World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:10, No:7, 2016

Screening of Factors Affecting the Enzymatic Hydrolysis of Empty Fruit Bunches in Aqueous Ionic Liquid and Locally Produced Cellulase System

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***Abstract*—**The enzymatic hydrolysis of lignocellulosic biomass is one of the obstacles in the process of sugar production, due to the presence of lignin that protects the cellulose molecules against cellulases. Although the pretreatment of lignocellulose in ionic liquid (IL) system has been receiving a lot of interest; however, it requires IL removal with an anti-solvent in order to proceed with the enzymatic hydrolysis. At this point, introducing a compatible cellulase enzyme seems more efficient in this process. A cellulase enzyme that was produced by *Trichoderma reesei* on palm kernel cake (PKC) exhibited a promising stability in several ILs. The enzyme called PKC-Cel was tested for its optimum pH and temperature as well as its molecular weight. One among evaluated ILs, 1,3-diethylimidazolium dimethyl phosphate [DEMIM] DMP was applied in this study. Evaluation of six factors was executed in Stat-Ease Design Expert V.9, definitive screening design, which are IL/ buffer ratio, temperature, hydrolysis retention time, biomass loading, cellulase loading and empty fruit bunches (EFB) particle size. According to the obtained data, IL-enzyme system shows the highest sugar concentration at 70 °C, 27 hours, 10% IL-buffer, 35% biomass loading, 60 Units/g cellulase and 200 µm particle size. As concluded from the obtained data, not only the PKC-Cel was stable in the presence of the IL, also it was actually stable at a higher temperature than its optimum one. The reducing sugar obtained was 53.468±4.58 g/L which was equivalent to 0.3055 g reducing sugar/g EFB. This approach opens an insight for more studies in order to understand the actual effect of ILs on cellulases and their interactions in the aqueous system. It could also benefit in an efficient production of bioethanol from lignocellulosic biomass.

***Keywords*—**Cellulase, hydrolysis, lignocellulose, pretreatment, stability.

I. Introduction

RECENTLY, ILs are gaining so much interests as highly promising solvent for lignocellulosic biomass pretreatment, due to their dissolution power for biopolymers [1].

As well known, the well-organized structure of the lignocellulose that consists of lignin and hemicellulose surrounding the cellulose limits the accessibility of cellulase to the cellulose units. And for that reason, enzymatic hydrolysis consumes longer time to be completed [2]. As reported, 1-ethyl-3-methylimidazolium dimethylphosphate showed the advantages of both good lignin-extraction capacity and hypotoxicity [3]. [DEMIM] DMP was used as well to dissolve cellulose followed by enzymatic hydrolysis in 10% IL [2]. However, the cost of enzymes is one of the obstacles that limit some of their applications. Thus, introducing microbial cellulase that is being secreted on abundant agricultural residues is one of the solutions that can be provided for efficiency and waste management concept.

In this study, the IL-pretreatment and enzymatic hydrolysis are conducted in a one-pot process. However, in order to apply this approach, it is required to find an enzyme-friendly IL that dissolve lignocellulose at the same time [4], [3]. The concept of the "in situ hydrolysis" provides the advantage of avoiding the extensive washing step with water, in which cellulase is introduced to the system in one vessel.

Definitive screening designs (DSD) are useful for active two-factor interactions, and are small designs, yet, conclusively identify which of several factors affect the response. Moreover, they help to identify the causes of nonlinear effects by fielding each continuous factor at three levels. DSD avoids the need for costly additional experimentation to resolve ambiguity from the initial results of standard screening designs.

In this study, 1, 3-diethylimidazolium dimethyl phosphate was investigated first in EFB dissolution capacity followed by enzymatic hydrolysis with a locally produced cellulase. The aim was to optimize several factors using DSD. The cellulase was produced locally throughout solid-state fermentation on PKC using *Trichoderma reesei.* Our enzyme showed promising results in terms of stability and hydrolysis capability.

II. Methods

A. Materials

PKC and EFB were collected from Sime Darby's West Mill Plantation in Carey Island, Malaysia. Samples were dried at 60 °C until constant weight obtained, then grinded to 1.0-2.0 mm and kept in air-tight containers. *Trichoderma reesei* (RUTC30) was purchased from American Type Culture

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Collection ATCC. Chemicals were obtained from Fisher Scientific, MERCK and Sigma-Aldrich, Malaysia.

B. Production of PKC-Based Cellulase

Cellulase was produced locally from the utilization of PKC as the basal medium. The fermentation was carried in solid-state fermentation (SSF). Crude enzyme was extracted with sodium citrate buffer (pH 4.8±0.2) followed by centrifugation, micro-and ultra-filtration. The retentate containing the enzyme was collected and tested for enzyme activity. The cellulase activity was determined using the method reported by [5].

C. PKC-Cel Stability in [DEMIM] DMP

The PKC-Cel was incubated in different concentrations of the IL, A. [DEMIM]DMP for certain periods of time, then, the enzyme activity was determined using carboxymethyl cellulose (CMC) [6]. The initial reading of PKC-Cel was taken as the control (100%) and subsequent readings were calculated as residual activities.

D. Screening of Factors Affecting the IL-PKC-Cel System in One-Step Hydrolysis

DSD was used at three levels on six factors: Reaction temperature, reaction time, enzyme loading, biomass loading, particle size and IL/buffer ratio as presented in Table II. Each experiment was conducted in triplicates and the average was taken.

The grinded EFB was sieved to three sizes of particles (200, 450 and 600 µm) as suggested by the design. EFB pretreatment was executed using various biomass loading in terms of percentage of volatile solids (%VS). To start the pretreatment, the required weight of the grinded EFB was taken into a micro-tube (2 ml) and 1.5 ml of the IL were added followed by incubation at 100 °C for 30 min. following that, the appropriate dilution (10% and 55%) was made with citrate buffer by transferring the content to 15 ml tubes. Using a thermo-mixer, the temperature was set according to the design and the agitation speed was fixed to 400 rpm for the specified time intervals. Upon incubation, the content was centrifuged at 8,000 rpm for 10 min. The supernatant was tested for total sugars and glucose concentrations using DNS method [5]. Stat-Ease Design-Expert 9.0.6 was used to analyse, evaluate and produce the graphs of the experimental design results.

III. Results and Discussion

A. Production of PKC-Based Cellulase

The enzyme activity was determined using both CMC assay and filter paper assay and resulted in 24.14±1.82 (filter paper unit per milliliter) FPU/ml (123.33±9.12 Unit (U)/ gram dry substrate (gds)) and 157.872±1.56 CMC unit/ml (789.386±7.8 U/gds) at the 7th day of the fermentation.

B. PKC-Cel Stability in [DEMIM] DMP

As noticed from Fig. 1, PKC-Cel retained its activity for the 6 hours in 10% IL and slightly had an activation effect. In 20 and 40%, PKC-Cel maintained at around 92%. Starting from higher concentrations, the enzyme activity started to drop; however, it maintained 79.48% in 60% IL and 49.26 at 80%. In pure IL, only 8.16% of the activity was maintained after 6 hours.

ILs having hydrophobic nature, less viscosity, kosmotropic anion and chaotropic cation usually stimulate the activity and stability of enzymes. However, the correlation could not be generalized because of many contradictory results [7]. [DMIM]DMP and [EMIM]Ac showed that ILs concentration higher than 40% resulted in cellulase deactivation. At 90% (v/v) of [DEMIM]DMP, endoglucanase retained roughly 50% of its activity [8].

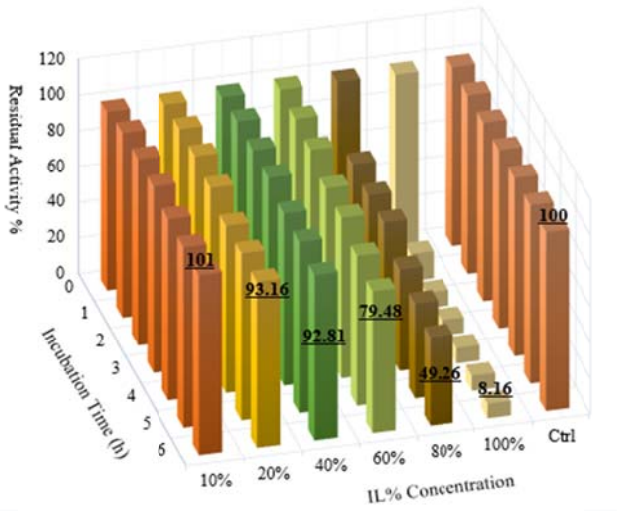

Fig. 1 Activity of the locally produced cellulase (PKC-Cel) from *Trichoderma reesei* in different concentrations of 1,3-diethylimidazolium dimethyl phosphate [DEMIM]DMP

C. Screening of Factors Affecting the IL-PKC-Cel System in One-Step Hydrolysis

As seen from Table II, the maximum sugar, 53.47± 4.59 g/L was obtained at the following parameters: 70 °C, 27 h hydrolysis, 60 units/g cellulase concentration, 200 µm particle size, 35% EFB in terms of (VS) of the EFB and 10% (IL/buffer ratio). The sugar produced is equivalent to 0.3055 g sugar/g EFB in total, where the minimum sugar concentration was recorded at the following parameters: 30 °C, 27 h hydrolysis, 10 units/g cellulase concentration, 600 µm particle size, 5% EFB in terms of (VS) of the EFB and pure IL (100%).

A second order equation which described the relation between the investigated factors and the reducing sugar released was obtained from the software by analyzing the experimental results. The final equation (1) in terms of coded factors:

$$In(Reducing\ sugars) = +1.24 + 0.12\,A - 0.093\,B - 1.17\,C + 0.59\,D + 0.93\,E - 0.54F - 0.45A^2 + 1.02\,B^2 + 0.27C^2 - 0.15\,E^2 - 0.31\,F^2 \quad (1)$$

where A is the temperature, B is the retention or (hydrolysis) time, C is the IL concentration, D is the biomass loading, E is cellulase loading and F is the particle size. The equation in

terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

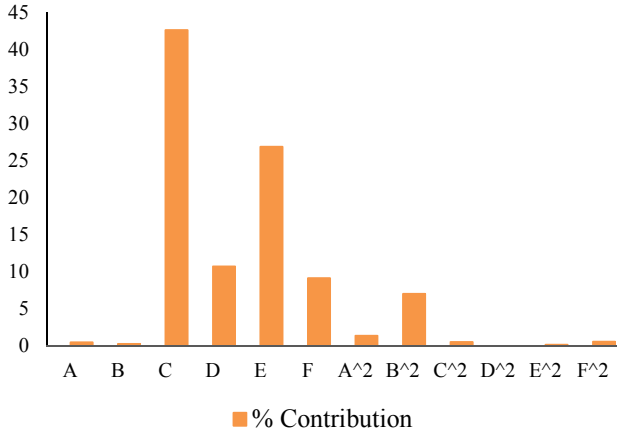

Fig. 2 Percentage of factors contribution to the model for production of reducing sugars from EFB in the IL-enzyme system

TABLE I
ANALYSIS OF VARIANCE FOR DSD FOR REDUCING SUGAR PRODUCTION FROM EFB BY APPLYING IN-SITU ENZYMATIC HYDROLYSIS

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	0.53	1	0.53			
Model	95.51	11	8.68	1142.19	< 0.0001	
A-Temperature	0.45	1	0.45	59.52	< 0.0001	
B-Retention Time	0.26	1	0.26	34.00	< 0.0001	
C-IL/Buffer	41.22	1	41.22	5422.72	< 0.0001	
D-Biomass Loading	10.36	1	10.36	1362.91	< 0.0001	
E-Cellulase loading	25.99	1	25.99	3418.41	< 0.0001	***significant***
F-Particle size	8.84	1	8.84	1162.99	< 0.0001	
A^2	1.33	1	1.33	174.36	< 0.0001	
B^2	6.78	1	6.78	891.29	< 0.0001	
C^2	0.49	1	0.49	64.88	< 0.0001	
E^2	0.14	1	0.14	18.87	0.0002	
F^2	0.55	1	0.55	72.24	< 0.0001	
Residual	0.22	29	7.602E-003			
Lack of Fit	5.459E-006	1	5.459E-006	6.933E-004	0.9792	***not significant***
Pure Error	0.22	28	7.873E-003			
Cor Total	96.27	41				

As revealed by the results, the contribution of factors is shown in Fig. 2 whereas IL concentration ratio was the most contributing factor. At positive level, factors that influenced the process can be ranked as followed: cellulases loading> biomass loading> temperature. On the other hand, at the negative levels, the most contributing factors were IL concentration> particle size> hydrolysis time. It was also observed that there was no interaction between the model terms.

D.Analysis of Variance (ANOVA) Analysis

The Model F-value of 1142.19 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, D, E, F, A^2-, B^2, C^2, E^2, F^2 are significant model terms.

Values greater than 0.1000 indicate the model terms are not significant.

The ""Lack of Fit F-value"" of 6.933E-004 implies the Lack of Fit is not significant relative to the pure error. There is a 97.92% chance that a ""Lack of Fit F-value"" this large could occur due" to noise. There was a reasonable agreement between the R(s) values. The Predicted R-Squared of 0.9951 is in reasonable agreement with the Adjusted R-Squared of 0.9968 with less than 0.2. The high value of R^2 (0.9977) implies a high degree of correlation between the experimental and the predicted values by the model. Adequate Precision value of 138.381 (greater than 4) indicates an adequate signal. Complete ANOVA analysis is presented in Table I.

As we observed from the experimental design, the integrated system performed better at elevated temperatures i.e., 70°C.

As has been reported, the highest yields of the reducing sugars were obtained at 70–90°C. With cholinium amino acids ILs, it appears that the range is enough to remove lignin. At lower temperature (50°C), less effective delignification led to low yields of sugars due to the low digestibility [9].

In this study, we have not performed any pretreatment prior to the hydrolysis step. Instead, we have investigated the performance of the IL-enzyme system in few variables. It is obvious that low temperature in this case gave lower sugar concentration. Moreover, low biomass loading at 30°C and smaller particle size (450µm) with the maximum cellulase loading but for longer time (48 h) gave approximately half the concentration obtained at 70°C at 27h and (200µm).

Reference [10] showed that wheat straw (<0.5 cm) was dissolved in 1-ethyl-3-methyl imidazolium diethyl phosphate to yield 54.8% of reducing sugars (RS) after 1 h treatment and 12 h hydrolysis. Although that the hydrolysis time showed to be the least contributing factor, however, it is seen from Table II that 6 hours were not sufficient to complete the enzymatic hydrolysis. As for the biomass, a higher biomass concentration under agitation allows more frequent contact and collision between the biomass particles and promote the cellulose dissolution from the efficiency [11]. An efficient conversion of pretreated cotton stalks to glucose at elevated substrate loadings as high as 15% (w/w) was linked to the disrupted crystalline structure of the biomass. It was reported that the disruption of the crystalline structure is more significant than biomass delignification [12] regardless of the applied substrate loading [13]. The enzymatic hydrolysis of bagasse was demonstrated i n presence of [EMIM] DEP in which the sugar yield reduced with IL concentration above 10%

because cellulase was partially inactivated. However, applying an integrated IL- system with enzyme reduced the temperature of treatment which reduces the energy consumption as well as enzyme inactivation [14].

TABLE II
DSD FOR SCREENING OF SEVEN FACTORS AT THREE LEVELS FOR REDUCING SUGAR PRODUCTION AFTER PRETREATMENT OF EFB

Run	A:Temperature °C	B:Retention Time hours	C:IL/Buffer %	D:Biomass Loading %	E:Cellulase loading Unit/g biomass	F:Particle size Micrometer (μm)	Released sugar g/L
1	70	6	100	35	10	450	1.65±0.28
2	50	6	10	5	10	200	10.77±0.96
3	70	48	100	5	35	200	1.82±0.06
4	30	6	10	35	35	600	19.56±1.81
5	30	6	100	20	60	200	6.76±0.85
6	70	48	10	20	10	600	3.91±0.03
7	30	27	100	5	10	600	0.07±0.01
8	70	27	10	35	60	200	53.47±4.59
9	30	48	10	5	60	450	22.01±0.96
10	50	27	55	20	35	450	3.35±39
11	70	6	55	5	60	600	3.97±0.15
12	30	48	55	35	10	200	3.84±0.14
13	50	48	100	35	60	600	6.04±0.51
14	50	27	55	20	35	450	2.94±0.26

The use of IL-water mixture can reduce the cost as well as the viscosity of the ILs, thus, reduces the impact of the ILs on the cellulases [15].

E. Optimization Solutions

The software provided a number of optimization solutions by setting the response (released sugar) at its maximum.

Temperature ranges between 50-70 °C whereas higher temperature requires less hydrolysis time. For temperatures lower than 55 °C, 48 hours will be required to achieve the maximum sugar concentration while temperature above 65° is predicted to consume 23 to 27 hours. Biomass loading ranges between 20 and 35%. Particle size was set at 200-250 μm, cellulase loading at 60 Units/g, and IL concentration at 10-15%.

IV. CONCLUSIONS

The PKC-Cel exhibited promising stability in the IL, [DEMIM] DMP as more than 90% activity was observed in 10 and 20% of the IL after 6 hours. Based on our findings, the optimum conditions to obtain 53.468±4.58 g/L g/L of sugar were identified. This study offered the utilization of two different abundant raw materials for both enzyme and sugar production towards an efficient approach for bioethanol production. More research is required in order to determine the best integration system for an efficient biomass conversion into biofuels.

ACKNOWLEDGMENT

This project is funded by FRGS-13-088-0329. We are grateful to the Department of Biotechnology Engineering and International Institute for Halal Research and Training (INHART) in IIUM and Centre of Research in ILs (CORIL) in University Technology Petronas for providing the lab facilities. Our gratitude is also extended to the West Oil Mill, Sime Darby Plantation for providing the experimental biomass samples for this project.

REFERENCES

[1] X.-D. Hou, N. Li and M.-H. Zong, "Significantly enhancing enzymatic hydrolysis of rice straw after pretreatment using renewable ionic liquid–water mixtures", *Bioresour. Technol.*, vol.136, no.0, pp. 469-474, Mar 2013.

[2] P. Engel, S. Krull, B. Seiferheld and A.C. Spiess, "Rational approach to optimize cellulase mixtures for hydrolysis of regenerated cellulose containing residual ionic liquid", *Bioresour. Technol.*, vol.115, no.0, pp.27-34, Jul 2012.

[3] J. Xu, B. Wu, L. Hu, Z. Wu, N. Xu, B. Dai and J. He, "Enzymatic in situ saccharification of lignocellulose in a compatible ionic liquid-cellulase system", *Chem. Eng. J.*, vol. 267, pp. 163-169, May 2015.

[4] H. Zhao, G.A. Baker, Z. Song, O. Olubajo, T. Crittle and D. Peters, "Designing enzyme-compatible ionic liquids that can dissolve carbohydrates", *Green Chem*., vol.10, no.6,pp. 696-705. Apr 2008.

[5] T. Ghose, "Measurement of cellulase activities", *Pure and Applied Chemistry*, vol. 59, no. 2, pp. 257-268, Jun 1987.

[6] Â.C. Salvador, M.d.C. Santos and J.A. Saraiva, "Effect of the ionic liquid [bmim] Cl and high pressure on the activity of cellulase", *Green Chem*., vol. 12, no. 4, pp. 632-635, Feb 2010.

[7] M. Naushad, Z.A. ALOthman, A.B. Khan and M. Ali, "Effect of ionic liquid on activity, stability, and structure of enzymes: a review", *Int. J. Biol. Macromol*, vol. 51, no.4, pp. 555-560, Nov 2012.

[8] R. Wahlström, S. Rovio and A. Suurnäkki, "Partial enzymatic hydrolysis of microcrystalline cellulose in ionic liquids by *Trichoderma reesei* endoglucanases", RSC Adv., vol. 2, no.10, pp. 4472-4480, Apr 2012.

[9] X.D. Hou, T.J. Smith, N. Li and M.H. Zong, "Novel renewable ionic liquids as highly effective solvents for pretreatment of rice straw biomass by selective removal of lignin", *Biotechnol. Bioeng*., vol. 109, no.10, pp. 2484-2493, Oct 2012.

[10] Q. Li, Y.-C. He, M. Xian, G. Jun, X. Xu, J.-M. Yang and L.-Z. Li, "Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment", *Bioresour. Technol*., vol. 100, no.14, pp. 3570-3575, Jul 2009.

[11] H.T. Tan, K.T. Lee and A.R. Mohamed, "Pretreatment of lignocellulosic palm biomass using a solvent-ionic liquid [BMIM]Cl for glucose recovery: An optimisation study using response surface methodology", *Carbohydr. Polym*., vol. 83, no.4, pp. 1862-1868, Feb 2011.

[12] N.I. Haykir, E. Bahcegul, N. Bicak and U. Bakir, "Pretreatment of cotton stalk with ionic liquids including 2-hydroxy ethyl ammonium formate to enhance biomass digestibility", *Ind. Crops Prod*., vol. 41, no.31, pp. 430-436, Jan 2013.

[13] N.I. Haykir and U. Bakir, "Ionic liquid pretreatment allows utilization of high substrate loadings in enzymatic hydrolysis of biomass to produce

ethanol from cotton stalks", *Ind. Crops Prod*., vol. 51, no.30, pp. 408-414, Nov 2013.

[14] S. Zhi, Y. Liu, X. Yu, X. Wang and X. Lu, "Enzymatic Hydrolysis of Cellulose after Pretreated by Ionic Liquids: Focus on One-pot Process", *Energy Procedia*, vol.14, pp. 1741-1747, Mar 2012.

[15] N. Kamiya, Y. Matsushita, M. Hanaki, K. Nakashima, M. Narita, M. Goto and H. Takahashi, "Enzymatic in situ saccharification of cellulose in aqueous-ionic liquid media", *Biotechnol. Lett*., vol. 30, no.6, pp. 1037-1040, Jun 2008.