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RESEARCH ARTICLE

Effect of cross-linked enzyme aggregates in hierarchically mesocellular mesoporous magnetic silica preparation conditions towards enzyme activity retention

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Abstract

This work aimed to optimize ten preparation factors that might influence the cellulase and xylanase activity retention of cross-linked enzyme aggregates in hierarchically mesocellular mesoporous magnetic silica. The factors were optimized using the fractional factorial design (2^{10-5}). The optimized output was occurred at 2 mL of enzyme amount, magnet-to-enzyme ratio of 1:0.15, enzyme adsorption at 26°C and 162 rpm in 40 min, enzyme-to-precipitant ratio of 1:11, 0.05% (v/v) of glutaraldehyde concentration, and cross-linking process at 37°C and 300 rpm in 2 h. The factors were examined to observe the effect of every factor towards cellulase and xylanase acrivity retention.

***Keywords*:** Fractional factorial design, optimization, magnetic silica, cellulase, xylanase

INTRODUCTION

Biocatalytic procedures are naturally well disposed, financially savvy and feasible. They have pulled in expanding consideration for over a century that keeps on growing (Jiang *et al.*, 2014). Nanobiocatalysis, fusing proteins into nanomaterials or incorporating the cross breed materials of catalysts and nanomaterials, is assembling increasing consideration due to the current accomplishments in balancing out the enzyme activities. Particularly, mesoporous materials have pulled in much more consideration due to their controlled porosity and high surface areas (Jun *et al.*, 2012). Affiliation of an enzyme with magnetic particles has an inalienable advantage of being effectively detachable (Gupta *et al.*, 2013). Recently, increasing attention is paid to magnetic mesoporous support as a result of the low enzyme activity retention on the ordinary magnetic beads (Sulaiman & Rahman, 2016). Different nanostructured materials, for examples magnetic nanoparticles, nanofibers, and mesoporous materials provide vast surface areas or high pore volume for enzyme immobilization that can prompt high catalyst loadings. Predominantly, mesoporous silica with a bottle-neck mesopore structure gives a novel chance to the catalyst adjustment through ship-in-a-bottle system (Kim *et al.*, 2013). Previous study proved that enzyme activity retention in magnetically separable hierarchically ordered mesocellular mesoporous silica could be improved by introducing precipitation step (Sulaiman & Rahman, 2016). In this study, the preparation conditions of cross-linked enzyme aggregates in hierarchically mesocellular mesoporous magnetic silica (CLEA-HMM-MS) have been optimized using the two-level fractional factorial design.The effect of preparation conditions towards enzyme activity retention would also be discussed. Cellulase and xylanase have been selected as the target enzymes for the immobilization due to their potential in a wide range of application.

Preparation of CLEA-HMM-MS involved various factors that affecting the cellulase and xylanase activity retention. The adsorptive conditions, type of precipitant, its concentration as well as the cross-linking agent give impact to phenylalanine ammonia lyase (Cui *et al.*, 2013). Time and temperature of glutaraldehyde are the most basic parameters in the cross-linking methods (Li *et al.*, 2012). This study was focused on optimization of ten different factors affecting the performance of enzymes; enzyme amount, HMM-MS-to-enzyme ratio, adsorption temperature, time and agitation, enzyme-to-precipitant ratio, cross-linker concentration, time, temperature and agitation.

The cellulase and xylanase activity retention should be high upon the preparation parameters optimization; thus, in this technique the design of experiment was applied to investigate the impact of the factors. The factors outcome, experimental design and mathematical equations or models are essentials in the design of experiment (DOE). This article was concentrated on the optimization, improvement and justification of a scientific strategy with DOE.

EXPERIMENTAL

Materials

The starting material, Pluronic® P-123 ($(EO)_{20}(PO)_{70}(EO)_{20}$, Sigma-Aldrich, average M_n ~5,800), acetic acid (CH_3COOH, AR, QRëC™), silica gel (SiO_2, FLUKA) and sodium hydroxide (NaOH, AR, QRëC™) were used to produce hierarchically mesocellular mesoporous silica (HMMS). Methanol (AR, RCI Labscan), iron (III) nitrate nonahydrate ($Fe(NO_3)_3.9H_2O$, AR, QRëC™) and propionic acid ($C_3H_6O_2$, Merck, 99%) were utilized for hierarchically

mesocellular mesoporous magnetic silica (HMM-MS) production. The enzymes used in this study were cellulase from Aspergillus sp. (Carezyme 1000L®, Sigma-Aldrich) and xylanase recombinant, expressed in Aspergillus oryzae (Pentopan Mono BG®, Sigma-Aldrich). Xylan from birch wood (Sigma-Aldrich) and filter paper Whatman No. 1 were used as substrate. Tert-butanol ($(CH_3)_3COH$, EMPLURA®, Merck) and glutaraldehyde ($C_5H_8O_2$, Fisher Scientific, 25%) were used in preparing cross-linked enzyme aggregates in HMM-MS (CLEA-HMM-MS).

Cellulase activity determination

For estimating cellulase activity, filter paper Whatman No. 1 was used as the substrate. One unit of enzyme activity could be described by the volume of enzyme needed to yield one µmol of reducing sugar per minute under assay conditions. Dinitrosalicyclic acid method was used to estimate the amount of reducing sugar (Ghose, 1987).

Xylanase activity determination

For estimating xylanase activity, xylan was used as the substrate (Ghose & Bisaria, 1987). One unit of enzyme activity could be described as the volume of enzyme needed to yield one µmol of reducing sugar per minute under assay conditions. Estimation on the amount of reducing sugar was done using the dinitrosalicyclic acid method (Ghose, 1987).

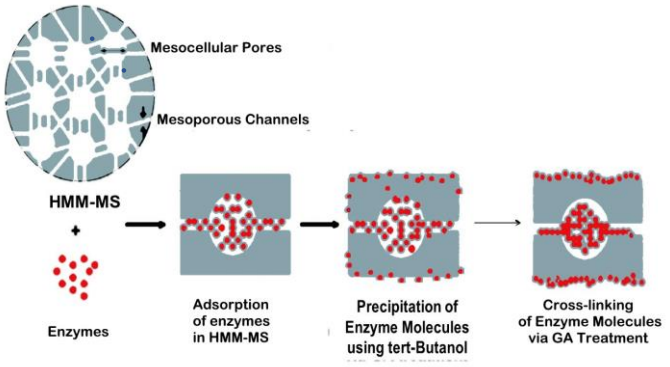

Fig. 1 Schematic diagram for preparation of CLEA-HMM-MS.

Synthesis of HMMS

A method from previous study was used to generate HMMS (Kim *et al.*, 2007). Regular preparation for HMMS was as the following: 200 mL of water was used to dissolve 9.7 g of P-123 and 4.48 mL acetic acid. The arrangement was heated to 60˚C and kept at that temperature for more than 1 h. 200 mL of water containing 16 mL sodium silicate (27% SiO_2, 14% NaOH) was dispensed into the readied arrangement and mixed well. The pH of the engineered arrangement was 6.3~6.4. The arrangement was further heated to 60˚C and matured at that temperature for 20 h, trailed by hydrothermal treatment at 100˚C for 24 h. Calcination of filtered materials at 550˚C produced HMMS.

Synthesis of HMM-MS

This synthesis step was contingent on past work (J. Lee *et al.*, 2009). Briefly, 0.5 g HMMS was doused with 3 mL methanol containing 1.34 g of $Fe(NO_3)_3.9H_2O$ and parched in a drying oven at 85˚C until methanol was completely dried. Propionic acid vapor was reacted with the $Fe(NO_3)_3$ soaked silica at 85˚C for more than 3 h under static vacuum to produce iron propionate complex. The composite was heated to 300˚C under air (1˚C/min) progressively to decay iron propionate compound and stayed at that temperature for 30 min. The subsequent magnetic nanoparticle absorbed HMMS was signified as HMM-MS.

Preparation of CLEA-HMM-MS

Preparation steps of CLEA-HMM-MS were shown in Fig. 1. HMM-MS was blended with equal amount of free cellulase and xylanase in a buffer solution (0.05 M sodium phosphate buffer, pH 4.8), vortexed for 30 s and brooded at designated temperature in a shaking condition. In following brooding for the adsorption of free enzymes into HMM-MS, tert-butanol was added as the precipitant. The resultant blend was mixed well to encourage the precipitation of the compound. At that point, the blend was incubated and agitated with glutaraldehyde (GA) solution. After the GA treatment, unreacted aldehyde groups of GA were further reacted with amino groups of Tris by incubating the specimens in 1 mL Tris-HCl buffer (100 mM Tris, pH 8.0) at 250 rpm for 30 min. After Tris-topping, the protein draining from HMM-MS into the supernatant was measured for the amount of enzyme activity retention, and was kept in storage at 4˚C.

Optimization of CLEA-HMM-MS preparation

Enzyme amount, HMM-MS-to-enzyme ratio, adsorption temperature, time and agitation, enzyme-to-precipitant ratio, cross-linker concentration, time, temperature and agitation played an important role in CLEA-HMM-MS preparation. Consequently, in the present study, these ten components were optimized using DOE. All of the parameters gave different effects on cellulase and xylanase activity retention. A large portion of the design investigations included only one variant at a time by keeping others as constant. With the assistance of the two-level fractional factorial design, investigators could study the impact of each elements that varied concurrently. The factorial design could consider the impacts created by autonomous factors and the associations between the individual factors (Kumar *et al.*, 2015). In the present work, ten independent factors were used, including enzyme amount (A), HMM-MS-to-enzyme ratio (B), adsorption temperature (C), adsorption time (D), adsorption agitation (E), enzyme-to-precipitant ratio (F), cross-linker concentration (G), cross-linking temperature (H), cross-linking time (J) and cross-linking agitation (K). The two level fractional factorial in resolution III with six center points and folded design were used in the study and were coded as -1, 0 and +1 for low, medium and high level, respectively. The total of 44 experimental runs were suggested by the software for analyzing the interaction of each level on formulation characters while the cellulase (R_1) and xylanase activity retention (R_2) were considered as response factors (dependent factors). Table 1 shows the factors chosen and different factor level settings.

RESULTS AND DISCUSSION

According to statistical analysis, two empirical equations were derived consisting coded unit of each parameters that involved in affecting cellulase and xylanase activity retention. The equations were as following:

Cellulase Activity =

+ 57.61	
+ 5.35	* A
+ 0.17	* B
+ 10.30	* C
+ 9.52	* D
- 11.97	* E
+ 8.81	* F
+ 13.88	* G
+ 12.26	* H
+ 6.57	* J
- 3.52	* K
- 1.50	* A * B
- 11.83	* A * C
- 3.38	* A * D
+ 1.86	* A * E
+ 0.45	* A * F
- 1.68	* A * G
+ 10.13	* A * H
- 5.16	* A * J
- 3.50	* A * K
- 1.40	* B * J
- 2.59	* B * K
- 4.46	* C * J
+ 5.43	* C * K
+ 9.19	* E * J
+ 0.82	* E * K

Table 1 2^{10-5} with 12 center points fractional factorial design for preparation study.

Run	Independent variables										Response	
	A	B	C	D	E	F	G	H	J	K	R_1	R_2
1	0	0	0	0	0	0	0	0	0	0	100	100
2	+1	+1	-1	-1	-1	+1	+1	-1	+1	+1	68.93	0
3	0	0	0	0	0	0	0	0	0	0	100	100
4	-1	+1	-1	-1	+1	+1	-1	+1	-1	-1	0	98.89
5	0	0	0	0	0	0	0	0	0	0	100	99.27
6	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	100	86.72
7	+1	+1	+1	-1	+1	-1	-1	-1	-1	+1	0	0
8	-1	-1	+1	+1	+1	-1	-1	+1	+1	+1	100	90.61
9	+1	-1	+1	-1	-1	+1	-1	+1	+1	-1	76.36	98.81
10	-1	+1	+1	-1	-1	-1	+1	+1	+1	-1	91.99	99.05
11	-1	-1	-1	+1	-1	+1	+1	+1	-1	+1	77.29	100
12	0	0	0	0	0	0	0	0	0	0	100	99.63
13	-1	-1	-1	-1	-1	-1	-1	-1	+1	+1	6.73	0
14	+1	-1	-1	+1	+1	+1	-1	-1	+1	-1	73.03	96.36
15	-1	-1	+1	-1	+1	+1	+1	-1	-1	+1	62.84	100
16	+1	+1	-1	+1	-1	-1	-1	+1	-1	+1	73.31	25.06
17	+1	-1	+1	+1	-1	-1	+1	-1	-1	-1	87	0
18	0	0	0	0	0	0	0	0	0	0	95.39	100
19	0	0	0	0	0	0	0	0	0	0	100	98.53
20	+1	-1	-1	-1	+1	-1	+1	+1	-1	-1	72.67	100
21	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	68.28	16.32
22	-1	+1	+1	+1	-1	+1	-1	-1	-1	-1	100	100
23	0	0	0	0	0	0	0	0	0	0	53.59	65.81
24	-1	+1	-1	+1	+1	-1	+1	-1	-1	+1	3.54	0
25	+1	+1	-1	-1	-1	+1	+1	-1	-1	-1	63.47	95.38
26	-1	-1	+1	+1	+1	-1	-1	+1	-1	-1	0	0
27	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0	6.21
28	0	0	0	0	0	0	0	0	0	0	53.59	65.81
29	-1	+1	+1	-1	-1	-1	+1	+1	-1	+1	80.63	44.39
30	+1	-1	+1	-1	-1	+1	-1	+1	-1	+1	96.03	95.83
31	-1	+1	-1	-1	+1	+1	-1	+1	+1	+1	0	0
32	-1	-1	+1	-1	+1	+1	+1	-1	+1	-1	72.72	99.26
33	0	0	0	0	0	0	0	0	0	0	53.59	65.81
34	0	0	0	0	0	0	0	0	0	0	53.59	65.81
35	0	0	0	0	0	0	0	0	0	0	53.59	65.81
36	-1	-1	-1	+1	-1	+1	+1	+1	+1	-1	85.23	95.45
37	+1	+1	-1	+1	-1	-1	-1	+1	+1	-1	87.38	74.96
38	+1	-1	-1	+1	+1	+1	-1	-1	-1	+1	0	100
39	+1	+1	+1	+1	+1	+1	+1	+1	-1	-1	100	93.1
40	-1	+1	+1	+1	-1	+1	-1	-1	+1	+1	86.94	97.96
41	+1	+1	+1	-1	+1	-1	-1	-1	+1	-1	0	0
42	+1	-1	+1	+1	-1	-1	+1	-1	+1	+1	32.13	8.69
43	+1	-1	+1	-1	+1	-1	+1	+1	+1	+1	77.15	69.39
44	0	0	0	0	0	0	0	0	0	0	53.59	65.81

Independent factors	Levels		
	-1	0	+1
Enzyme amount (mL)	1.50	1.75	2.00
HMM-MS: Enzyme ratio (1mg: mL)	0.150	0.325	0.500
Adsorption temperature (°C)	4.0	20.5	37.0
Adsorption time (min)	10	725	1440
Adsorption agitation (rpm)	125.0	162.5	200.0
Enzyme: Precipitant ratio (1 mL: mL)	1	7	13
Cross-linker concentration (%v/v)	0.0050	0.0275	0.0500
Cross-linking temperature (°C)	4	27	50
Cross-linking time (h)	2	13	24
Cross-linking agitation (rpm)	200	250	300

Xylanase Activity =

+ 59.14
- 0.12 * A
- 7.15 * B
+ 4.26 * C
+ 2.44 * D
+ 0.28 * E
+ 25.72 * F
+ 3.85 * G
+ 14.13 * H
- 0.79 * J
- 7.97 * K
- 4.97 * A * B
- 15.39 * A * C
- 0.85 * A * D
+ 8.90 * A * E
- 1.46 * A * F
- 6.20 * A * G
+ 7.34 * A * H
- 3.86 * A * J
- 2.83 * A * K
- 4.32 * B * J
- 12.25 * B * K
+ 10.03 * C * J
+ 2.44 * A * B * J
- 14.28 * A * B * K
- 6.26 * A * C * J
- 2.80 * A * C * K
- 0.91 * A * E * J
- 2.37 * A * E * K

(Equation 1)

+ 10.10 * C * K
- 1.29 * E * J
+ 4.40 * E * K
+ 2.49 * A * B * J
- 12.78 * A * B * K
- 4.71 * A * C * J
+ 0.63 * A * C * K
+ 0.87 * A * E * J
+ 2.24 * A * E * K (Equation 2)

These equations could be utilized to discover the desired values of the ten components to be set in order to achieve the desired enzyme activity retention. In a typical circumstance, an enzyme activity retention of 100% with the lowest amount of material should be focused on and related optimum values of the remaining factors should be found.

The accompanying data on the model showed that R^2 for both responses were 0.9996 and 1.000 (Table 2 and 3), respectively. These indicated that both models were significant and could be describe as 99.96% and 100% of the variable content affecting the responses. Higher R^2 indicates the validity of the model in predicting the optimum input and output. The model F-values of 818.82 and 11610.16 for cellulase and xylanase activity retentions were also implied the significance of the models. This suggested that this model could properly illustrate the effect of all parameters towards cellulase and xylanase activity retentions.

Table 2 The results of analysis of variance (ANOVA) on cellulase activity retention in CLEA-HMM-MS.

Factors	F Value	Prob > F	R^2
Model	818.82	< 0.0001	
A	517.6	< 0.0001	
B	0.49	0.4983	
C	1917.27	< 0.0001	
D	1637.26	< 0.0001	
E	2590.94	< 0.0001	
F	1403.52	< 0.0001	
G	3479.94	< 0.0001	
H	2717.4	< 0.0001	
J	778.83	< 0.0001	
K	223.76	< 0.0001	
AB	40.4	< 0.0001	
AC	2527.51	< 0.0001	
AD	206.31	< 0.0001	
AE	62.83	< 0.0001	
AF	3.62	0.0861	0.9996
AG	50.71	< 0.0001	
AH	1855.19	< 0.0001	
AJ	48.92	< 0.0001	
AK	221.78	< 0.0001	
BJ	35.65	0.0001	
BK	121.35	< 0.0001	
CJ	359.87	< 0.0001	
CK	531.84	< 0.0001	
EJ	1526.95	0.0058	
EK	12.18	< 0.0001	
ABJ	107.55	< 0.0001	
ABK	1005.31	< 0.0001	
ACJ	708.29	< 0.0001	
ACK	141.94	< 0.0001	
AEJ	14.97	0.0031	
AEK	101.25	< 0.0001	

The analysis as shown in Table 2 and 3 indicated that each factor aside from HMM-MS-to-enzyme ratio on cellulase activity retention and enzyme amount on xylanase activity retention as profoundly significant. Each one of those factors has small P-values. Since the P-values of the HMM-MS-to-enzyme ratio and enzyme amount were larger than the picked value of α=0.05 for the analysis, it was negligibly affected the responses of the activity retention.

The Normal Plot of Effects (Fig. 2) additionally helps to decide the size and the significance of a parameter. The irrelevance of factor B on cellulase activity retention and factor A on xylanase activity retention could likewise be reasserted from the normal plot, in which, the factors that did not fall close to the fitted line were vital. The elements that have insignificant impact on the yield reaction have a tendency to be smaller and ~~are~~ revolved around zero.

Table 3 The results of analysis of variance (ANOVA) on xylanase activity retention in CLEA-HMM-MS.

Factors	F Value	Prob > F	R^2
Model	11610.16	< 0.0001	
A	2.66	0.1337	
B	9455.17	< 0.0001	
C	3360.95	< 0.0001	
D	1099.63	< 0.0001	
E	14.18	0.0037	
F	1.224E+005	< 0.0001	
G	2735.69	< 0.0001	
H	36920.18	< 0.0001	
J	115.45	< 0.0001	
K	4563.52	< 0.0001	
AB	4563.52	< 0.0001	
AC	43799.51	< 0.0001	
AD	132.28	< 0.0001	
AE	14654.61	< 0.0001	
AF	397.02	< 0.0001	1.0000
AG	7120.81	< 0.0001	
AH	9959.31	< 0.0001	
AJ	2759.75	< 0.0001	
AK	1486.1	< 0.0001	
BJ	3457.23	< 0.0001	
BK	27759.1	< 0.0001	
CJ	18595.6	< 0.0001	
CK	18858.5	< 0.0001	
EJ	309.32	< 0.0001	
EK	3577.21	< 0.0001	
ABJ	1149.8	< 0.0001	
ABK	8241	< 0.0001	
ACJ	4108.05	< 0.0001	
ACK	72.69	< 0.0001	
AEJ	139.01	< 0.0001	
AEK	929.21	< 0.0001	

According to equation 1, it was shown that factor G (glutaraldehyde concentration) has the highest coefficient, indicating that it has the most significant effect towards cellulase activity retention as described in previous study (Sukri & Munaim, 2017). Meanwhile Equation 2 indicated that factor F (enzyme-to-precipitant ratio) coefficient value was the highest, signifying substantial effect towards xylanase activity retention. The obtained results were in agreement with earlier study which depended on ethanol concentration for protein yield (Taskila *et al.*, 2017).

Determination of the optimal enzyme amount was crucial since it was the subject of this work; enzyme immobilization. Thus, the effect of cellulase and xylanase amounts on cellulase and xylanase activity retentions was done in the range of 1.5-2 mL. From Equation 1, it showed that the coefficient for enzyme amount (A) was positive which indicated that factor A as having positive effect towards cellulase activity retention. Therefore, cellulase activity retention was increased as the enzyme amount was also increased. The result obtained might be due to the cellulase having no difficulty for transporting inside the HMM-MS structure. However, our result gave no significant increment in activity retention of xylanase as shown in Table 3. It was reported that after an initial rapid phase of enzyme loading, the rate of loading would tend to be stagnant, entering a saturated phase (Salwanee *et al.*, 2013). At this point, increasing the amount of enzyme used could not result in higher degree of loading rate as the concentration of enzyme available in the HMM-MS might become the limiting factor. This might also be due to the different transfer rate of cellulase and xylanase However, further studies were needed in order to determine the validity of this possibility. Subsequently, based on the result obtained for enzyme amount, 2 mL of enzyme amount was chosen to be fixed for the next experiment.

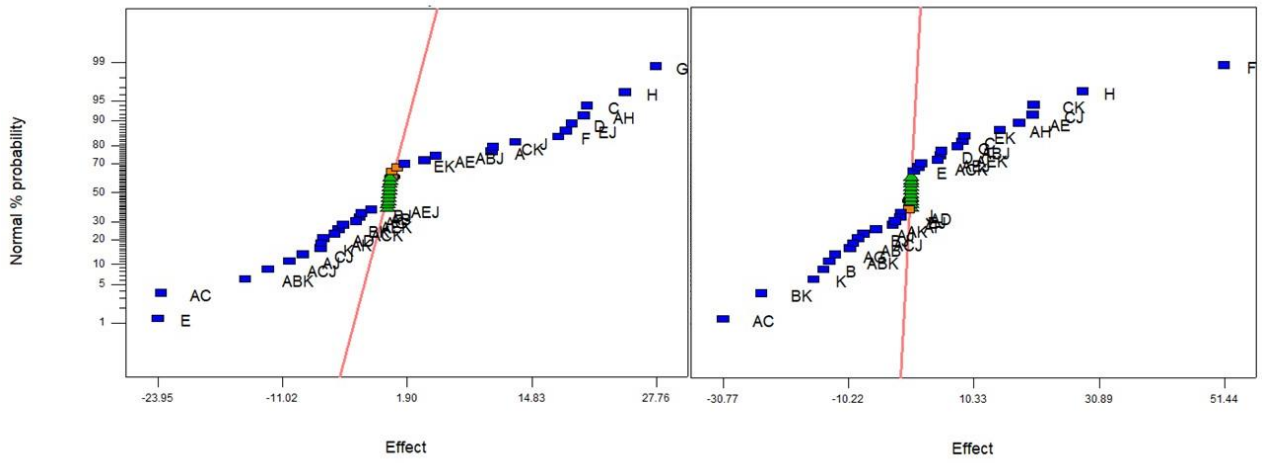

Fig. 5 Normal plot, cellulase(left) and xylanase (right).

As the density of immobilized enzymes within a support plays an important role in enzyme activity, different HMM-MS-to-enzyme ratios were investigated for cellulase and xylanase activity retentions. For cellulase (Equation 1), activity retention was remained relatively constant throughout 1:0.15-1:0.5 of HMM-MS-to-enzyme ratio, indicating the factor effect to be negligible. This might indicate that for cellulase, increase or decrease ratio would give the same retention, leading to a constant overall activity retention. This finding was different from the previous study where the ratio gave negative effect towards glucose oxidase (Kazenwadel *et al.*, 2015). For xylanase activity retention, HMM-MS has negative coefficient which indicated for negative effect towards the output. Thus, increase in HMM-MS-to-enzyme ratio could decrease the xylanase activity retention as the HMM-MS structure was saturated at high xylanase concentration and has no more space for xylanase loading (Baig *et al.*, 2016). This result might explain why higher ratios were not considered to be economical due to the loss of free enzymes. Regarding the relationship between the overall activity retention and adsorption yield, a HMM-MS-to-enzyme ratio of 1 mg:0.15 mL was found to be optimal.

Temperature is one of vital aspect in adsorption. This section would present some results of temperature influence on adsorption. For the temperature range of 25°C to 45°C, Kim *et al.* (1992) adsorbed purified components (CBHs and EGs) of cellulases on Avicel PH 101 and CMC. Increasing temperature could decrease the amount of cellulase components to be adsorbed. Increase in temperature from 55°C to 60°C resulted in maximum components activity reported in the investigation of temperature effect on all the components of an enzyme-mixture (Kim *et al.,* 1992). Solka floc SW-40 was used as the support for cellulase adsorption and the profile of the adsorbed cellulase at 4°C was closely parallel to that at 50°C (Lee, 1999). Changing the temperature from 4 to 50°C resulted in only a small effect on the adsorption by some reports (Zheng *et al.*, 2013). There were contradictory reports on the temperature effect on cellulase adsorption; some groups of researchers said it was expanded with expanding temperature and others said it was diminished with it (temperature?) and there were some researchers who asserted that temperature has irrelevant impact on adsorption. In this study, the cellulase and xylanase activity retentions were increased with increasing adsorption temperature (4-37°C) and 26°C was chosen as the optimal temperature.

Coefficient of factor D represented the effect of adsorption time on the activity retentions of cellulase and xylanase. The time of adsorption was varied in the range between 10 min and 24 h. It could be observed that activity retention of the immobilized enzyme was increased upon adsorption time. This showed that there was no hindrance for the enzyme to be adsorbed into HMM-MS eventhough there was accumulation of enzyme inside HMM-MS pores upon time. This finding was contradicted with the previous work that resulted in the decrease of enzyme activity due to the blocking of pores of chitosan/TiO2 composite beads by enzyme molecules (Deveci *et al.*, 2015). From the optimization result, 40 min was chosen as the optimum time for the enzymes to be adsorbed.

The results on the adsorption agitation coefficient, given in Equation 1 and 2, showed that the adsorption of cellulase was decreased as the adsorption agitation was increased from 125 to 200 rpm while for xylanase, it was the other way around. This suggested that the action of cellulase was highly reversible at higher level of adsorption agitation (Azevedo *et al.*, 2000). The increasing amount of xylanase in the HMM-MS with higher level of mechanical agitation could be due to the mechanical agitation action on the HMM-MS structure, creating more spaces for other xylanase molecules to enter the pores, thus indicating the reversibility of xylanase adsorption to be negligible (O'Neill *et al.*, 2007). Design Expert suggested that 162 rpm as the optimum adsorption agitation.

Higher tert-butanol concentration would lower the solubility of the enzymes, hence inducing the precipitation of cellulase and xylanase (Arakawa *et al.*, 2007). Furthermore, higher tert-butanol concentration could lower the dielectric of the solution, turning into poorer solvent for cellulase and xylanase, and consequently increasing the relative favorability of cellulase-cellulase and xylanase-xylanase interactions, making the enzymes to precipitate (Kramer *et al.*, 2012). Besides that, it could also increase the hydrophobic interactions, causing the enzyme stability to decrease (McCue, 2009). Special care must be taken for ensuring that the enzymes did not unfold after reaction with tert-butanol (Wang *et a*l., 2016).

After precipitation of enzymes, it was presumed that enzymes were located close to each other and they were cross-linked by glutaraldehyde on the surface and inside HMM-MS. It is well known that in most cases, glutaraldehyde cross-linking can cause notable decrease in the catalytic activity of enzymes, which mostly influenced by the time of treatment and the concentration of glutaraldehyde (Radva *et al.*, 2011). Somehow, in this study, the increase in glutaraldehyde concentration resulted in the increase of cellulase and xylanase activity retentions. This was due to glutaraldehyde that could stabilize the structure of enzymes and decrease the possibility of desorption by cross-linking the enzymes that previously adsorbed and precipitated on the surface and inside the support (Melo *et al.*, 2017).

The cross-linking process was optimized at different temperatures in the range of 4-50°C. Resultant coefficients of cross-linked temperature were shown in Equation 1 and 2 as factor H. The enzyme activity retention was increased as the temperature was increased. The optimal temperature of cross-linked was decided at 37°C due to HMM-MS that has a protecting effect at high temperatures, in which enzyme deactivation could take place. The conformational flexibility of the enzyme was affected by immobilization. Immobilization of cellulase and xylanase in HMM-MS caused an increase in the enzyme rigidity

which was commonly reflected by the increase in stability towards denaturation by raising the temperature (Cetinus & Öztop, 2003).

Cellulase and xylanase were cross-linked for 2-24h. The activity retention for cellulase was increased as the cross-linked time was increased. For xylanase, the enzyme activity retention was decreased as the cross-linked time was increased. The reasons were that the cross-linked time for cellulase was too short and the level of crosslinking was insufficient, causing the cross-linked enzyme in carrier was less and prompting low immobilized enzyme activity (Chen *et al.*, 2013). The enzyme activity was increased along with the extension of the cross linking time, enabling the reaction to be carried out gradually to completion. At the point when the cross-linking time was too long, the arrangement of the xylanase might change altogether due to excessive cross-linkimg that caused the denaturation of enzyme protein (Radva *et al.*, 2011).

Agitation speed could also affect the activity retention of cellulase and xylanase in HMM-MS and the optimum speed was selected at 300 rpm (Table 4). According to Agyei & He (2015), aggregates that prepared at slower agitation (<100 rpm) speed has a coarse appearance as compared to aggregates that prepared at higher agitation speed (>100 rpm) which has a finer consistency and appearance. Unquestionably, higher agitation speeds would enhance the appearance of aggregates. However, beyond a critical value there was the possibility of excessive cross-linking of the aggregates which could lead to enzyme rigidification (Illanes, 2008). This could compromise the recovery of enzyme activity as occurred to the activity retention of cellulase and xylanase in this study.

For optimization of the activity retention, optimized values of the reaction were calculated. The desirability of each of the anticipated values indicated its closeness to the target value on a scale of 0 to 1 (Table 4). It showed that these models were valid since the actual response was in good agreement with the predicted response.

Table 4 Optimum condition for desired output.

Factors	Optimum condition	
A	2 mL	
B	1 mg:0.15 mL	
C	26°C	
D	40 min	
E	162 rpm	
F	1 mL:11 mL	
G	0.05 (%v/v)	
H	37°C	
J	2 h	
K	300 rpm	
	Predicted responses	**Actual responses**
R_1	99%	93%
R_2	100%	98%
Desirability	0.992	

CONCLUSION

In this study, it was observed that cellulase and xylanase immobilization in CLEA-HMM-MS could be optimized to 100% activity retention through fractional factorial design. The models were also suitable to predict the responses since the actual responses were comparative to the predicted value. It could be concluded that all of the factors involved in this study were vital in influencing the recovery of cellulase and xylanase activity.

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