Enzyme Kinetics Study for Heterogeneous System of Pretreated Kenaf Hydrolysis

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ABSTRACT

The peculiarity of spatially restricted diffusion and molecular collision processes results in considerable contrast in a reaction between the reactant and catalyst in the heterogeneous system from its corresponding homogeneous structure. The identification of the enzymatic hydrolysis process of pre-treated kenaf and to convert it into simple sugars employing a systematic kinetic investigation is the aims of this study. The influence of substrate concentration on xylanase hydrolysis was performed in water bath shakers. In-house recombinant xylanase expressed in *Pichia pastoris* was used for the hydrolysis at pH 4.0 in 50 mM sodium citrate buffer with 200 rpm agitation. Modified Prout-Tompkins equation was used for the heterogeneous substrate hydrolysis. The results obtained show that temperature simultaneously influenced the time dependency of the reducing sugar yield. Dependence of the enzymatic rate of reaction can be calculated effectively on the conversion of substrates over different temperatures. The activation energy needed for pretreated kenaf hydrolysis was among the least compared to other lignocelluloses, which was only 25.15 kJ/mol. In conclusion, the exponential kinetic equation by the Modified Prout-Tompkins equation offers a solid understanding of xylanase hydrolysis on the pretreated kenaf. Thus, the prediction

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of the degree of hydrolysis required at the predetermined temperature and time values used can be quickly and precisely determined.

Keywords: Enzymatic hydrolysis, hemicellulose, heterogeneous system, kenaf, kinetic study

INTRODUCTION

Kenaf (*Hibiscus cannabinus L.*) is lignocellulosic biomass capable of generating bioenergy, xylooligosaccharides (XOS), and other value-added biomolecules. In the long term considering lignocellulosic biomass is another option for crop source, the economical, substantial, and renewable raw material (Mandelli et al., 2014). Despite cellulose, lignin, and a low number of extractives, it is a rapidly growing biomass and rich in hemicelluloses (Azelee et al., 2014). The hemicellulose is arbitrary and does not have a defined form that can be promptly hydrolyzed by dilute acid or base along with other various hemicellulolytic enzymes (De Menezes et al., 2009). The employment of enzyme technology in bioconversion processes provides advantages over conventional chemical technology in reducing the complexity of the carbohydrate–lignin complex.

Current research has raised huge concerns about the development of XOS from lignocellulose (rather than xylose) for the prebiotics, nutraceuticals, and food industries in particular. Attributable to this goal, the enzyme complex ratio developed needs to be wisely optimized, and its efficiencies tested. Enzymatic hydrolysis generally associates a buffer solution with complicated interfaces between the enzyme(s), substrate, and reaction environment. The reciprocal interaction between various enzyme activities is profiting a heterogeneous reaction of xylan or xylooligomer enzymatic hydrolysis. Plus, the kinetic view of reactions has become more complicated due to the existence of a broad range of xylooligomer chain lengths. Xylan, which degrades in the enzymatic media, is similar to other hydro-associated cleavages of heterocyclic ether bonds by the hydronium-catalyzed approach (Garrote et al., 2002). Xylan's enzymatic hydrolysis obeys the kinetic principles of insoluble substrates, where the assessment of the activation energy is vital to acquiring effective hydrolysis.

Enzyme kinetic is a catalyst analysis of quantitative enzyme which gives data regarding reaction rates. The understanding of the way of the enzyme acts and the actions of enzymes during the reaction are the fundamental grounds in the enzyme kinetics research. Enzyme kinetics studies calculate the enzyme affinity to substrate inhibitors and provide details on mechanisms of the reaction (Cinar et al., 2020; Valchev et al., 1998). The kinetic parameters also indicate the substrate's affinity to the enzyme. Enzyme kinetics generally involves the assessment of the enzyme-mobilized response rates at a varying concentration of substrates and enzymes.

The explanation on the kinetic process needs to consider most heterogeneous equations relating to other activity of heterogeneous catalytic, topochemical and diffusion (Valcheva

et al., 2000), and most heterogeneous conditions kinetic research applied the first method of hydrolysis velocity as the parameter of the primary concentration of enzymes (Chrastil, 1988; Nill et al., 2018). Due to its significant correlation variable ($R^2 = 0.9974$), the revised Prout-Tompkins equation, notably, best described the heterogeneous enzyme hydrolysis kinetics (Valtcheva et al., 2003)

Towards this end, this study aims to understand the hydrolysis mechanism on the pretreated kenaf by xylanase (the main hemicellulosic xylan degrading enzyme) by a systematic kinetic study into simple reducing sugars. It is of practical significance as it can predict the reaction rate, enzyme concentration, and the hydrolysis intensity needed at a certain temperature that has been determined beforehand. Figure 1 shows the general hydrolysis mechanism of xylanase on the pretreated kenaf.
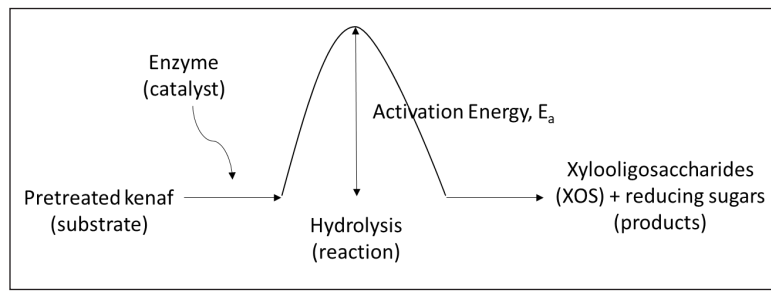

Figure 1. Hydrolysis mechanism of xylanase on the pretreated kenaf

MATERIALS AND METHODS

Preparation of the Raw Substrate

A company that is involved with kenaf processing in Bachok, Kelantan (North-East, Peninsular Malaysia) had generously provided the fresh whole kenaf stems including the core and the bast which were aged 3 to 4 months old. The constant weight of the kenaf stems was achieved by oven-dried for 24 h (105°C). The kenaf stem's moisture content was about 10 % (w/w). To acquire 40–60 mesh particle size, the dried kenaf stem was then hand-cut into tiny chunks and grounded using a mechanical grinder (Model RT-20, China). Eventually, before proceeding with the pretreatment procedures outlined below, the dried-ground kenaf stem was stored in a sealed container.

Pretreatment of Kenaf

The kenaf underwent 2-stages pretreatment with 7% $Ca(OH)_2$ for 1.5 hour, followed by 20% peracetic acid (PPA) for subsequent 2 h (Azelee et al., 2014)

Kinetic Study

The system of hemicellulose hydrolysis is never constructed precisely for the sake of its complicated nature and varying reaction conditions. Following the experimental result, a batch kinetic system was modelled and carried out for the hydrolysis of enzymatic hemicellulose. Based on the heterogeneous equation of the modified Prout-Tompkins equation as presented in (Equation 1), the kinetic model for the reaction of xylanase to the insoluble kenaf substrate was constructed in this analysis. The enzymatic hydrolysis of hardwood pulp (Valchev et al., 1998) and the steam-exploded wheat straw had widely employed the model previously (Radeva et al., 2012).

In this kinetic study of the enzyme, three different temperatures (30, 40, and 50°C) were used. Temperature variance was examined using the Arrhenius equation in the apparent rate constant, k. The equation of Arrhenius (Equation 2) provides the reliance of the rate constant, k of a chemical reaction on the absolute temperature T (in Kelvin), where A is the pre-exponential factor (or merely the *prefactor*), E_a is the activation energy, and R is the Universal gas constant ($8.31446 \frac{J}{mol.K}$). The rate, v was measured in (Equation 3) as a function of the degree of substrate conversion, $\propto$ at varying temperatures, and the graph was eventually constructed.

$$\frac{\propto}{(1 - \propto)} = (k\ t)^{x} \qquad \text{(Equation 1)}$$

$$k = A e^{\frac{-E_a}{RT}} \qquad \text{(Equation 2)}$$

$$v = x\ k\ \left[\frac{\propto}{(1 - \propto)} \right]^{\frac{(x - 1)}{x}} (1 - \propto)^{2} \qquad \text{(Equation 3)}$$

Enzyme Kinetic by Prediction and Experimental Values

Given this test, the kinetic variable was dimensional quantity α, the amount of reduced sugar which moved across the solution at a particular moment (Rs) and the overall saturated quantity, Rs_{max} $\propto = \frac{Rs}{Rs_{max}}$. Rs is the present value of reduced sugars, whereas Rs_{max} is the utmost amount of reduced sugars produced during the treatment of enzymes. The Rs_{max} values for various temperatures are therefore calculated by the equation derived (as in Equation 4). The detailed mathematical derivation of the formula is shown in the supplementary data.

$$R\ s_{max} = \frac{2\ R\ s_1 \left[\left(\frac{t_1}{t_2} \right)^{x} - 1 \right]}{\left[2 \left(\frac{t_1}{t_2} \right)^{x} - 1 \right]} \qquad \text{(Equation 4)}$$

However, another set of kinetic calculation (for the E_a and A values) was performed based on the experimental values obtained directly from the graph of reducing sugars (RS) versus time. Moreover, a comparison was made to determine the most suitable method (by prediction or experimental) for the calculation of the kinetic study of xylanase hydrolysis on the pretreated kenaf stem.

RESULTS AND DISCUSSION

Comprehending the hydrolysis process on the pretreated kenaf by xylanase (the main hemicellulosic xylan degrading enzyme) into simple reducing sugars by a structured kinetic investigation was the purpose of this kinetics analysis of enzymatic hydrolysis. It was of significance as it might predict the degree of hydrolysis needed at specified temperature levels, the concentration of the enzyme, and the time required to accomplish the reaction.

Figure 2 demonstrates the experimental results about the variation of reducing sugars (Rs) with the time (t) in the temperature range studied. After the first exponential increase, the mechanisms decelerate throughout every temperature from the kinetic curves. Consequently, a test with a prolonged period of incubation is usually required to determine the maximum amount of sugar reduction (Rs_{max}). Furthermore, the measurement of substrate concentration in terms of its molecular weight is hard to achieve in the study, attributable to the laborious and unmeasurable method to determine the concentration of the insoluble substrate required in the heterogeneous reaction. Within the sense of the prominent Michaelis-Menten equation, which is widely applied for homogeneous reactions and is not restricted to a time constraint, these descriptions and the configuration of the kinetic curves do not imply the likelihood of their mathematical explanations (Radeva et al., 2012).
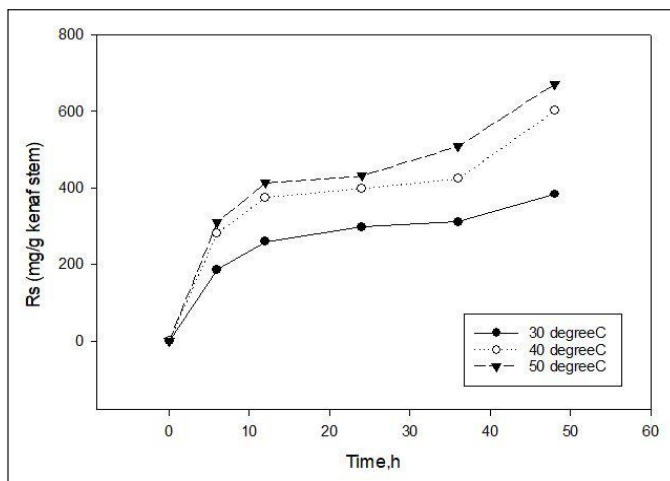

Figure 2. Kinetic curves (at the exponential phase) of the enzymatic xylanase hydrolysis of the pretreated kenaf stem at different temperatures from 0 h until 48 h. Other operating conditions were set at the optimum value of the xylanase hydrolysis (T = 40°C, pH 4.0, substrate loading = 2% and xylanase loading = 400 U).

Although the xylanase kinetic behaviour matches the Michaelis–Menten framework on clearly identified soluble oligosaccharides but this traditional Michaelis–Menten method is insufficient to explain xylanase activity on insoluble hemicellulose (xylan) (Fialho & Carmona, 2004). The renowned Michaelis-Menten principle is not fit to be generalized for heterogeneous structures that have an uncertain amount of substrates. Thus, derivations of kinetic formula relating to dispersion, topochemical and alternative forms of heterogeneous processes were investigated by prior researchers for a kinetic description (Valchev et al., 1998; Valtcheva et al., 2003)

The ability of the components to move within the system is part of the significant dissimilarities between homogeneous and heterogeneous kinetics. Although reactant molecules inhomogeneous environments are typically accessible to each other, solid-state reactions also take place within crystal lattices or with molecules that have to pass through lattices where mobility is limited and possibly rely on lattice deformities. If the rate of the process is regulated by the reaction interface movement of the reactants or products, a product layer can increase (Khawam & Flanagan, 2006; Welch, 1955).

Therefore in this analysis, the enzyme kinetics study utilizing xylanase (the key xylan backbone degrading enzyme) was analyzed based on the modified Prout-Tompkins formula for the processing of simple reducing sugars. Similar findings for the xylanase activity on kraft pulp have also been documented (Valcheva et al., 2000).

Thermogravimetry is often used to study reaction kinetics in the solid-state, although other appropriate analytical approaches are applicable for the assessment of kinetic reactions (Cai et al., 2018). The determined parameter must be converted into a fraction of conversion ($\propto$) that can be employed for every single analytical process in kinetic equations. A dimensionless quantity ($\propto$) is recognized as a kinetics variable used in (Equation 1), which is the proportion of the amount of sugar reduction that has been transferred at a particular point in time (Rs) and its maximum values beneath the saturation level, (Rs_{max} $\propto = \dfrac{Rs}{Rs_{max}}$). Besides, $\propto$ also feasible to facilitate the degree of conversion of the substrate or also to explain the degree of hydrolysis, where x is an unvarying constant features of the model or power factor, and k is a constant rate of reaction calculated in reciprocal time units (Valchev et al., 1998). The activity of xylanase on pretreated kenaf can be defined as a topochemical process, meaning that the reaction occurs at the interphase limit. The rate of reaction also relies on the size and shape of the shifting interaction surface result from the substrate's heterogeneous structure (Valchev et al., 1998). Owing to this complex heterogeneous composition of the pretreated kenaf matrix, it specifies the reaction zones and their topochemical mechanism. It is well established that the following Equation 1 can be implemented efficiently to topochemical chain mechanism reactions and diffusion regulated heterogeneous processes.

Temperature Dependence of the Hydrolysis Process

In the current study, the kinetics of kenaf stem hemicellulose hydrolysis was studied at temperatures of 30ºC, 40ºC, and 50ºC, under optimized xylanase hydrolysis conditions (2% substrate concentration, 400 U of xylanase activity, and in a pH 4.0 system of sodium acetate buffer). Experimental results on the total value of reducing sugar, Rs, produced after enzymatic hydrolysis was recorded to acquire the kinetic curves, as demonstrated in Figure 2. They are reducing sugar yield that can be observed increasing with time when the temperature rises from 30°C to 40°C but is not preferred at 50°C. The thermal inactivation of the enzyme may contribute to the decrease of the reducing sugars at 50°C at a longer hydrolysis time. (Valcheva et al., 2000).

At the start of the process, it was presumed that at low concentrations of reducing substances ($\alpha \ll 1$), Equation 1) was reduced to Equation 5:

$$\alpha = (k\ t)^{\chi} \qquad \text{(Equation 5)}$$

Therefore,

$$\frac{R\ s}{R\ s_{max}} = (k\ t)^{\chi} \qquad \text{(Equation 6)}$$

The logarithmic form of (Equation 6), is presented as in (Equation 7)

$$ln\ R\ s = ln\ (Rs_{max}) + \chi \cdot lnk + \chi \cdot lnt \qquad \text{(Equation 7)}$$

Dependence of reducing sugars over time at different temperatures in logarithmic coordinates is presented in Figure 3. By utilizing the experimental kinetic information, $Rs = f(t)$, at the initial stage of the process it facilitates the determination of the coefficient χ at a specified temperature. The data in Figure 3 shows that the coefficient χ is temperature independent and the average result is 0.2746.

It is also possible that from Equation 7, a maximal value of the reducing substances can be measured with a value for χ from Equation 8. It may assist to provide an accurate and better regression for Rs_{max}. Thus, the Rs_{max} values are estimated for various temperature adopting the Equation 8

$$Rs_{max} = \frac{2\ Rs_1 \left[\left(\frac{t_1}{t_2} \right)^{x} - 1 \right]}{\left[2 \left(\frac{t_1}{t_2} \right)^{x} - 1 \right]} \qquad \text{(Equation 8)}$$
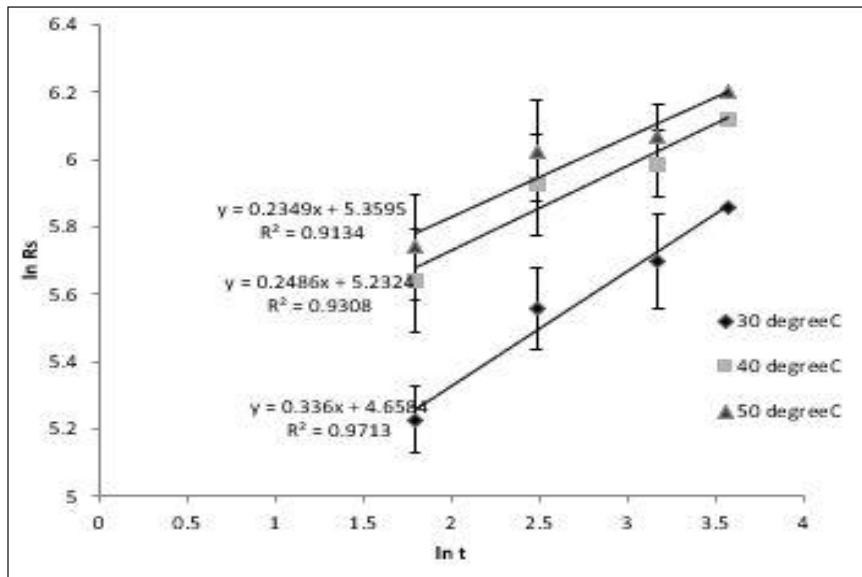
Figure 3. The dependences of the amount of reducing sugars on time in logarithmic coordinates at various temperatures (30°C, 40°C and 50°C)

The predicted Rs_{max} values for three temperatures are briefed in Table 1 below. The detailed calculation for each temperature is shown in the supplementary data.

Table 1
Rs_{max} values at different temperatures

Temperature, ºC	Rs_{max}
30	650.173
40	980.036
50	1084.04

Significantly higher Rs_{max} was obtained from the study compared to the one achieved by Valcheva et al. (2000) where only 37.5 mg/g of sugar was obtained from kraft pulp xylanase hydrolysis. The high yield of the Rs_{max} achieved in the study showed the high efficiency of xylanase hydrolysis on the pretreated kenaf stem. This method and the equation were developed for Rs_{max} substantial contributions to related process kinetics being studied. Further analysis of the kinetic data was performed on the basis of the modified Prout-Tompkins (Equation 1).

From the logarithmic form of Equation 1, the apparent rate constant, k was found and is presented in Equation 9.

$$ln \frac{\propto}{1 - \propto} = \chi \ .ln \frac{\propto}{1 - \propto} = \chi \ .ln \qquad \text{(Equation 9)}$$

In addition, linear relations for three temperatures following Equation 9 are shown in Figure 6. The calculated constants k and χ were proofs that the values of χ from Equation 7 and Equation 9 are in strong compromise to one another (90 % confidence level). In this study, the pre-treated kenaf stem used is categorised as the harder to degrade material (hardwood) due to the crystalline structure of the lignocellulose (Rowell & Rowell, 1996). A prior study by Zhang et al. (1999) reported that the degree of hydrolysis (α) of the harder-to-degrade material was more susceptible temperature than the rate for the easier-to-degrade material. On that account, the temperature rises from 30°C to 40°C is, therefore, necessary to achieve the optimum hydrolysis of xylanase for the pretreated kenaf stem. On the contrary, a rise in temperature from 40°C to 50°C is required for steam-exploded wheat straw as it is categorized as easier to degrade material (Radeva et al., 2012).

In Figure 4, the inhomogeneity coefficient, $\propto$ is predicted from the gradient of the straight lines. For temperature readings of 30°C, 40°C and 50°C, it was discovered to be equal to 0.229. In instances where it is independent of temperature, just entropy inhomogeneity is accounted for by the coefficient of $\propto$ (Radeva et al., 2012).

In Figure 5, linear relations are presented in accordance with Equation 9 for three varying temperatures. The linear relationship is significant for the calculations of the kinetic constant of χ and k (Table 2). Valcheva et al. (2000) had also performed a similar study with four different temperatures (20 to 50°C) on kraft pulp and all of them showed a straight line with high correlation coefficient. In this analysis, the correlation coefficient, R, and standard deviation calculated by the Revised Prout-Tompkins equation is 0.9097 and 0.119, respectively. The values of χ and k for each temperature (Table 2) are acquired from the plot of $ln\frac{[\propto}{(1-\propto)]}$ versus $ln\ t$ in Figure 5.
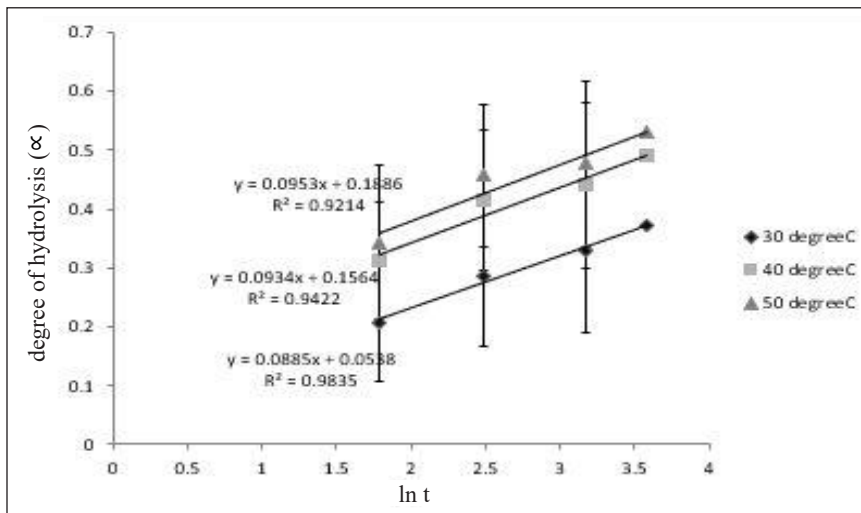

Figure 4. The linear dependences of the kinetic variables (degree of hydrolysis ($\propto$) against different time (t) in logarithmic coordinates) at given temperatures for 2% of pretreated kenaf stem
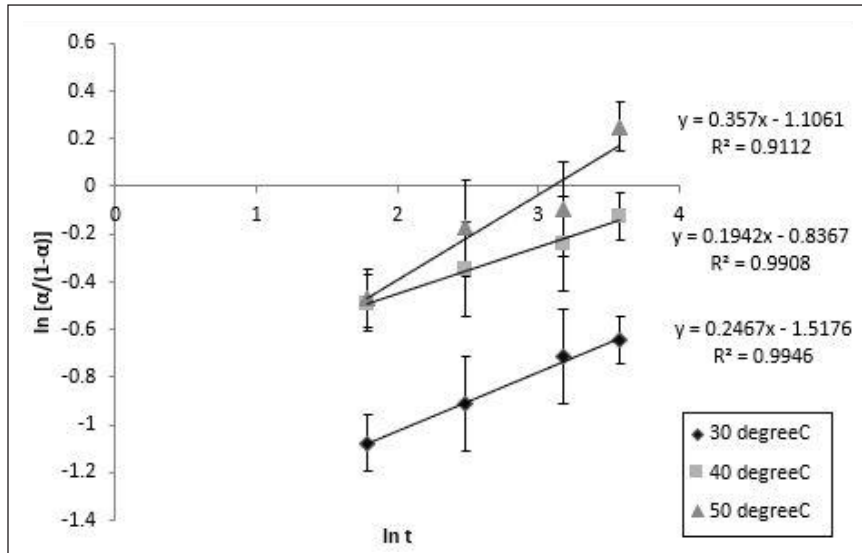
Figure 5. The linear form of Prout-Tompkins equation for different temperatures

Table 2
The values of χ and k at different temperatures

Temperature, °C	x	k
30	0.2467	0.00737
40	0.1943	0.00969
50	0.3570	0.00915

Research on hardwood pulp by Valchev et al. (1998) offers a different value of χ and k for temperature 20 to 40°C. The values to other substrates would be slightly different. These values are crucial for investigating variations in temperature using the Arrhenius equation (Valcheva et al., 2000).

The Determination of Activation Energy from the Enzymatic Hydrolysis of the Pretreated Kenaf using the Predicted Values of Rs_{max}

To attain the kinetic parameters (model, E_a, A), the temperature variation of the apparent rate constant, k was studied using the Arrhenius equation (Khawam & Flanagan, 2006). The Arrhenius equation gives dependence on the rate constant, k of a chemical reaction on the absolute temperature T (in Kelvin), where A is the pre-exponential factor (or simply the *prefactor*), E_a is the activation energy, and R is the Universal gas constant (8.31446 $\frac{J}{mol.K}$). The Arrhenius equation and its derivation are shown in Equations 10 and 11.

$$k = A \ e^{\frac{-E}{RT}} \qquad\qquad \text{(Equation 10)}$$

$$ln \ k = \frac{-E_a}{RT} + ln \ A \qquad\qquad \text{(Equation 11)}$$

As shown in Figure 6 at the grounds of the statistically significant correlation between $ln \ k$ and $\frac{1}{T}$, the activation energy, E_a and the pre-exponential factor A was revealed to be: $E_a = 8.95 \ \frac{J}{mol.K}$ and $A = 0.271$. The pre-exponential factor units, A, are analogous to the rate constant units and should differ according to reaction order. Supposing that the reaction is first order, then the unit is s^{-1}. Since the concentration of the reactant in this study is constant (because the concentration of enzymes does not rise or drop with time, the concentration of one of the reactants remains constant because it is distributed in vast quantities and its concentration can be absorbed within the constant rate), the order of this reaction is known as a pseudo-first-order reaction. This pseudo-first-order reaction is very beneficial, as it significantly simplifies the quantification of the kinetics of the reaction. It can be complicated to observe a second-order reaction mostly because the two reactants associated must be calculated concurrently. Other complications may occur because it requires a certain amount of each reactant to calculate the reaction rate, for example, which can make one's experiment uneconomical if one or both of the reactants required are costly. The majority of kinetic findings recognize the restricted parts of the kinetic curves generally apply pseudo-first or second-order equations (Valtcheva et al., 2003).
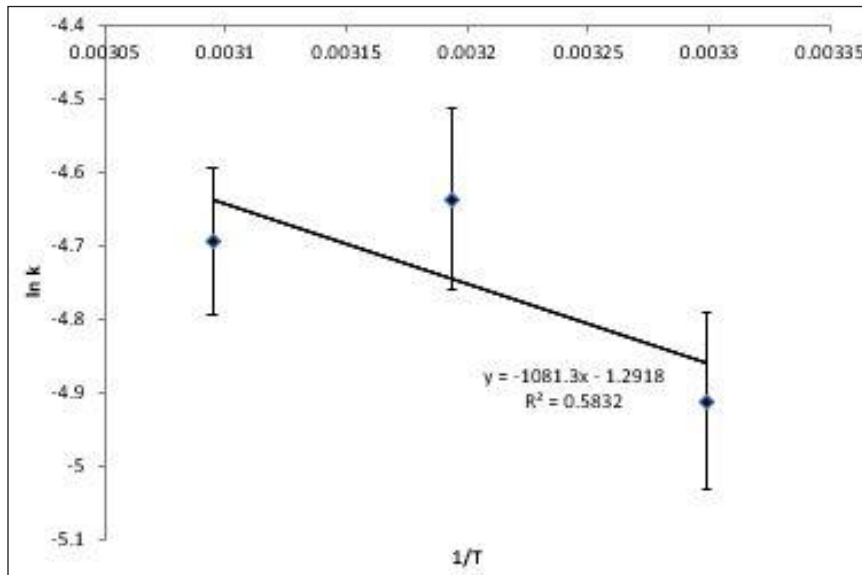

Figure 6. Linear relation between $ln \ k$ and $\frac{1}{T}$ from the Arrhenius equation. The activation energy was calculated to be 8.95 kJ/mol

By using the above equation with identified values of k and χ, the rate v can be determined for other ∝. The rate v is shown in Figure 7 as a function of the degree of substrate conversion, ∝ at different temperatures. At temperature 40ºC, absolute values of the rate and their variations with ∝ in the entire process are the highest while the lowest rate is at 30ºC. The pre-exponential factor, A, which decreases when ∝ increases contribute to the rate decreases with time. The measurements of the reaction area and its availability may be related to the pre-exponential component A, which is developed during the process on the basis of xylan-enzyme complexes and modifications. The application of the topochemical kinetic model to the evaluation of the reaction may also be taken into consideration. The process initially occurs on the kenaf's most accessible outer surface and eventually permeates into the kenaf fibre matrix. The consistent activation energy indicates that on the surface and within the capillary system, the energy properties of the xylan-enzyme complex are the same, i.e. the enzyme has a chemical interaction with the same xylan only.
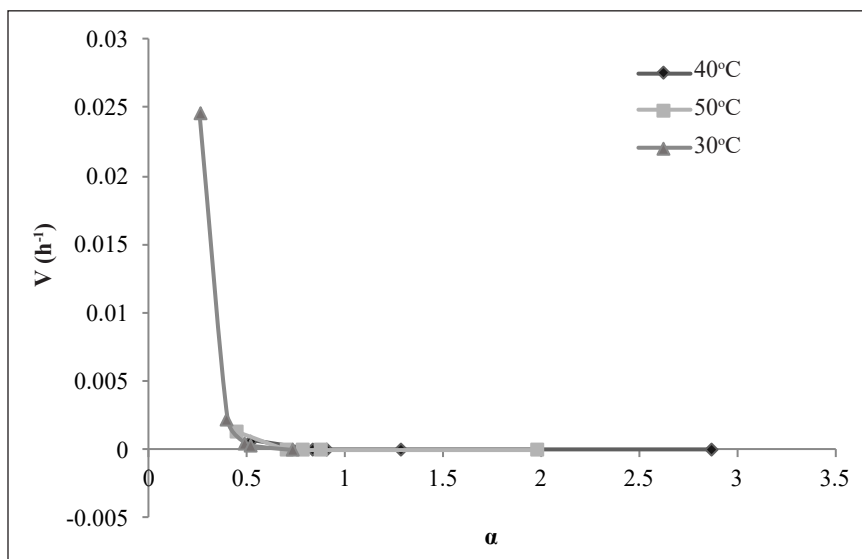

Figure 7. The present rate of hydrolysis in enzymes, v reliance on the degree of substrate conversion, ∝ for different temperatures

The Determination of Activation Energy from the Enzymatic Hydrolysis of the Pretreated Kenaf using the Actual Values of Rs_{max} Obtained from the Experiment

As the rate of hydrolysis, v decreased dramatically to 0 when ∝ > 0.5, the temperature variation of hydrolysis rate, v which at the given degree of substrate conversion, ∝ did not satisfy the Arrhenius equation. To obtain an excellent v profile, the values of Rs_{max} from the experimental result were used to rectify the previously projected values of the Rs_{max}. Therefore, an amended assessment of ∝ was conducted by using the Rs_{max} from

experimental data. The values of Rs_{max} for respective temperature were gained from the graph in Figure 8 and are shown in Table 3.

In Figure 8, the reaction is labelled as a burst phase at around 40 hours when the graph grows exponentially for all three temperatures. This type of graph obtained in the study can be called as pre-steady-state kinetics or may be appealed as Burst kinetics. Burst kinetics is a form of enzyme kinetics which pertains to the initial high enzyme turnover velocity when the enzyme is introduced to the substrate. This initial phase of product development at high velocity is termed the "Burst Period". This duration is examined as the enzymes become saturated with substrates up to saturation of all enzymes. After the saturation of all enzymes, the Burst Phase leads to a linear velocity of reaction (Praestgaard et al., 2011). The Burst kinetics accounts well for the experimental data that have successfully used the Burst kinetics model for the hydrolysis of Cellobiohydrolases (exo-cellulases) on cellulose. Before reaching the steady-state kinetics interests in the development and utilization of intermediate enzyme-substrate up till their steady-state concentrations are attained. The rate subsequently decreases as it enters a steady state. A single enzyme turnover was analyzed during the rapid burst period of the process.

This nonlinearity of the heterogeneous reaction kinetics could be clarified either one or more among this considerations: inactivation of the enzyme, inhibition of the product, or heterogeneity of the substrate (Zhang et al., 1999). The restriction of the mass transfer suggests that the rate of reaction is not entirely determined by the catalytic capacity of the enzyme. The mass-transfer rates of the substrate(s) from the bulk reaction medium to the
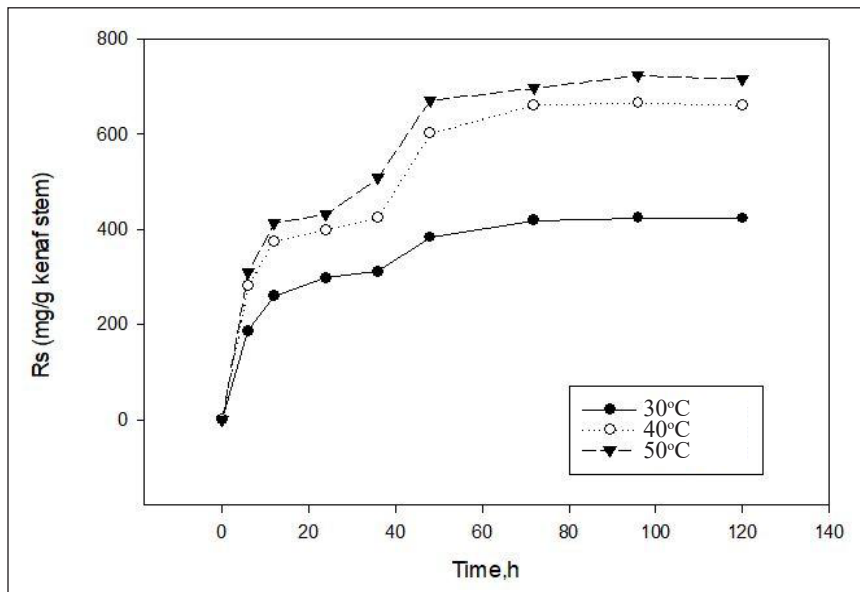

Figure 8. Overall kinetic curves of enzyme action for different temperatures.

enzyme site as well as the mass-transfer rates of the product(s) from that channel back to the reaction medium need to be taken into consideration (Illanes et al., 2013). Due to the virtue of the structural changes inside the substrate, kinetic action will not intrinsically be similar to the free enzyme.

Based on the graph obtained by the experiment, the Rs_{max} values for each temperature (30°C, 40°C and 50°C) are shown in Table 3 below.

Table 3
Rs_{max} values for respective temperature (based on the experimental research)

Temperature, ˚C	Rs_{max}
30	550
40	680
50	723

Figure 9 describes the linear dependencies derived using the experimental result. To obtain the inhomogeneity coefficient, $\propto$ each value of Rs is divided at each time with the median value of Rs_{max} gained from the respective temperature. The determination of $\propto$ is essential to be used in the next step of determining the kinetic values of χ and k. The linear form of Prout-Tompkins equation using the value of $\propto$ (initially determined) for vary temperatures are also presented in Figure 10.
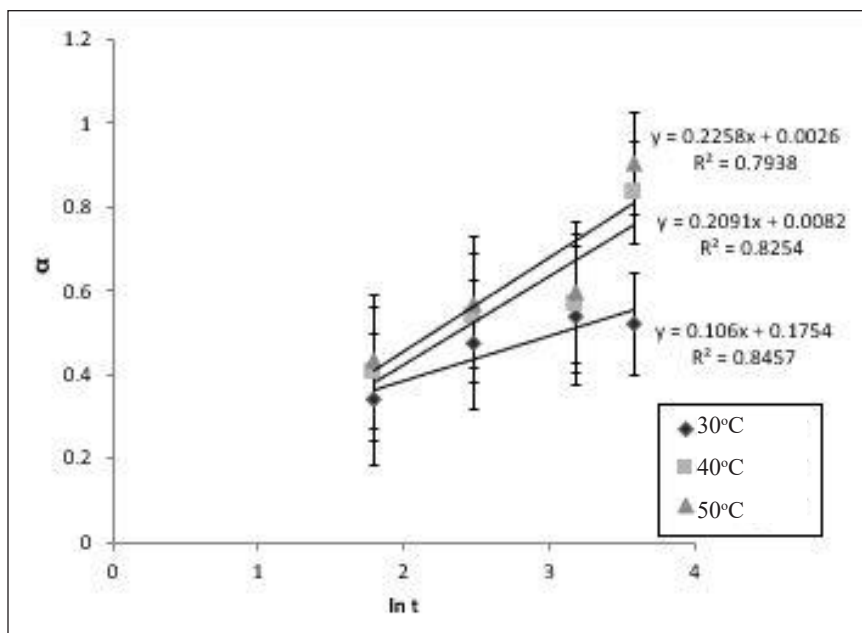

Figure 9. Linear dependences of the kinetic variable, $\propto$ vs. $ln\ t$ (experimental work)
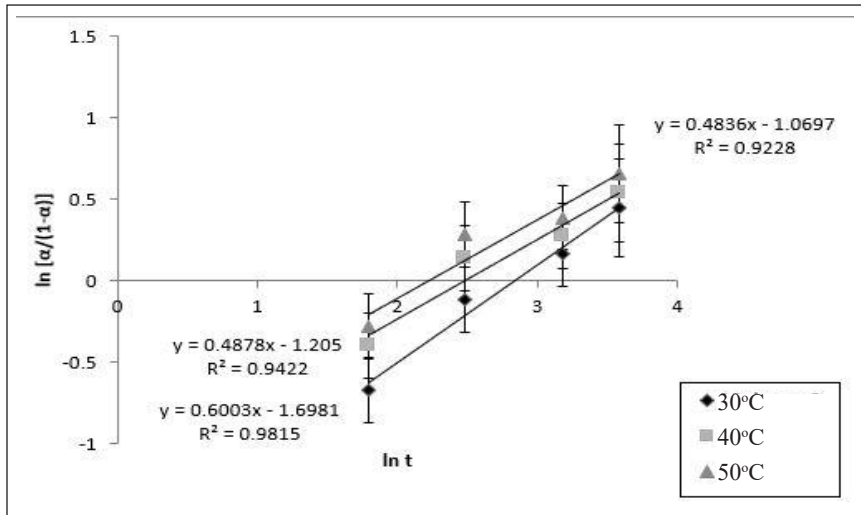
Figure 10. The linear form of Prout-Tompkins equation for vary temperatures (experimental work)

In the analysis, the correlation coefficient R and standard deviation for using the Revised Prout-Tompkins equation are respectively 0.9064 and 0.0532. The values of χ and *k* acquired for respective temperature from the plot of $ln\frac{\alpha}{1-\alpha}$ versus $ln\ t$ are recorded in Table 4.

Table 4
The values of χ and k at different temperatures (gained from the experimental values)

Temperature, °C	χ	k
30	0.6005	0.0590
40	0.4878	0.0846
50	0.4836	0.1090

The values of activation energy, E_a and pre-exponential factor, A are determined by plotting the values of $ln\ k$ against $\frac{1}{T}$ with T in Kelvin (Figure 10).

The applicability of the logarithmic form of Equation 4 approved by the linear behaviour. Based on the linear relation between $ln\ k$ and $\frac{1}{T}$ (shown in Figure 11), the activation energy, E_a and the pre-exponential factor, A in the analysis it was identified that: $E_a = 25.15\ \frac{J}{mol.K}$ and $A = 1290.648$ respectively. The R² value is above 0.90, which is 0.9945 proving that the obtained result is reliable.

For comparison, Leszczynski and Shukla (2012) had reported that the energy needed for the activation of glycosidic C-O bond in the absence of a catalyst was estimated to be $41.83\ \frac{J}{mol.K}$ which was relatively higher than what had been obtained in the study. Hence, with the addition of xylanase enzyme as the bio-catalyst to hydrolyse the pretreated kenaf stem has managed to reduce the amount of activation energy required successfully. Smaller
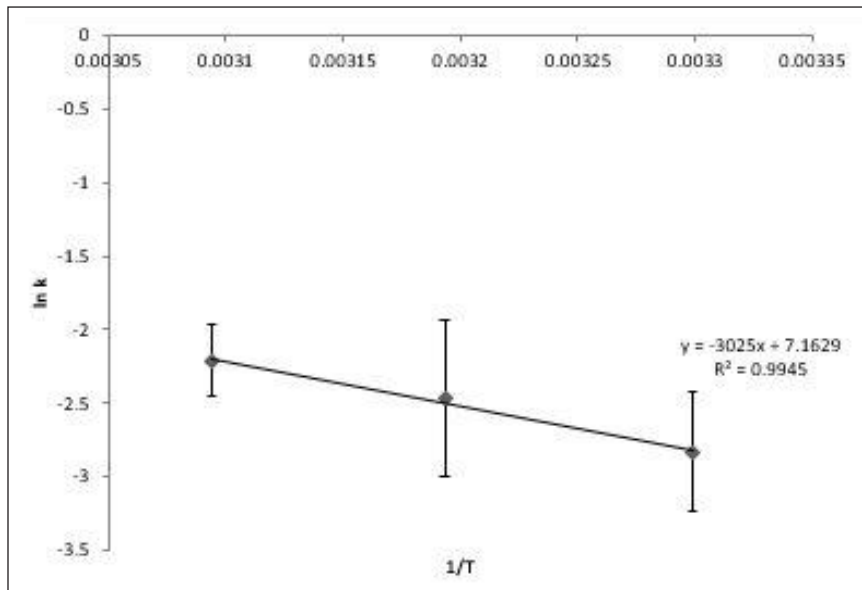
Figure 11. Linear relation between $ln\ k$ and $\frac{1}{T}$ (from the experimental values)

activation energy is preferred as a more effective way to bring about a substrate's reaction or hydrolysis. The presence of xylanase enables a more significant percentage of reactant molecules to gain sufficient power for transit through the state of transition and become products.

The breakdown of this C-O glycosidic bond demanded less energy to activate than the C-N glycosidic bond where the breakdown of the C-N bond was expected to be 100.79 $\frac{J}{mol.K}$ (Leszczynski & Shukla, 2012). Besides, hydrothermal treatment has measured the activation energy usually required for the cleavage of hardwood xylan (*Eucalyptus saligna*) glycosidic bonds is 125.6 $\frac{J}{mol.K}$. Model studies proposed that the degradation rate differs subjected to classification of the structure for the lignocellulosic bonds even though they are chemically labile in acid (Christopher, 2012). The energy of 129.2 $\frac{J}{mol.K}$ is required for activation as well as the frequency variables within 2.56 x 10^{15} to 2.57 x 10^{14} for acid hydrolysis of Beechwood hemicellulose from *Fagus crenata*. Whereas, multiple reports have demonstrated a broad range of activating energy for the hydrolysis of acid (106.2–159.7 $\frac{J}{mol.K}$). Xylobiose which is the analog from the acid hydrolysis of Cellobiose reveals an activating energy of 137 $\frac{J}{mol.K}$. This outcome is approximately similar to the glucans cleavage energy (other lignocellulose conformation) (Dumitriu, 1998). Conversely, hardly any kinetic reaction constants are identified in alkaline hydrolysis literature (Dumitriu, 1998).

The present predicted rate of the hydrolysis process is at varying values. The temperature outcome is graphed in Figure 12. The process rate is seen to be the highest at $\propto$ values below 0.4. It then declines substantially, most likely due to the exertion of the active site available on the substrate region (Radeva et al., 2012; Valtcheva et al., 2003). Earlier research reported that standard hydrolysis cellulose process began with proportionately steady momentum, then the rate of hydrolysis declined, and usually concluded with incomplete hydrolysis of the substrate later when enormous doses of the enzyme were introduced. This circumstance can arise with the disappearance of enzyme stability or activity, the consequence from the hindrance of the reaction output, a possible elevation in the recalcitrance of the substrate from the hydrolysis process, or rivalry amidst various enzyme elements on the substrate's active sites (Chandra et al., 2011). The difficulty in comparing the speed and degree of hemicellulose hydrolysis by different xylanases is the result of the non-linear system. This non-linearity also affects the process of hemicellulose hydrolysis as the rate declines and usually halts before all substrate is metabolized. The finding supports the theory which, over time, a decline in the rate of xylanase activity of kenaf hemicellulose is because of a reduction in the simple degradable substrate. The absent of β-xylosidase stimulation for xylanase activity dismisses the idea of inhibition of substances being the reason for the reported non-linearity.

Table 5 below demonstrates a table of contrast on the activation energy required for hydrolysis of varying substrate. Particularly in comparison with some of the other substrates, the xylanase hydrolysis on the pretreated kenaf stem has shown to have a rather low activation energy. This means that with a slow step with less energy barrier, the xylanase
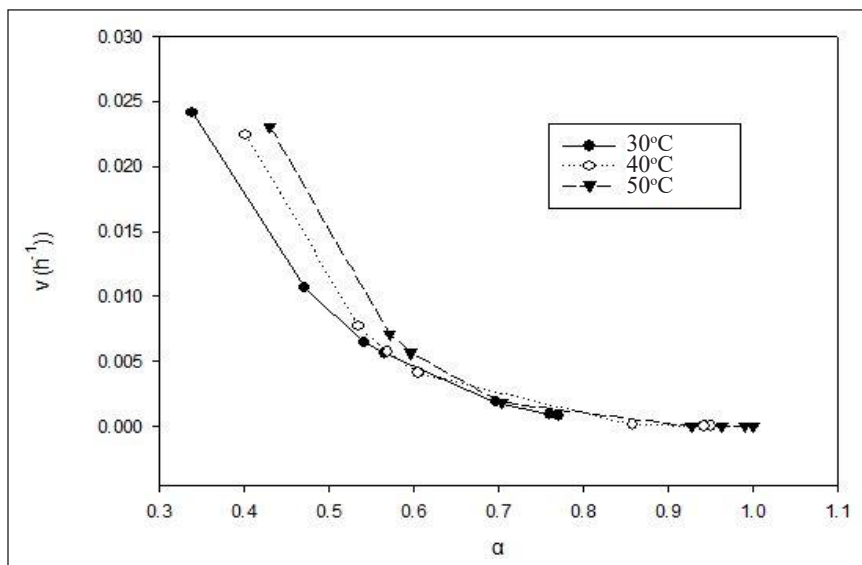

Figure 12. Dependence of the enzymatic hydrolysis current rate, *v* on the degree of substrate conversion, $\propto$ at various temperature values (30°C, 40°C and 50°C).

enzyme was able to shift the reaction pathway to another. The value of activation energy obtained in the study is within the common range used by other hydrolysis enzymes.

Table 5
Comparison between various substrate hydrolysis and the activation energy required for the process

Substrate	Activation energy, $E_a(\frac{J}{mol.K})$	Reference
Kenaf	25.15	This study
Hardwood kraft pulp	34.1	(Valchev et al., 1998)
Kraft pulp	34.6	(Valcheva et al., 2000)
Pulp	22.2	(Valtcheva et al., 2003)
Wheat straw	20	(G. Radeva, 2011)
Wheat straw	46.7	(Radeva et al., 2012)

CONCLUSION

The xylanase hydrolysis kinetics of the pretreated kenaf was evaluated after pretreatment with alkali-acid (Ca$_2$(OH)–PPA). It has been discovered that the exponential kinetic equation offers an excellent clarification on the behaviour of xylanase, and the activation energy needed for hydrolysis is lower than some lignocellulose. Temperature influences the time dependence of the reduced sugar yield. It is of practical significance since it gives prediction on the degree of hydrolysis demanded at predetermined temperature values and the time spent. Moreover, the reaction rate depends on the conversion of the substrate and the value can be estimated at different temperature levels.

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REFERENCES

Azelee, N. I. W., Jahim, J. M., Rabu, A., Murad, A. M. A., Bakar, F. D. A., & Illias, R. M. (2014). Efficient removal of lignin with the maintenance of hemicellulose from kenaf by two-stage pretreatment process. *Carbohydrate Polymers, 99*, 447-453. doi:10.1016/j.carbpol.2013.08.043

Cai, J., Xu, D., Dong, Z., Yu, X., Yang, Y., Banks, S. W., & Bridgwater, A. V. (2018). Processing thermogravimetric analysis data for isoconversional kinetic analysis of lignocellulosic biomass pyrolysis: Case study of corn stalk. *Renewable and Sustainable Energy Reviews, 82*, 2705-2715. doi:10.1016/j.rser.2017.09.113

Chandra, R. P., Au-Yeung, K., Chanis, C., Roos, A. A., Mabee, W., Chung, P. A., ... & Saddler, J. N. (2011). The influence of pretreatment and enzyme loading on the effectiveness of batch and fed-batch hydrolysis of corn stover. *Biotechnology Progress, 27*(1), 77-85. doi:10.1002/btpr.508

Chrastil, J. (1988). Determination of the first order consecutive reaction rate constants from final product. *Computers & Chemistry, 12*(4), 289-292. doi:10.1016/0097-8485(88)80002-0

Christopher, L. (Ed.) (2012). *Integrated forest biorefineries: Challenges and opportunities*. Cambridge, United Kingdom: Royal Society of Chemistry. doi: 10.1039/9781849735063

Cinar, K., Gunes, G., & Gulec, H. A. (2020). Enzymatic synthesis of prebiotic carbohydrates from lactose: Kinetics and optimization of transgalactosylation activity of β-galactosidase from Aspergillus oryzae. *Journal of Food Process Engineering*, *43*(8) e13435. doi:10.1111/jfpe.13435

De Menezes, A. J., Siqueira, G., Curvelo, A. A., & Dufresne, A. (2009). Extrusion and characterization of functionalized cellulose whiskers reinforced polyethylene nanocomposites. *Polymer, 50*(19), 4552-4563. doi:10.1016/j.polymer.2009.07.038

Dumitriu, S. (Ed.) (1998). *Polysaccharides: Structural diversity and functional versatility*. Boca Raton, Florida: CRC Press. doi: 10.1021/ja0410486

Fialho, M. B., & Carmona, E. C. (2004). Purification and characterization of xylanases from *Aspergillus giganteus*. *Folia Microbiol (Praha), 49*(1), 13-18. doi:10.1007/bf02931639

Garrote, G., Domínguez, H., & Parajó, J. C. (2002). Autohydrolysis of corncob: Study of non-isothermal operation for xylooligosaccharide production. *Journal of Food Engineering, 52*(3), 211-218. doi:10.1016/s0260-8774(01)00108-x

Illanes, A., Wilson, L., & Vera, C (2013). Enzyme kinetics in a heterogeneous system. In Illanes, A., Wilson, L., & Vera, C. (Ed.), *Problem solving in enzyme biocatalysis* (pp. 87-140). New Jersey, USA: John Wiley and Sons Ltd. doi: 10.1002/9781118341742.ch03

Khawam, A., & Flanagan, D. R. (2006). Solid-state kinetic models: Basics and mathematical fundamentals. *The Journal of Physical Chemistry B, 110*(35), 17315-17328. doi:10.1021/jp062746a

Leszczynski, J., & Shukla, M. (Eds.) (2012). *Practical aspects of computational chemistry II: An overview of the last two decades and current trends*. New York, London: Springer. doi: 10.1007/978-94-007-0923-2

Mandelli, F., Brenelli, L., Almeida, R., Goldbeck, R., Wolf, L., Hoffmam, Z., ... & Squina, F. (2014). Simultaneous production of xylooligosaccharides and antioxidant compounds from sugarcane bagasse via enzymatic hydrolysis. *Industrial Crops and Products, 52*, 770-775. doi:10.1016/j.indcrop.2013.12.005

Nill, J., Karuna, N., & Jeoh, T. (2018). The impact of kinetic parameters on cellulose hydrolysis rates. *Process Biochemistry, 74*, 108-117. doi:10.1016/j.procbio.2018.07.006

Praestgaard, E., Elmerdahl, J., Murphy, L., Nymand, S., McFarland, K. C., Borch, K., & Westh, P. (2011). A kinetic model for the burst phase of processive cellulases. *The FEBS Journal, 278*(9), 1547-1560. doi:10.1111/j.1742-4658.2011.08078.x

Radeva, G., Valchev, I., Petrin, S., Valcheva, E., & Tsekova, P. (2012). Kinetic model of enzymatic hydrolysis of steam-exploded wheat straw. *Carbohydrate Polymers, 87*(2), 1280-1285. doi:10.1016/j.carbpol.2011.09.012

Radeva, G., Valchev, I., Petrin, S., Valcmeva, E., & Tsekova, P. (2011). Comparative kinetic analysis of enzyme hydrolysis of steam-exploded wheat straw. *Cellulose Chemistry and Technology, 46*(1-2), 61-67.

Rowell, R. M., & Rowell, J. (1996). Chemical composition of fibers. In J. Stein (Ed.), *Paper and composites from agro-based resources* (pp. 83-134). Massachusetts, USA: CRC Press.

Valchev, I., Yotova, L., & Valcheva, E. (1998). Kinetics of xylanase treatment of hardwood pulp. *Bioresource Technology, 65*(1–2), 57-60. doi:10.1016/s0960-8524(98)00020-0

Valcheva, E., Veleva, S., Valchev, I., & Dimitrov, I. (2000). Kinetic model of xylanase action on kraft pulp. *Reaction Kinetics and Catalysis Letters, 71*(2), 231-238. doi:10.1023/A:1010310706612

Valtcheva, E., Veleva, S., Radeva, G., & Valtchev, I. (2003). Enzyme action of the laccase-mediator system in the pulp delignification process. *Reaction Kinetics and Catalysis Letters, 78*(1), 183-191. doi:10.1023/A:1022538621950

Welch, A. J. E. (1955). *Solid-solid reactions*. New York, NY: Academic Press

Zhang, S., Wolfgang, D. E., & Wilson, D. B. (1999). Enzyme action of the laccase-mediator system in the pulp delignification process. *Biotechnology Bioengineering, 66*(1), 35-41. doi: 10.1023/A:1022538621950