring of SW with proper regulation of the variables. Incorporating the use of sulfuric acid in microwave irradiation pretreatment for starchy lignocellulosic waste could minimize the said problem with promising reducing sugar production at relatively low power and short reaction time, leading to cost and energy savings.
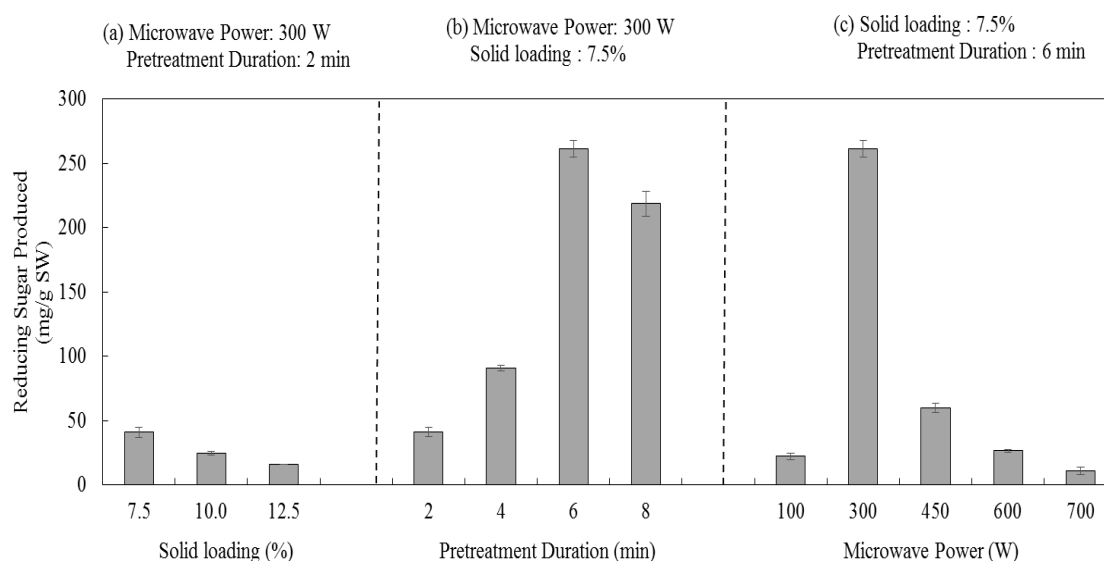

Fig. 2. Reducing sugar produced from microwave irradiation-pretreated SW at various pretreatment conditions

Chemical Composition Analysis

Apart from the comparison study on the yield of reducing sugars using different pretreatment techniques, a comparison of the chemical compositions of untreated and microwave-pretreated SW was performed, as shown in Table 5. MI pretreatment with sulfuric acid successfully hydrolyzed total carbohydrate components (starch, cellulose, and hemicellulose) in SW, from 88.6% to 18.5%. There is a noticeable decrease in starch content in SW, from 88.3% to 2.9%, after SW was pretreated with microwave irradiation.

The combination of the hydrolyzing power of sulfuric acid and the structural disruption ability of microwave irradiation pretreatment effectively hydrolyzed approximately 97% of the starch content. This is likely associated with the branched polysaccharides of starch, which are easier to be hydrolyzed than cellulose that consists of uniform and linear reducing sugar polymers. The structure of cellulose acts as a stabilizing network between cellulose molecules to form stronger intra- and inter-chain secondary forces as well as to create straight and recalcitrant supramolecular fibres (Richardson and Gorton 2003). Hence, 3.8% cellulose and 11.8% hemicellulose remained unhydrolyzed after microwave irradiation pretreatment. In spite of its hydrolyzing power, sulfuric acid did not seem to have any significant lignin removal ability. This explains the presence of approximately 66% of lignin as the main constituent of pretreated SW.

Table 5. Chemical Composition of Untreated and Pretreated SW

Components	Untreated SW	Pretreated SW
Starch*	88.3%	2.9%
Cellulose*	0.1%	3.8%
Hemicellulose*	0.2%	11.8%
Lignin	5.0%	66.0%
Ash	6.5%	15.5%

*Total carbohydrate (starch, cellulose and hemicellulose) for untreated SW is 88.6% and pretreated SW is 18.5%.

Field Emission Scanning Electron Microscopy (FESEM)

The degree of structural disruption of microwave irradiation pretreatment on SW was analyzed by FESEM. The efficiency of microwave irradiation pretreatment can be reflected by the severity of structural disruption on the surface of SW. Figure 3 shows the structures of untreated and microwave irradiation-pretreated SW. It is obvious that pretreatment induced physical impairment of SW; the pretreated SW had a rough and uneven surface, whereas the surface of the untreated SW was smooth and intact.

Close examination revealed that microwave irradiation pretreatment had created some cavities on the surface of the SW. This confirms the implosion of biomass during MI pretreatment had favorably imparted a more porous surface (as highlighted in Fig. 3) for a better hydrolysis process.
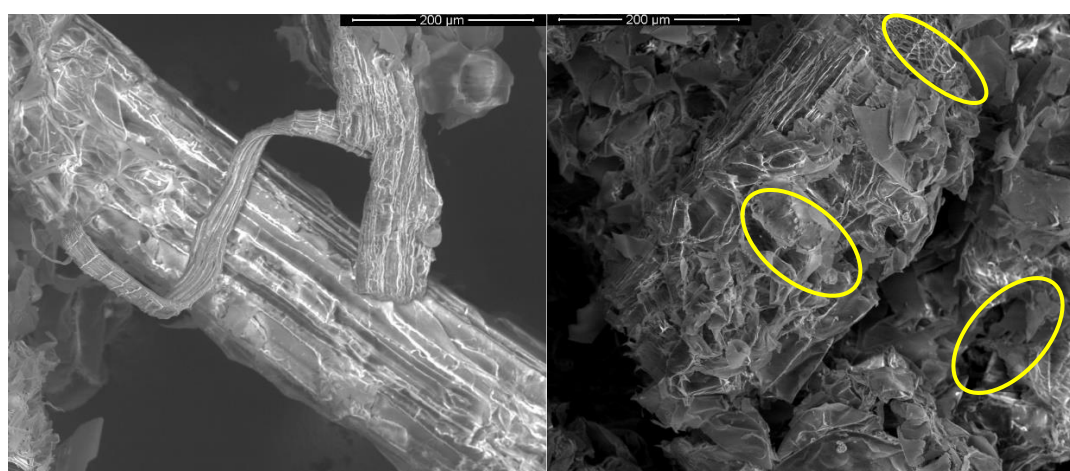

Fig. 3. FESEM images of (left) untreated SW and (right) microwave irradiation-pretreated SW at 7.5% solid loading, 6 min pretreatment duration, and 300 W microwave power

Fermentation of Pretreated Sago Waste

As discussed before, incorporation of sulphuric acid into microwave irradiation pretreatment effectively disrupted the structure of SW and promoted hydrolysis of starch content into reducing sugar. The reducing sugar produced was then fermented using *ragi tapai* for ethanol production.

The fermentation performance was assessed based on the ethanol and reducing sugar concentration profiles illustrated in Fig. 4.
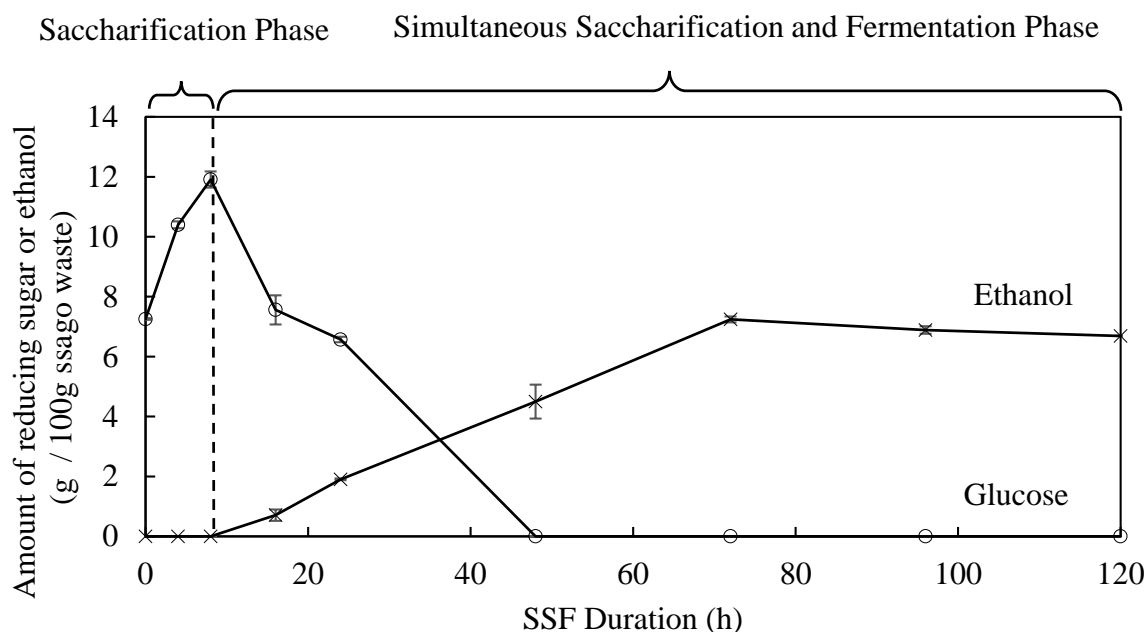
Saccharification Phase   Simultaneous Saccharification and Fermentation Phase

Fig. 4. Ethanol and reducing sugar concentration for the *ragi tapai* SSF

During fermentation of the pretreated SW, two distinct phases were observed. A sole-saccharification phase was observed within 0 to 8 h after *ragi tapai* was inoculated, as 4.66 g reducing sugar/100 g SW was produced without any trace of ethanol. The fermentation of reducing sugar into ethanol by *ragi tapai* only started after 8 h of inoculation. The findings signifies that *ragi tapai* played dual roles; initially saccharifying MI-pretreated SW into reducing sugar and subsequently saccharifying and simultaneously fermenting the intermediate products into ethanol.

During the saccharification phase of *ragi tapai* fermentation, the initial reducing sugar level was registered at 7.25 g reducing sugar/100 g SW from the previous MI pretreatment, and the quantity had increased to 11.91 g reducing sugar/100 g SW after 8 h. The increase in reducing sugar level could be a result of the excretion of enzymes such as α-amylase and glucoamylase by *Aspergillus* and *Rhizopus* groups (Wu *et al.* 2015) in *ragi tapai* that might have saccharified any unhydrolyzed starch in pretreated SW to reducing sugar. This also implies that the structural disruption by MI pretreatment promoted enzymatic attack by microbes during fermentation process. Moreover, *ragi tapai* has an affinity for gelatinized starch than raw starch to be saccharified into reducing sugar (Prasad *et al.* 2007).

There was a substantial decrease of 4.35 g reducing sugar from 11.91 g reducing sugar/100 g SW at 8 h to 7.56 g reducing sugar/100 g SW at 16 h during *ragi tapai* fermentation. The maximum theoretical yield of the amount of reducing sugar consumed should produce 2.22 g ethanol/100 g SW. However, ethanol obtained in this study was only 0.71 g of ethanol/100 g SW (32% of the maximum theoretical value). This suggests that a large quantity of reducing sugar probably had been utilized for cell growth, maintenance and other product formation by the co-culture strains in *ragi tapai*.

When fermentation was progressed to 48 h, higher microbial activity was observed in the fermentation in which the reducing sugar in the system (7.56 g reducing sugar/100 g SW) was completely consumed to yield 4.50 g ethanol/100 g SW. The ethanol yield achieved was 98% of the maximum theoretical value. However, only at 72 h of fermentation had reducing sugar reached a maximum ethanol yield of 7.24 g/100

g SW. One possible explanation for the observation could be that the unhydrolyzed starch during the saccharification phase of *ragi tapai* fermentation continuously produced reducing sugar for ethanol production.

In the fermentation of starchy biomass, good coordination of the two reactions is important, as reducing sugar produced at different saccharification rates can significantly influence the microbial growth and ethanol conversion rate (Wu *et al.* 2015). Thus, employing *ragi tapai* could provide an effective way to prevent accumulation of reducing sugar and end-product inhibition while producing ethanol (Schell *et al.* 1999; Balat 2011). Most importantly, *ragi tapai* demonstrated the role in saccharification and fermentation equally efficient at a compromised temperature of 25 °C. This is because it is a challenge to find an operation temperature that suits both processes with different optimum temperature (Sun and Cheng 2002).

Attributable to the dual role of *ragi tapai*, ethanol produced from MI-pretreated starchy lignocellulosic biomass in this study was considerably higher than those from conventional pretreated lignocellulosic biomass, *e.g.*, 4.0 g ethanol/100 g corn fiber (Rasmussen *et al.* 2010) and 1.48 g/100 g sugarcane bagasse (Yoon *et al.* 2012).

Kinetic Study on *Ragi Tapai* Fermentation

To study the kinetics of product formation, models that can be used include the logistic model, the logistic incorporated Leudeking-Piret model, the modified Gompertz model and *etc*. The requirement of detailed growth kinetic data in certain models such as the logistic model and the logistic incorporated Leudeking-Piret model imposes difficulty in kinetic study due to the presence of multi-strains in *ragi tapai*. Considering that, a simpler model that does not require accounting for the relationship between product formation and microbial growth was applied. Ethanol production during *ragi tapai* fermentation was modelled into the modified Gompertz model by using Matlab software as shown in Fig. 5.
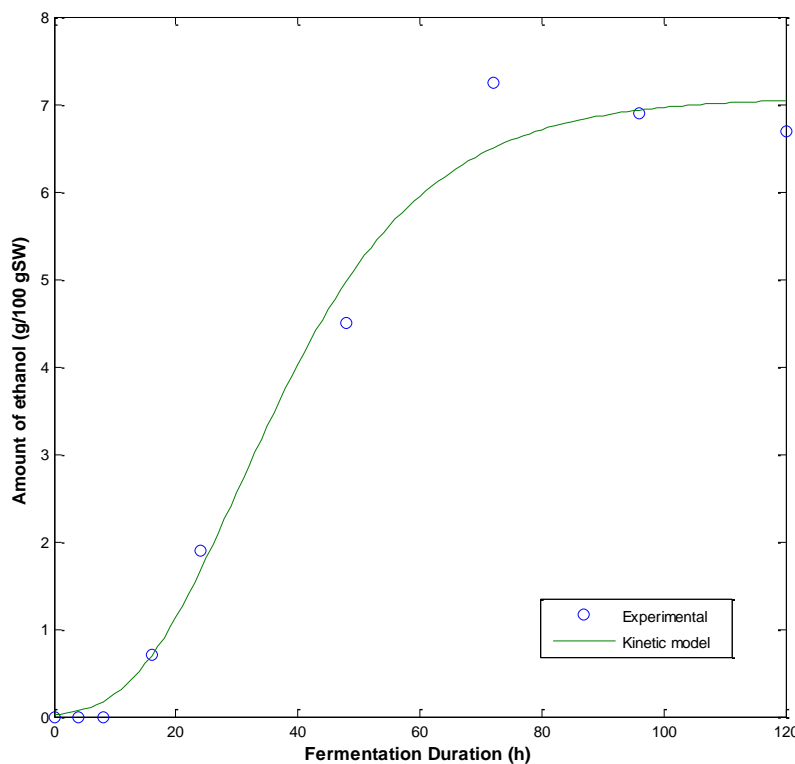

Fig. 5. The fitted results of ethanol production based on the modified Gompertz model

An overall good model fitting was achieved, with $R^2 = 0.9879$. The model predicted a maximum production rate of ethanol, $r_{p,m}$ of 0.15 g ethanol/ (100 g SW. day) and a lag phase, t_L of 13.29 h, which is longer compared to the experimental finding of 8 h. This suggests that the lag phase predicted does not necessary reflect the actual lag phase, as *ragi tapai* is comprised of multiple strains that have different lag phases. Moreover, as indicated by the two phases exhibited during the fermentation process previously, certain microbial strains present in *ragi tapai* had dominated the saccharification phase for the first 8 h of the fermentation to produce sufficient reducing sugar which might have encouraged the production of ethanol sooner than the predicted time. On the other hand, although the modified Gompertz Model had been widely applied in kinetic study for product formation in fermentation such as ethanol production from mix natural microflora (Zajšek and Goršek 2010) and hydrogen production by mixed anaerobic cultures (Mu *et al.* 2006), the design of the lag phase prediction by the model did not take into account the saccharification phase, the type of microbial and the unhydrolyzed starchy substrate. However, the predictability of the model registers its accuracy once the ethanol production by *ragi tapai* in the fermentation phase had taken place. As a result, the maximum amount of ethanol predicted (7.08 g ethanol/100 g SW) did not deviate much from the experimental value (7.24 g ethanol/100 SW). This signifies that the modified Gompertz model was able to describe the ethanol production from the fermentation but not the lag phase of the multiple strains of *ragi tapai*.

CONCLUSIONS

1. Microwave irradiation pretreatment with water on starchy lignocellulosic biomass to produce reducing sugar resulted in undesirable starch gelatinization.

2. Employment of sulfuric acid in the MI pretreatment improved reducing sugar production and minimized starch gelatinization.

3. Chemical composition analysis and FESEM confirmed the effectiveness of MI-acid pretreatment in structural disruption and carbohydrate hydrolysis.

4. The fermentation of pretreated SW by *ragi tapai* produced 7.24 g ethanol/100 g SW.

5. In the fermentation of MI pretreated SW, *ragi tapai* simultaneously converted the unhydrolyzed starch into reducing sugar and produced ethanol.

6. The modified Gompertz model can only accurately describe the ethanol production by *ragi tapai* but not the lag phase of the fermentation.

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