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Conversion of Fibrous Sago (*Metroxylon sagu*) Waste into Fermentable Sugar via Acid and Enzymatic Hydrolysis

[1,2]A.C. Kumoro, [2]G.C. Ngoh, [2]M. Hasan, [2]C.H. Ong and [2]E.C. Teoh
[1]Department of Chemical Engineering, Faculty of Engineering, Diponegoro University, Prof. H. Sudharto, SH Road, Tembalang Campus Semarang, Indonesia
[2]Department of Chemical Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia

Abstract: The hydrolysis of dried-powdered fibrous sago waste by sulphuric acid and glucoamylase was studied. Both studies were carried out in an Erlenmeyer flask placed in a controlled temperature water bath. Samples were taken from the reaction flask at every 30 min interval for reducing sugar determination. The optimum condition for acid hydrolysis was found to be at 90°C, using 1.5 M acid concentration and reaction time of 120 min yielding 0.6234 g glucose g^{-1} waste. The kinetic parameters of acid hydrolysis in the Saeman's model, were the rate constant (k_1 = 0.01405 (1/min)), activation energy (Ea =120.40 (kJ mol^{-1})) and pre-exponential factor ($A = 9.52\times10^{16}$ (1/min)). The optimum condition for enzymatic hydrolysis using glucoamylase was found to be at enzyme concentration of 6 AGU mL^{-1} and reaction time of 30 min, yielding 0.5646 g glucose g^{-1} waste. The kinetic parameters in the competitive inhibition model corresponding to the optimum condition, namely the equilibrium constant for enzyme-inhibitor complex, Michaelis-Menten constant and maximum velocity, are 1.4727, 0.24175 and 1.35460 g L^{-1} min, respectively.

Key words: Hydrolysis, lignocellulosic waste, sulphuric acid, glucoamylase, kinetic modelling

INTRODUCTION

Sago palm (*Metroxylon* sp.) is one of the few tropical crops with the ability to thrive in the harsh swampy peat environment (Johnson, 1977; Ruddle, 1977). In Malaysia, this type of land covers an area of 1.5 million ha or about 12% of Sarawak's total land area (Tie and Lim, 1977). Cecil *et al*. (1982) reported that sago starch accumulates in the pith core of the stem of the sago palm. They also reported that the chemical analysis of pith showed about 6-12% of soluble solids (dry substance) and 1-3% of ash, besides from 79-88% of apparent starch plus sugar content. The sago pith also contains most of the constituents in any other plant materials namely fibre, hemicelluloses, other cell structural materials, soluble solids and unidentified traces of other substances.

Beside wastewater and bark, residual solid waste is produced by sago processing plant daily. The residual solid waste is the fibrous residue obtained after the starch has been washed out of the rasped pith of the sago palm. Due to the presence of the lignocellulosic fibrous material, this refuse is a strong pollutant with no significant industrial application except as animal feed supplement and chipboard production. However, dried fibrous sago waste has been found to

Corresponding Author: Dr. Andri Cahyo Kumoro, Department of Chemical Engineering, Faculty of Engineering, Diponegoro University, Prof. H. Sudharto, SH Road, Tembalang Campus Semarang, Indonesia Tel: (+62)-24-7460058 Fax: (+62)-24-7460058

Table 1: Component analysis of dried fibrous sago waste

	Content (% w/w)	
Components	Vickineswary and Shim (1996)	Present study
Apparent starch	65.70	70.00
Crude fibre	14.80	15.00
Crude protein	1.00	0.98
Fat	ND	ND
Ash	4.10	4.16
Moisture	5.91	6.10

ND: Not Detected

contain about 60-70% dry weight starch although this depends mostly on the quality of the extraction process (Vickineswary and Shim, 1996). The approximate components of dried fibrous sago waste obtained from nine major factories in Sibu, Sarawak are shown in Table 1.

In view of the above, the starch presents in the fibrous sago waste can be hydrolysed into useful glucose (Pei-Lang *et al*., 2006) to be used as low cost nutrient source in fermentation processes for the biotechnology industry. Two methods, namely the acid hydrolysis and the enzymatic hydrolysis were used to hydrolyse fibrous sago waste into useful glucose in this study. In the acid hydrolysis, the acid acts as the catalyst to break the starch's glycosidic bonds to produce dextrin, maltotriose, maltose and glucose depending on the relative location of the bond under attack as counted from the end of the chain. While in the enzymatic hydrolysis, enzymes such as glucoamylase, act as the catalyst to break the starch glycosidic bonds to produce useful glucose.

KINETIC MODELLING

Kinetic Study of Sulphuric Acid Hydrolysis

According to Rodriguez-Chong *et al*. (2004), Saeman's model can be applied to describe hydrolysis of homopolymers and heteropolymers such as starch and hemicellulose. The generalised Saeman's model for starch hydrolysis can be written as:

$$\text{Polymers} \xrightarrow{k_1} \text{Monomers} \xrightarrow{k_2} \text{Decompositions products} \quad (1)$$

The concentrations of reducing sugar (M) as function of time can be calculated using the following equation:

$$M = Po\frac{k_1}{k_2 - k_1}\left(e^{-k_1 t} - e^{-k_2 t}\right) \quad (2)$$

where, P_o, k_1 and k_2 are the potential reducing sugar (%), the hydrolysis and decompositions reaction rate constants (1/min), respectively.

The effect of temperature and acid concentration on the reaction rate constant can be assumed to follow modified Arrhenius correlation by Carrasco and Roy (1992):

$$k_1 = e^{A} C^{N} e^{-\frac{E_a}{RT}} \quad (3)$$

where, A and E_a are the pre-exponential factor specific to particular reaction (1/min) and activation energy (kJ mol^{-1}) in the original Arrhenius equation, while N and C are the regression parameter and acid concentration in % (w/w), respectively.

Kinetic Study of Enzymatic Hydrolysis

The competitive inhibition model was chosen to study the kinetic of enzymatic hydrolysis of fibrous sago waste. Liew (2007) found that the competitive inhibition model is the most suitable model for kinetic study of enzymatic hydrolysis of sago hampas. In competitive inhibition model, the inhibitor binds to the active site and prevents binding of the substrate (Segel, 1975).

The model of an enzymatic hydrolysis with a reversible competitive inhibitor is shown as follows:

$$\begin{array}{l} E + S \underset{}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \\ + \\ I \underset{}{\overset{k_3}{\rightleftharpoons}} EI \end{array} \quad (4)$$

where, E, S, ES, P, I and EI are the enzyme, substrate, instantaneous enzyme-substrate complex, product, inhibitor and instantaneous enzyme-inhibitor complex, respectively. The rate constants of this enzymatic hydrolysis are k_1, k_{-1}, k_2 and k_3. By solving the model using quasi-steady-state approximation and taking the closed form solution, the time dependent equations for product formation are as follows:

$$[P](t) = [S_o] - [S](t) - [ES](t) \quad (5)$$

Where:

$$[ES](t) = \frac{[E_o][S](t)}{[S](t) + K_M\left(1 + \frac{[I_o]}{K_i}\right)} \quad (6)$$

and

$$[S](t) = K_M\left(1 + \frac{[I_o]}{K_i}\right) W\left(\left[\frac{[S_o]}{K_M\left(1 + \frac{I_o}{K_i}\right)}\right] \frac{[S_o] - V_{max}t}{K_M\left(1 + \frac{[Io]}{Ki}\right)}\right) \quad \text{W is the Lambert function} \quad (7)$$

where, [P], $[S_o]$, [S], $[E_o]$, $[I_o]$ and [ES] are the concentration of instantaneous product, initial substrate, instantaneous substrate, initial enzyme, initial inhibitor and instantaneous enzyme-substrate complex, respectively, in g L^{-1}. $K_M = (k_{-1} + k_2)/k_1$ is the Michaelis-Menten constant in g L^{-1}, $K_i = k_{-3}/k_3$ is the equilibrium constant for enzyme-inhibitor complex in g L^{-1} and $V_{max} = k_2[E_o]$ is the maximum velocity in g L^{-1} min. The k_1, k_{-1}, k_2, k_3 and k_{-3} are the reaction rate constants in (1/min) and $[E_o]$ is the initial enzyme concentration in g L^{-1}.

MATERIALS AND METHODS

Materials

Dried Sago (*Metroxylon sagu*) fibrous waste was obtained as a donation from CL Nee Sago Industries Sdn. Bhd. Malaysia. It was milled and sieved to obtain 300 μm particle size in the Bioprocess Laboratory, Department of Chemical Engineering, University of Malaya, Kuala Lumpur-Malaysia. The debranching enzyme, glucoamylase (AMG 300 L, EC 3.2.1.3), was from Novozyme A/S, Denmark and is produced by *Aspergillus niger*. One AGU will hydrolyse 1 μmole of maltose per minute at pH 4.3 and temperature 25°C.

Characterization of Fibrous Sago Waste

The moisture, ash, crude protein, crude fat and crude fibre contents of starch were determined using the oven method (Pearson, 1976), AOAC method, microKjeldahl method and Soxhlet method respectively. The starch content was determined by the glucoamylase (AOAC, 1990), where glucose, liberated by the action of the enzyme on starch, was measured using glucose oxidase. Three replicates were used for the determination of each constituent.

Determination of Reducing Sugars

The reducing sugars content of the hydrolysis products was carried out by the method of Miller (1959). Reducing value determines the concentration of reducing groups as they bind to 3, 5-dinitrosalicylic acid (DNS) to form DNS conjugates.

Acid Hydrolysis

Acid hydrolysis of fibrous sago waste was carried out in 250 mL Erlenmeyer flasks.

Five percent weight to volume (%, w/v) of fibrous sago waste slurry (a mixture of 7.5 g of dried-ground fibrous sago waste with 150 mL of 0.5 M sulfuric acid) was prepared in a 250 mL Erleumeyer flask. The solution was stirred for 1-2 min and was then placed into a water bath at 75°C. The samples were collected at every 30 min interval and the solution was then cooled in an ice-water bath to allow settling. After reaction was completed, solids were separated from aqueous solution by filtration. Fifty millilitres of the filtrate was taken and 25 mL of 2 M sodium hydroxide was added for neutralization. The sample was then submerged in ice-water bath to immediately absorb the heat of neutralization to avoid further decomposition of reducing sugar. The sample was centrifuged at 10,000 rpm and 20°C for 10-15 min after neutralization. The sample was then stored and kept in an incubator at 4°C in order for the measurement of reducing sugar. The experiments were also conducted at other sulphuric acid concentration (1.0 and 1.5 M) and temperature (90°C). The percentage of fibrous sago waste conversion into reducing sugar was later calculated using the following equation:

$$M = \frac{\text{Total amount of reducing sugar (g)}}{\text{Initial amount of fibrous sago waste (g)}} \times 100\% \quad (8)$$

Enzymatic Hydrolysis

Five percent weight to volnme (%, w/v) of fibrous sago waste slurry (a mixture of 5 g of dried-ground fibrous sago waste in 100 mL of 0.1 M KH_2PO_4 buffer solutions at pH = 4) was prepared in a 250 mL Erleumeyer flask. The flask was then submerged in a water bath at temperature of 75°C for 15 min for gelatinization process and then further submerged in an ice-water bath to cool down to 60°C. A 2 AGU mL^{-1} (6.7 μL) of enzyme glucoamylase from *Aspergillus niger* (AMG 300 L, EC 3.2.1.3) was added into the mixture after cooling. The fibrous sago waste solution was then submerged in a water bath at temperature of 60°C for 10 min. The flask was submerged in an ice-water bath to cool to around 20°C for settling and prevent further hydrolysis. The sample was labelled and kept in an incubator at 4°C for determination of the reducing sugar. The experiments were also conducted at different enzyme concentrations (4 AGU mL^{-1} (13.3 μL) and 6 AGU mL^{-1} (20 μL) of AMG 300 L) and at different reaction times (20, 30, 60, 90 and 120 min).

RESULTS AND DISCUSSION

Acid Hydrolysis

From Fig. 1, it is observed that the yield obtained from acid hydrolysis increases with the increase of hydrolysis duration at both temperatures 75 and 90°C. The hydrolysis yield also increases with the

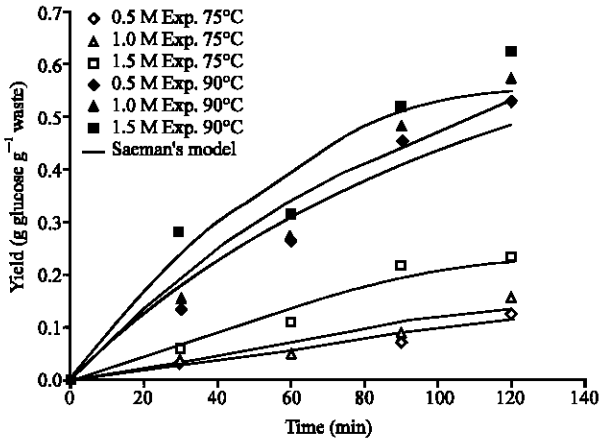

Fig. 1: Experimental and predicted yields of sago waste obtained from acid hydrolysis using various sulfuric acid concentration hydrolysis at 75 and 90°C

increase of sulphuric acid concentration. When hydrolysis was conducted at 75°C using 1.5 M H_2SO_4, highest yield was achieved at 0.2339 g glucose g^{-1} waste. However, this value is still very low. Therefore, a higher temperature (limited by maximum temperature of water bath, which is ouly about 100°C) should be applied to improve the yield of hydrolysis.

Hydrolysis of fibrous sago waste at 90°C using 1.75 M H_2SO_4 gave the highest yield, which was 0.6996 g glucose g^{-1} waste. It was obvious, that the effect of acid concentration was more pronounced than hydrolysis duration at 75 and 90°C. It seemed to be possible for hydrolysis at 90°C to achieve higher yield if the hydrolysis duration was extended. This is because the reaction yield profile showed an increasing trend. The yield obtained from the reaction using 1.75 M acid concentration at 90°C did not increase further after 100 min. Therefore, the use of higher acid concentration to enhance the yield is more effective rather than prolong the hydrolysis duration. On the other hand, the yield of acid hydrolysis increased with the increase of temperature from 75-90°C. The higher temperature provides greater energy to break down the linkage of fibrous sago waste, which leads to the achievement of higher yield.

Kinetic Study of Sulphuric Acid Hydrolysis

A Matlab version 7.0 code was developed to carry out the optimisation of Eq. 2 by selecting the value of k_1 and k_2 to obtain minimum difference between experimental and calculated percentage of fibrous sago waste conversion (M). In the initial calculation we found that the decomposition rate constant, k_2 is very small ($\approx 10^{-07}$-10^{-10}) compared to the hydrolysis rate constant, k_1. This indicates that the rate of hydrolysis is much faster than the decomposition rate of reducing sugars. This behaviour is very common in acid hydrolysis as observed by Garrote *et al.* (2001). Therefore, it is plausible to neglect k_2 for further calculation. Table 2 and 3 show the calculation results of the kinetic modelling of sulphuric acid hydrolysis. The reaction rate constant for hydrolysis, k_1 increases as temperature increases from 75-90°C for all acid concentrations. Besides, k_1 also increases as with the increasing sulfuric acid concentration at 90°C. The increases of k_1 with both temperature and concentration of acid agree well with Aguilar *et al.* (2002). The potential reducing sugars, Po obtained from the calculation were about 70%, which is comparable to the analysis result in this work as well as by Vickineswary and Shim (1996).

From Table 4, it can be seen that the average values of E_a was 120.4 kJ mol^{-1}, while the individual E_a values are in agreement with those reported by other researchers. The E_a value for birch

Table 2: Kinetic and statistical parameters for conversion of sago waste into reducing sugar by sulphuric acid hydrolysis by optimizing P_0, k_1 and neglect k_2 at 75°C

Sulphuric acid concentration (M)	P_0 (%)	k_1 (1/min)	R^2
0.50	69.96000	0.00147	0.95285
1.00	69.96000	0.00176	0.91927
1.50	69.95990	0.00350	0.96194

Table 3: Kinetic and statistical parameters for conversion of sago waste into reducing sugar by sulphuric acid hydrolysis by optimizing Po, k_1 and neglect k_2 at 90°C

Sulphuric acid concentration (M)	P_0 (%)	k_1 (1/min)	R^2
0.50	69.96000	0.01006	0.96285
1.00	69.96000	0.01110	0.94783
1.50	69.96000	0.01405	0.94708
1.75	69.96000	0.02073	0.95420

Table 4: Activation energies for hydrolysis of sago waste into reducing sugar at different sulphuric acid concentration

Sulphuric acid concentration (M)	A (1/min)	E_a (kJ mol^{-1})	R^2
0.5	2.45×10^{17}	134.8	1
1.0	4.05×10^{16}	129.1	1
1.5	1.43×10^{12}	97.4	1
Average	9.52×10^{16}	120.4	

wood was 127 kJ mol^{-1} (Maloney *et al*., 1985). For agricultural wastes, the E_a values were found to be between 80.3 and 92.3 kJ mol^{-1} (Eken-Saracoglu *et al*., 1998). The value of E_a obtained in this study also agreed well with those reported using other kinetic models. For instances, E_a values of 65.4 kJ mol^{-1} (Veeraraghavan *et al*., 1982) and 172 kJ mol^{-1} (Bhandari *et al*., 1984) were found using other kinetic models. The activation energy of acid hydrolysis of fibrous sago waste decreases as the acid concentration increases from 0.5-1.5 M and the pre-exponential factor also shows the same trend. It proves that at higher acid concentration, sulphuric acid which acts as catalyst lowers the activation energy of reaction so that the reaction occurs faster. As a result, the rate of hydrolysis or the conversion of sago waste into reducing sugar increases with acid concentration.

Using the values of previously obtained for k_1 and applying a non-linear regression analysis to correlate k_1 with H_2SO_4 concentration and temperature. The value of the regression parameter, A is the average of the pre-exponential values and Ea, were 9.52×10^{16} (1/min) and 120.40 kJ mol^{-1}, respectively as shown in Table 4. The value of N is found to be 0.34. For similar lignocellulosic materials: N = 1.55 (Eken-Saracoglu *et al*., 1998), N = 0.80 (Veeraraghavan *et al*., 1982) and N = 0.66 (Kim *et al*., 2000).

Enzymatic Hydrolysis

In this study, pH = 4.0 and temperature at 60°C were used as recommended optimum conditions for glucoamylase to perform as catalyst by the manufacturer. The milled fibrous sago waste was initially cooked at 75°C for 15 min for gelatinisation process, which was crucial in order to obtain high hydrolysis yield. During the gelatinisation process, the intermolecular bonds of starch molecules broken down in the presence of water and temperature and allowing the hydrogen bonding sites (the hydroxyl hydrogen and oxygen) to engage more water. This penetration of water increases randomness in the general structure and decreases the number and the size of the crystalline region. The crystalline region does not allow water entry. When heat is applied, this region will be diffused, so that the chains start to pull out from each other. The region is thus called amorphous. Therefore, the fibrous sago waste become softened and susceptible to be attacked by enzyme in breaking down α-1,4 and α-1,6 glucosidic bond and then convert them into reducing sugar.

From Fig. 2, it can be observed that the conversion of fibrous sago waste into reducing sugar increased steadily at duration of hydrolysis from 10-120 min. From Fig. 2, it was also observed that higher enzyme concentration led to the achievement of higher yield. The highest enzyme concentration,

Table 5: Kinetic and statistical parameters for hydrolysis of sago waste into reducing sugar at different glucoamylase concentration by using competitive inhibitor model

Glucoamylase concentration (AGU mL^{-1})	I_0 (g L^{-1})	V_{max} (g/L.min)	K_M (g L^{-1})	K_i (g L^{-1})	k_2 (1/min)	R^2
2	1.2361	0.1121	0.6396	2.0716	0.01401	0.8706
4	1.3206	0.1517	0.4217	1.9016	0.00948	0.9673
6	1.0690	1.3546	0.2472	1.4727	0.05644	0.9946

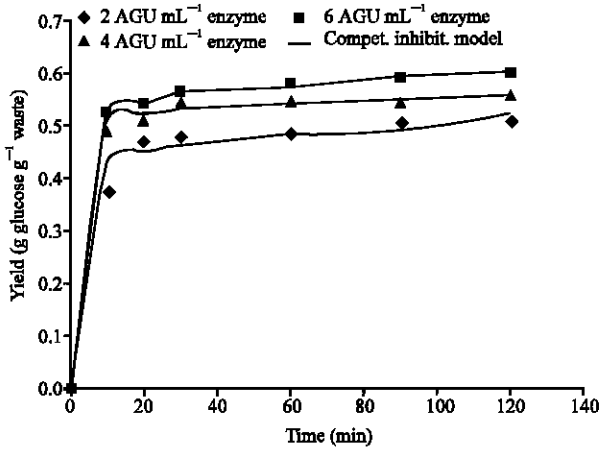

Fig. 2: Experimental and predicted yields of sago waste obtained from enzymatic hydrolysis using various enzyme concentrations 60°C

which was 6 AGU mL^{-1} gave the highest yield of 0.5976 (g glucose g^{-1} waste). The effect of enzyme concentration is more pronounced than the hydrolysis duration. The increase of enzyme concentration from 2-6 AGU mL^{-1} improved the hydrolysis yield. However, it is difficult to achieve higher yield although tremendous increase in enzyme concentration (double time concentration), this may be caused by the inhibition of polyphenols in the sago waste (Ansharullah, 1997); accumulation of branched chain oligosaccharides (consist mostly of α-1,6-linkages) which are hydrolyzed more slowly as compared to straight chain oligosaccharides (α-1,4-linkages) (Polakovic and Bryjak, 2004); product inhibition (Kusunoki *et al*., 1982); substrate inhibition of the glucoamylase at the beginning of the hydrolysis (Lopez *et al*., 2006) and the decrease in the affinity of glucoamylase towards the substrate, mostly short chain maltooligosaccharides with lower DP value (Polakovic and Bryjak, 2004).

Kinetic Study of Enzymatic Hydrolysis

A Matlab version 7.0 program based on Eq. 5, 6 and 7 was used to carry out the curve fitting to find K_M, V_{max}, k_2 and K_i. From Table 5, the goodness of fit increases as the glucoamylase concentration increased from 2-6 AGU mL^{-1} with R^2 increased from 0.8706 to 0.99457. This model fits very well to the experimental data at high concentration of glucoamylase (6 AGU mL^{-1}). The maximum velocity, V_{max} increases with concentration of glucoamylase, which indicates that the rate of hydrolysis increases when concentration of enzyme increases. Besides, K_i decreases with concentration of glucoamylase from 2-6 AGU mL^{-1} indicate that k_3 increased with concentration of glucoamylase. It also indicates a competitive inhibition when high concentration of glucoamylase was used. The rapid accumulation of product (product inhibition) which acts as a competitive inhibitor to the glucoamylase when higher concentration of glucoamylase was used (Kusunoki *et al*., 1982).

CONCLUSIONS

Acid hydrolysis at 90°C with acid concentration of 1.5 M for 120 min hydrolysis duration was found as the optimum condition which gave the yield of 0.6234 g g^{-1}. The acid concentration of 1.75 M at the same condition was not considered as optimum conditions due to its corrosiveness, which can lead to increase of overall cost of equipment and shorten life span of equipment. The reaction rate constant, activation energy and pre-exponential factor obtained at the optimum condition were 0.01405 (1/min), 97.40 kJ mol^{-1} and 1.43×10^{12} (1/min), respectively. The optimum condition for enzymatic hydrolysis of fibrous sago waste was at 60°C and 30 min using enzyme concentration of 6 AGU mL^{-1} to obtain 0.5646 (g glucose g^{-1} waste) yield. The kinetic parameters corresponding to enzyme concentration of 6 AGU mL^{-1} were K_i = 1.4727, K_M = 0.24175 g L^{-1} and V_{max} = 1.35460 g L^{-1} min. Thus it is to be mentioned that under controlled treatment conditions, fibrous sago waste can be fruitfully utilised as a potential source of glucose, which can be a starting raw material for the production of various chemicals, especially by microbial conversion process.

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