VOL. 12, NO. 18, SEPTEMBER 2017 ISSN 1819-6608
ARPN Journal of Engineering and Applied Sciences


www.arpnjournals.com

EVALUATION OF THE ENZYMATIC HYDROLYSIS PROCESS OF OIL PALM EMPTY FRUIT BUNCH USING CRUDE FUNGAL XYLANASE

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ABSTRACT

Oil Palm empty fruit bunches (OPEFB) is the solid waste produced from crude palm oil industries. It comprises of cellulose, hemicellulose and lignin. The major component of hemicelluloses, xylan, is a complex heteropolysaccharide with a β-1,4-xylose backbone. In the utilization of xylan, it must first be hydrolyzed into its components, that are the 5 carbon atom sugar xylose and arabinose. Endoxylanase (xylanase) is the primary enzyme that attacks the backbone structure of hemicellulose by random cleavage of internal xylosidic linkages to produces the xylose hydrolisate. Further xylose can be used as raw material for the production of a wide variety of chemicals such as xylitol. Enzymatic hydrolysis of xylan to xylose using xylanase offers an environmentally friendly biotechnological process beside performed at ambient temperature and pressure, it also has high specificity and low cost. Previous research has shown that fungal species *Trichoderma* is a good producer of lignocellulosic enzymes including xylanase. This article describes the study of enzymatic hydrolysis of OPEFB using crude xylanase extract produced by *Trichoderma viride* ITB CC L67. The study foccused on the optimation of hydrolysis process in terms of temperature and pH. The optimization was done based on Response Surface Methodology (RSM) to give either the optimum xylose concentration or xylose to glucose concentration ratio. Further, the kinetics of the enzymatic hydrolysis process of OPEFB was studied. The enzymatic hydrolysis can be well approached by the Michaelis Menten kinetic model, and kinetic parameters were obtained from experimental data.

Keywords: enzymatic hydrolysis, oil palm empty fruit bunches, optimization, xylanase and xylose.

1. INTRODUCTION

The utilization of lignocellulosic materials such as agricultural waste to value added products of chemicals, food and fuels are interesting as they are low cost, renewable and available in huge quantity in nature. One of the potential lignocellulosic materials is oil palm waste. Currently, Indonesia is the largest exporter of palm oil in the world commercial market. In the processing of crude palm oil, oil palm empty fruit bunch (OPEFB) is generated as waste. Approximately 20-22% of fresh fruit bunch is converted into OPEFB during the extraction of fresh fruit bunches [1].

The OPEFB comprised of cellulose (43%), hemicellulose (23%) and lignin (20%) and other extractive [2].From 1970s through the late 1980s, many research and development efforts were conducted to improve the saccharification of cellulose in lignocellulosic feedstocks. These materials must be broken down through hydrolysis for the production of glucose, which can then be converted intoethanol [3]. The hemicellulose, which is a branched polysaccharide consisting of the pentoses (D-xylose and L-arabinose) and hexoses (D-galactose, D-glucose, and D-mannose), is potential raw material for the production of various bioproducts. In agricultural residues the hemicellulose is composed mainly of xylan, made up of xylose units [3]. Hemicellulose may be hydrolized to produce xylose for the production of xylitol, a sugar alcohol, which is a low calorie sugar and has been used in various food applications [4]. This hydrolysis process could be performed chemically, at high pressure and temperature using acid or alkaline as the catalyst or at ambient condition using biological xylanolytic enzim as the catalyst[5].

The xylanolytic enzymes or the so called xylanases are produced by a wide variety of microorganisms, among those are the filamentous fungi such as *Trichoderma viride, Aspergillus niger,* and *Penicillium*. The production of hemicellulase by these fungi are particularly interesting as they secrete the enzymes into medium or the so called extracellular enzymes and provide xylanase activities that are much higher than those found in yeast and bacteria [6], [7]. In our previous research [8], we identified *A.niger ITBCC*L51, *T.viride* ITBCCL67 and *Penicilliumsp.* ITBCC L96, to be potential xylanase producers. The enzyme was produced via solid state cultivation of these fungal species using OPEFB as the substrate. Among them, *T.viride* ITBCC L61 was found to be the best xylanase producer giving xylanase activity of 5093.5 U/ g substrate [8].

Factors affecting the enzymatic hydrolysis of lignocellulosic materials are substrates, enzyme activity, and reaction conditions (temperature, pH, as well as other parameters) [9]. To improve the yield and rate of the enzymatic hydrolysis of OPEFB, in particular the hydrolysis of its hemicellulose component, it is necessary to find the optimal condition of enzymatic hydolysis including temperature, pH and substrate concentration.

It is the objective of this research to optimize the hydrolysis process of OPEFB using crude xylanase produced by *T.viride*ITBCCL67. The research included the search for the optimum condition of enzymatic hydrolysis of OPEFB in term of pH and temperature, the influence of substrate to the enzymatic hydrolysis process, the dynamics of hydrolysis process and following to study

the kinetics of this process. The xylose hydrolysate will further be used for xylitol production.

2. MATERIAL AND METHOD

a) Raw materials

OPEFB was collected from Padang palm oil mill, Indonesia. OPEFB was sun dried, cleaned, oven dried at 105°C overnight and grinded before used. Only OPEFB of small size, max. 80 mesh were used. OPEFB composition has been previously determined following standard method of NREL and TAPPI [8].

b) Production of crude xylanase

Crude xylanase was produced from solid state cultivation of *T.viride* ITBCC L67 on OEFB, as was described in [8].The cultivations of *T.viride*ITB CCL.67was carried out in 300 mL shake flasks on the media that composed of OPEFB and mineral solution[10].Inoculum was prepared by suspending the spores in sterile physiological solution to give a final spore count of 1×10^6 spores/mL. The cultivation was conducted at its optimum temperature 33 °C and substrate solid to liquid ratio 0.63 gr OPEFB/mL during 36h cultivation time. Enzymes were extracted from the fungal cultivation by adding distilled water (four time volume of liquid medium) to the cultivation solution. Subsequently the solution were stirred with a sterile glass stick and then shaked at 100 rpm for 1 hour at room temperature. The crude enzyme was obtained by separating the solid (left over OPEFB and fungal) from the solution by centrifugation. The supernatant was then analyzed for enzymatic activity following the DNS method using larchwood xylan (Sigma Co., USA) as substrate.

c) Enzymatic hydrolysis of OPEFB

The enzymatic hydrolysis of OPEFB was performed in a 300 mL shake flask containing100 mL working volume with 3 % of OPEFB or 5.88 g/L of xylan (sterile 3 g OPEFB in 25 mL acetate buffer 0.05M pH, and 75 mL crude fungal xylanase) for 4 h in incubator shaker that was set at 150 rpm. The xylanase activity (U/mL) of the crude enzyme used in the experiments was measured to be750 U/mL, giving the total enzyme activity for each batch of experiment 75000 U/100 mL. In order to assess the effects of temperature and pH on the enzymatic hydrolysis process, the experiments were designed following the Central Composite Design (CCD) with two factors, five levels, 2 replicates at factorial point, 2 replicates at star point and 5 replicates at the center point. The temperature was varied in the range of 30-50 °C, whereas the pH was varied between 4-6. The center point was set up at 40 °C and pH 5. Table-1 shows the factors and variation of conducted experiments.

Table-1. Design experiment in CCD.

	(-α)	(-)	(0)	(+)	(+α)
pH	3,5	4	5	6	6,5
T (°C)	26	30	40	50	55

The hydrolysate was obtained by separating the solid containing OPEFB left over from the solution by vacuum filtration and centrifugation. The hydrolysate was then analyzed for its sugars composition including xylose and glucose.

d) Analysis

The xylanase activity was determined using Oat spelts xylan (Sigma Co., USA) as the substrate according to [11]. The produced reducing sugars produced were quantified by the dinitrosalicylic acid method using D-xylose as standard [11].

Xylose and other sugar concentration produced by enzymatic hydrolysis was measured by HPLC and calculate the hydrolysis yield using HPX-87H Biorad column and Refractive Index Detector (RID) at the following conditions: mobile phase: 0.005 M sulfuric acid, flow rate: 0.6 mL/minute, column temperature:60 °Cand detector temperature: 40 °C.

3. RESULT AND DISCUSSIONS

a) Optimization of enzymatic hydrolysis conditions

In order to produce the hydrolysate for xylitol fermentation, both xylose and glucose concentration in the xylitol fermentation medium is a critical factor that regulates the xylitol production by yeasts. Xylose is used as the main substrate for xylitol production. Xylose is reduced to xylitol by the xylose reductase using NADPH as the coenzyme. Part of the xylitol produced is converted to xylulose through the action of NAD^+ dependent xylitol dehydrogenase, to be metabolized further in the central carbon metabolism to generate cell mass and energy for maintenance, whereas the rest is excreted out of the cells. Glucose is used for generate energy and biomass directly from glucose via glucose-6-phosphate (Glu-6P) throught hexose monophosphate (HMP) pathway. On the other hand, the presence of glucose may repress the activity of the xylose reductase that is involved in the xylose conversion into xylitol resulting in low yields of the product [12]. Therefore, the OPEFB hydrolysis process needs to be conditioned as such that produced xylose is maximum and there is just enough glucose for support yeast growth but not inhibiting the xylitol production process. The objective of our research is to maximize xylose concentration and in the same time minimize the glucose concentration produced in the enzymatic hydrolysis process. The optimization process in this research was observed using three responses. They were xylose concentration, ratio of xylose over glucose concentration and both, or the sum of xylose concentration and the ratio response. Table-2 shows factors and results of the experiments.

The significance of each variable in the CCD experiment the working parameters were calculated. The generated response surface graphs, the anova analysis for each objective function are shown in Table-3. The accuracy and general ability of the quadratic model could be evaluated by the determination coefficient (R^2).The obtained experimental data was proceesed using multiple regression analysis and the data were fit to quadratic model. The fitted equations for enzymatic hydrolysis for each responses were presented succeedingly in Equations 1-3. Y_1 is xylose concentration, Y_2 is ratio xylose to glucose and Y_3 is the sum of xylose concentration and ratio of xylose to glucose concentration. The Student's *t*-distribution and the corresponding *P*-value, along with the parameters was shown in Table-3. The *P*-values are used as a tool to check the significance of each coefficient, which will help to explain the pattern of mutual interactions between the best variables. The parameter coefficient and the corresponding *P*-value suggested that, pH do has significant effect on all three responses (*P*-values<0.05). Whereas only for xylose concentration response analysis, temperature significantly influenced these response. The sample 3D response surfaces plots were employed to illustrate the interaction of temperature and pH and their effects onenzymatic hydrolysis result (Figure-1).The estimated optimum condition for enzymatic hydrolysis for each objective function were presented in Figure-1.

For the first response, that is xylose concentration, the maximum xylose concentration was obtained at pH 4.7 and temperature 41.8 ºC, giving xylose concentration of 2.1 g/L, which corresponded to hydrolysis yield of 31%. Theoretical xylose yield of xylanase hydrolysisof hemicellulose was reported to be 88% [13]. This might be caused by the hydrolysis time that was set to be 4 h, such that during the time course of hydrolysis experiment the obtained hydrolysis yield was lower than the literature. It is important to note that the purpose of this resarch was not to maximize the hydrolysis yield, but to determine the optimum hydrolysis process conditions. On the other hand, the lowest hydrolysis yield was obtained at pH 6 and temperature 30 ºC. In this case only 0.3 g/L xylose was released from 30 g/L substrate of OPEFB and the alkaline and the low temperature condition of the hydrolysis that was not enough to break the cell wall fiber of the substrate.

Table-2 also showed that increasing of xylose released, sometimes followed by increasing of glucose concentration. For the second response, that is ratio of xylose to glucose concentration, the maximum ratio was obtained at pH 4.4 and temperature 41.5 ºC, giving the ratio of xylose to glucose concentration of 3.19, which corresponded to hydrolysis yield of 32%. The obtained yield is, however, higher than the maximum yield obtained in the first response (xylose concentration).

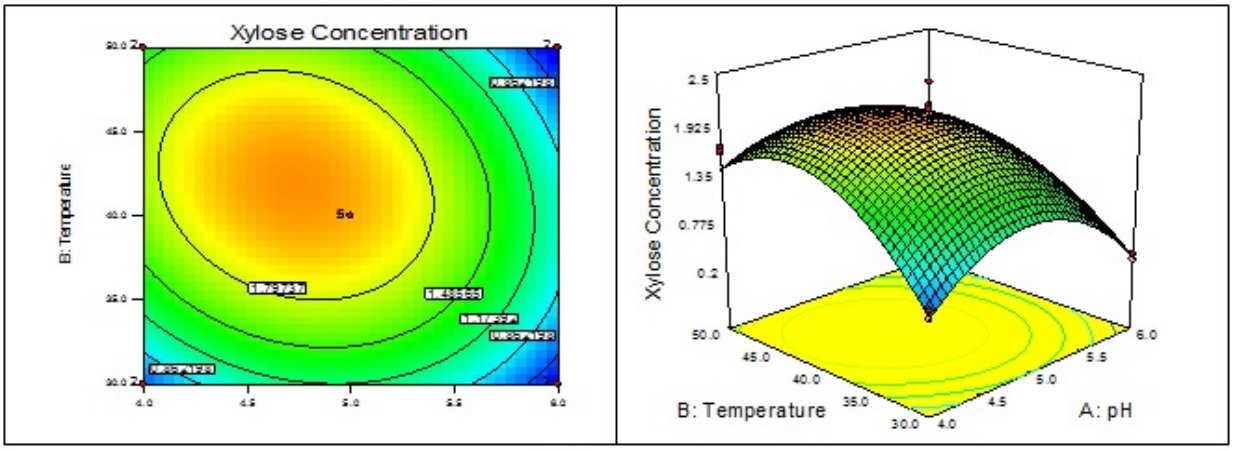
Table-2 also showed that for the third response, that is the sum of xylose concentration and ratio of xylose to glucose concentration, the maximum response was obtained at pH 4.6 and 41.7 ºC giving xylose concentration of 2.4 g/L and the ratio of xylose to glucose concentration of 3.16, which corresponded to hydrolysis yield of 35%.

If we compared, the optimization with multi objective functions , that were the xylose concentration and the ratio ofxylose-glucosegiving the maximum hydrolysis yield.

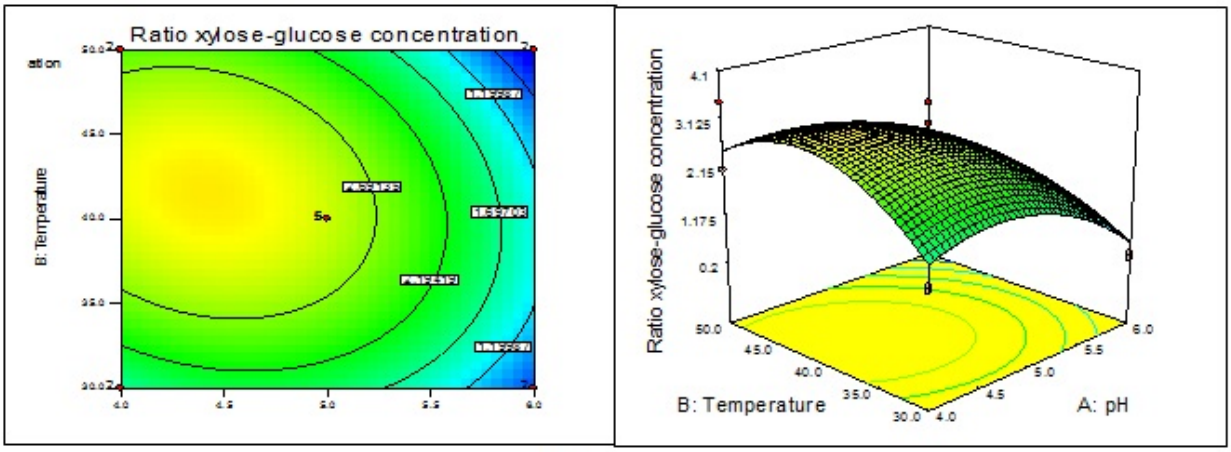
If we compared the obtained results with the hydrolysis of OPEFB using commercial xylanase (Accelerase XY produced by Genecor) (Mardawati *et al*., [2]), the optimized hydrolysis condition using commercial enzyme was obtained at similar pH, but higher temperature, 60 ºC. This showed that commercial enzyme has higher tolerance to high temperature.

Table-2. Factors and result of experiment in CCD design.

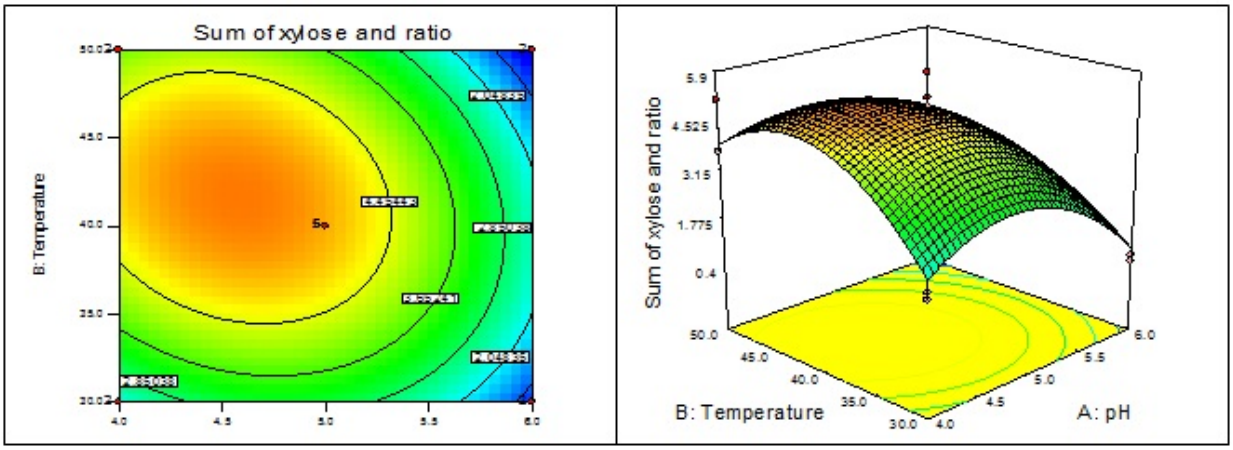
Run	Factor A	Factor B	Response 1	Response 2	Response 3	Hydrolysis yield (%)
	pH	Temperature (°C)	Xylose concentration (g/L)	Ratio xylose-glucose	Sum of xylose and ratio	
1	5	26	0.478	1.709	2.187	7.125
2	5	40	2.044	2.654	4.697	30.485
3	6.2	40	0.460	0.556	1.016	6.862
4	5	40	2.140	3.061	5.201	31.914
5	4	30	0.533	1.184	1.717	7.957
6	5	40	2.404	3.470	5.874	35.850
7	5	40	1.530	2.592	4.122	22.817
8	6	30	0.303	0.290	0.593	4.514
9	5	40	2.088	2.863	4.950	31.138
10	5	55	0.535	0.458	0.994	7.984
11	4	50	1.592	2.127	3.720	23.753
12	6	50	0.468	0.665	1.133	6.983
13	3.5	40	1.217	4.051	5.269	18.155
14	5	26	0.439	1.322	1.760	6.541
15	6.2	40	0.505	0.764	1.269	7.530
16	4	30	0.629	1.271	1.900	9.377
17	6	30	0.369	0.399	0.769	5.511
18	5	55	0.565	0.563	1.129	8.430
19	4	50	1.651	3.477	5.128	24.623
20	6	50	0.390	0.696	1.085	5.810
21	3.5	40	0.373	1.147	1.521	5.569



(a)



(b)



(c)

Figure-1. 3D graph and countour of the effect temperature and pH on the enzymatic hydrolysis of OPEFB for each reponses.

a. Xylose concentration,
b.Ratio xylose to glucose concentration ,
c. Sum of xylose concentration and ratio of xylose to glucose concentration).

The obtained optimum condition of hydrolysis using crude enzyme extract is near with the optimum hydrolysis conditions of corn cob as reported by [14]. The result showed that 45 ºC was the optimal temperature for all commercial enzyme tested and the range pH optimal is 4-5, resulted the xylose concentration up to 24 g/L or 80% of hydrolysis yield.Reference [15] reported that the enzymatic hydrolysis from sugarcane bagasse by xylanase of *Thermoascus aurantiacus* conducted at optimal condition at 50 ºC and pH 5, the maximum conversion to xylooligosaccharides (37.1%) was obtained with 2.6% of substrate and xylanase load of 60 U/g, xylose released 5.8 g/L. The result was reach higher than this research because the enzyme used has the higher activity and hydrolysis time was longer, it was 96h.

$$Y1 = -30.95 + 7.87\,pH + 0.69\,Temp - 0.02\,pH * Temp - 0.73\,pH^2 - 0.007\,Temp^2 \quad (1)$$

$$Y2 = -33.09 + 7.92\,pH + 0.91\,Temp - 0.003\,pH * Temp - 0.75\,pH^2 - 0.009\,Temp^2 \quad (2)$$

$$Y3 = -64.04 + 15.79\,pH + 1.59\,Temp - 0.005\,pH * Temp - 1.48\,pH^2 - 0.016\,Temp^2 \quad (3)$$

Table-3. Varians analysis of the experiment in CCD design with kuadratic model for response.

Source	p-value Prob > F for response			
	1	2	3	
Model	< 0.0001	0.0010	< 0.0001	Significant
A-pH	0.0002	0.0005	0.0003	
B-Temperature	0.0134	0.5791	0.235	
AB	0.0263	0.2513	0.125	
A^2	< 0.0001	0.0070	0.0003	
B^2	< 0.0001	0.0007	< 0.0001	
Residual				
Lack of Fit	0.213	0.233	0,256	not significant
Pure Error	< 0.0001	0.0010		
Cor Total	0.0002	0.0005		
R^2	89%	72%	80%	

b) Kinetic study of enzymatic hydrolysis

The data for kinetic study of the hydrolysis process was obtained from hydrolysis experiment at the optimum hydrolysis temperature and pH. The observation of performed for 4h, during which samples were taken periodically. Overall result is presented in Figure-2.

Figure-2 shows that maximum xylose concentration was achieved at the end of observation, 150 min on 9.8 g/L xylan in OPEFB substrate. At this condition, the maximum xylose concentration was measured to be 2.85 g/L. Figure-2 also show that at the beginning of hydrolysis process (0-20min), there is lower xylose produced. At the time of 0-20 minutes, there are barriers to transfer of substrate, so that the data is not used in the estimation of parameters.

The effect of OPEFB concentration on hydrolysis rate was shown in Figure-3. The rate of enzymatic hydrolysis was calculated from xylose concentration at 20-90 min, within which the initial rate was approximately constant. After this period, the rate of hydrolysis was observed to slow.

Figure-3 shows that the initial rate of enzymatic hydrolysis increased with the increase ininitial substrate concentrations which represented xylan concentration as converted by OPEFB concentration. However, at high initial substrate concentration a saturation trend was observed. This phenomena is consistent with the Michellis Menten kinetics model, which mathematically described as follows [16]:

$$V = Vm[S]/(Km + [S]) \quad (4)$$

V is Enzymatic reaction rate (g/L/min)
V_m is Maximum rate of enzymatic reaction (g/L/min)
K_m is Michaelis Menten constant (g/L)
S is Substrate concentration (g/L)

Followingly, the kinetics parameters of OPEFB hydrolysis with crude xylanase were estimated via the linearisation of Michellis Menten model, following Lineweaver-Burk plot method as is shown in Figure-4.

According to Figure-4, the apparent V_{max} and K_m values for the enzymatic hydrolysis were calculated to be K_m = 6.986 g/L and V_m = 0.045 g xylan/L/min with R^2=98%. Overall, the obtained results showed that although the enzymatic hydrolysis process not only involving the liquid-liquid medium, it still can be approached using Michellis Menten kinetics model, on the condition of regime transport limitation is eliminated first. This shows that the process could be well modelled following model after 20 min hydrolysis time.

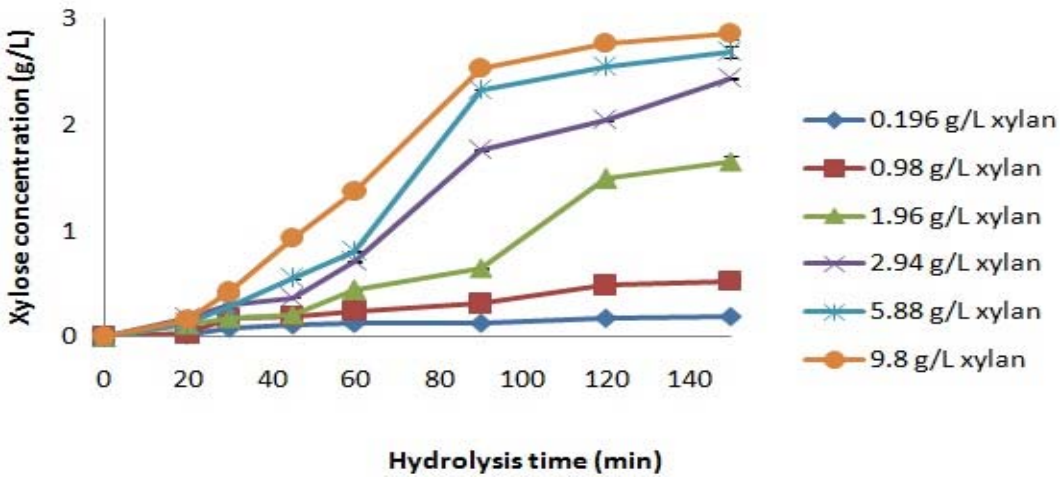


Figure-2. The dynamic profile of xylose concentration during the enzymatic hydrolysis of OPEFB at various initial xylan concentration (g/L).

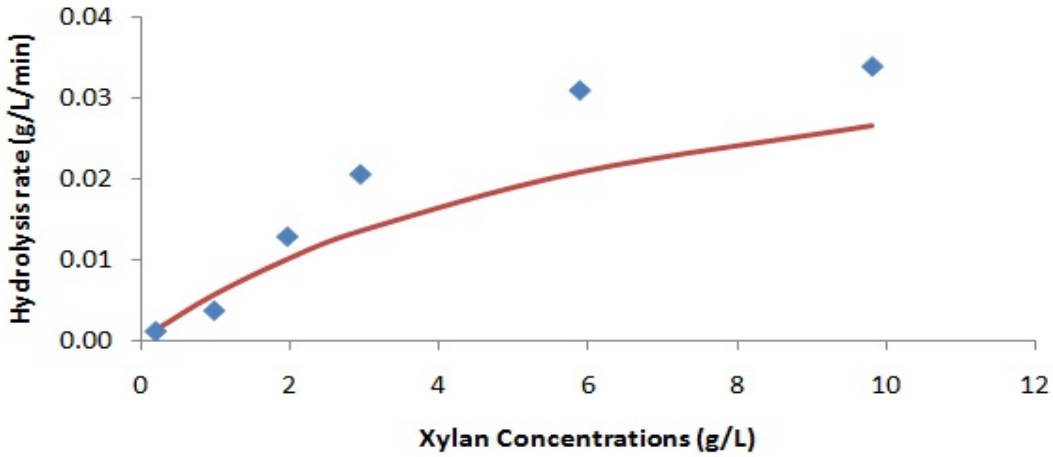


Figure-3. Effect of xylan concentrations on the intial enzymatic hydrolysis rate.

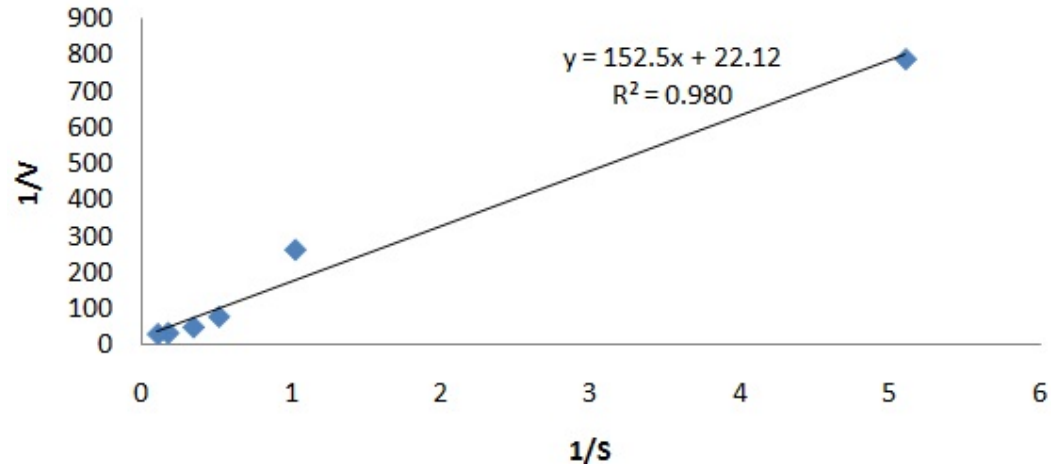


Figure-4. Lineweaver-Burke plot correlation between substrate (1/S) and reaction rate (1/V) in the enzymatic hydrolysis OPEFB.

4. CONCLUSIONS

Temperature and pH significantly influenced xylose concentration on the hydrolysis process of OPEFB. The optimization of enzymatic hydrolysis of OPEFB was observed using three methods, single objective optimization using xylose concentration or ratio xylose-glucose concentration as the response, and multi objective optimization using sum of xylose and ratio as the response. The results of optimization of the three methods are similar in temperature, whereas for pH sufficiently different. To obtain the optimum condition to reach the high of xylose and the lower of glucose concentration in hydrolysate, it was obtained at condition 41.6 ºC, pH 4.6 with sum of xylose and ratio concentration as response in multi objective optimization method. Since it involves solid-liquid phase, at the start of the reactionupto20minutes, there are barriers transport. Thus modeling the kinetics of enzymatichydrolys is reactions could be well approached by the Michaelis Menten kinetic modelby eliminating the transport limitations first. The parameter are K_m = 6.986 g/L and V_m = 0.045 g/L/min with R^2 = 98%.

ACKNOWLEDGEMENTS

This research was funded by Directorate of Higher Education, Indonesian Ministry of National Education (DIKTI) under the scheme of Desentralisation Research 2015 “The Advance Development of Production ‘Green’ Xylitol: Integrated Convertion of Biomass Waste of Palm Oil into Bioethanol and Valuable Chemicals”.

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