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Combination of Entrapment and Covalent Binding Techniques for Xylanase Immobilisation on Alginate Beads: Screening Process Parameters

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Xylanase are responsible enzyme for hydrolysis of xylan into many beneficial products such as xylose, xylitol and xylooligosaccharides. Due to industrial potential of xylanase, a large number of studies have become interested in their immobilisation to reduce the cost of enzyme. Immobilised enzymes are currently the object of interest due to its benefits over soluble or free enzyme applied in enzymatic hydrolysis. The aims of this study were to determine the suitable method for xylanase immobilisation and to identify the significant parameter which affecting the immobilisation yield by fractional factorial design (FFD). Immobilised xylanase was prepared using a single immobilisation techniques of entrapment and covalent binding and also a combination of entrapment and covalent binding techniques. The immobilisation conditions for xylanase which includes of sodium alginate concentration, calcium chloride concentration, agitation rate and enzyme loading were screened using FFD experimental design to determine the most significant parameters in affecting the efficiency of immobilised xylanase. The analysis of xylanase activity was determined using dinitrosalicyclic acid (DNS) method. The xylanase enzyme was successfully immobilised by entrapment in sodium alginate beads and covalent binding on the surface of beads by glutaraldehyde. The combination of entrapment and covalent binding showed the highest immobilisation yield of 65.81 % compared to a single technique which contributes only 31.99 % and 48.53 %. Glutaraldeyhde concentration showed the most significant parameters compared to the others parameter which gives about 69.01 % of contribution on the xylanase immobilisation yield. The study shows the efficiency of enzyme immobilisation could be improved by a combination of immobilisation techniques and determination of the most significant factors for xylanase immobilisation.

1. Introduction

Xylanase are enzyme that catalyse the endohydrolysis of 1,4-β-D-xylosidic linkage in xylan (Collins et al., 2005). Xylan is the major structural polysaccharides in plant cells and known as the second most abundant polysaccharides in nature after cellulose where approximately accounts for one-third of all renewable organic carbon on earth (Prade, 1995). Wide application of xylan hydrolysis into beneficial sugar such as xylose, xylitol and xylooligosaccharides by enzymatic reaction of xylanase has encouraged the development and research to improve the xylan degradation efficiency. Enzyme immobilisation particularly for xylanase could offer considerable advantages with the high possibility of continuous processing and enzyme reuse. Moreover, immobilisation also eliminates the need for an inactivation process because immobilised enzyme can be easily removed by filtration, thus facilitating a better control of the hydrolysis process and prevents contamination of enzyme and the products. In contrary with free enzyme, it always presents problems such as low stability, low product, and inability to reuse (Bhushan et al., 2015).
Numerous establish immobilisation methods are available but the effectiveness of each one depends upon reaction conditions, process of product formation and its cost evaluation (Bhushan et al., 2015). The immobilisation procedure by entrapment on alginate beads is not only inexpensive but also provides extremely mild conditions, so that the potential for industrial application is considerable (Ortega et al., 2009). Enzyme entrapment in alginate beads cross-linked with glutaraldehyde (Bhushan et al., 2015) improved the desirable characteristics such as thermostability and operational utility of the enzyme. It also avoids the leakage of enzyme

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entrapped in alginate matrix. The immobilisation by activation of alginate beads with glutaraldehyde prior to enzyme addition by covalent binding showed improvement in thermal stability (Ortega et al., 2009) and higher efficiency with high immobilisation yield (Pal and Khanum, 2012). The entrapment of enzyme in alginate beads and the activation of the beads with glutaraldehyde for enzyme attachment on the surface of beads by covalent binding have not been reported.

In order to obtain a better immobilisation yield, the immobilisation parameter such as sodium alginate, calcium chloride concentration, glutaraldehyde and agitation rate were screened to identify the significant variables that influence the immobilisation yield. The use of screening process is able to reduce the number of parameters in order to reduce the experiment time and process cost (Bouchekara et al., 2011). In this work, using of design of experiments (DOE) approach to reduce the number of parameters is explored. This approach is presented to access the effects of the different parameters and their interaction involved in xylanase immobilisation process. The aims of this study were to evaluate the effects of process parameters on xylanase immobilisation by combination of entrapment and covalent binding and identified which parameter are the key factors that give significant impact on the immobilisation yield.

2. Materials and Methods

2.1 Materials

The enzyme used for immobilisation process was xylanase from Thermomyces lanuginosus, purchased from Sigma-Aldrich. Sodium alginate, calcium chloride and glutaraldehyde were supplied by Merck Sdn. Bhd. All other chemicals and reagents used were purchased locally and were analytical grade.

2.2 Preparation of entrapment immobilisation of xylanase on alginate beads

Xylanase dilution from stock solution was immobilised by entrapment in sodium alginate beads. Xylanase dilution with specific activity was mixed in equal amount with ratio 1 : 1 of 2 % w/v sodium alginate solution. The mixture then was added drop wise into calcium chloride solution (0.3 M) with continuous stirring for 30 min for hardening process. The process will form a hydrogel beads with an entrapped enzyme inside. The beads were washed with distilled water until no enzyme activity was detected.

2.3 Activation of xylanase immobilisation by covalent binding

Prepared entrapment beads then were activated with xylanase outside the surface of beads by covalent binding using glurataldehyde. The beads were dipped in 6 % w/v glutaraldehyde solution in citrate buffer (0.05 M, pH 4.8). The activation was carried out at a room temperature under orbital stirring, 150 rpm for 3 h. The ratio between the beads and glutaraldehyde solution should be kept at 1 : 10 (w/v). The complete immobilised xylanase beads were filtered and washed with distilled water to remove unbound glutaraldehyde. The beads were stored at 4 °C until further use. Activated beads were coupled with xylanase by covalent binding on the surface of beads. Xylanase dilution with specific activity was added to the activated beads and 2nd stage of immobilisation was carried out at room temperature under orbital stirring 200 rpm for 100 min. During the coupling reaction, a ratio of 1 : 1 (w/v) should be kept between the enzyme solution and activated beads. The beads were washed with distilled water until no enzyme activity was detected in washing.

2.4 Xylanase activity assays

Free and immobilised xylanase were measured according to (Bailey et al., 1992) using substrate xylan from beechwood. The activity of xylanase was determined based on the production of reducing sugar by dinitrosalicyclic acid (DNS) method (Miller, 1959). The amounts of xylose as a reducing sugar were determined by reading the absorbance of the sample using UV-VIS spectrophotometer at wavelength of 575 nm. One unit of enzyme activity (U) is defined as the amount of enzyme which releases one µmol of reducing sugar refer as xylose per minute under the assay conditions. Triplicate measurements were performed for each assay to obtain the mean value and standard deviation. The immobilisation yield is calculated according to the following (Eq(1)) (Pal and Khanum, 2011).

$$\text{Immobilisation yield (IY) (\%)} = \left(\frac{A}{A_o}\right) \times 100\ \% \quad (1)$$

where *A* is total activity recovered on beads and *Ao* is the total activity offered for immobilisation.

2.5 Experimental design and parameter screening by FFD

The parameters for obtaining the best conditions of immobilised xylanase were screened using two-level factorial of design expert. Five parameters which include of sodium alginate concentration (X_1), enzyme loading (X_2), agitation rate (X_3), $CaCl_2$ concentration (X_4) and glutaraldehyde concentration (X_5) were screened resulted in 19 runs of experiments included of three center point with various parameters values. Immobilisation yield was taken as the response variable of the factorial design experiments. The screening process by factorial design was used to determine which independent factors give the most significant contribution to the

immobilisation process. It also explained the interaction between each factor and determined the optimized range for each factor. The range and values of the studied variables in experiment are summarised in Table 1.

Table 1: Independent variables and their levels used for screening

Independent variable	Symbol	Coded variable levels		
		-1	0	+1
Sodium alginate concentration (% w/v)	X_1	2.00	3.00	4.00
Enzyme loading (U)	X_2	100.00	200.00	300.00
Agitation rate (rpm)	X_3	50.00	150.00	250.00
Calcium chloride concentration (M)	X_4	0.20	0.30	0.40
Glutaraldehyde concentration (% w/v)	X_5	3.00	7.50	12.00

3. Results and Discussion

3.1 Comparison of Immobilisation techniques for xylanase

Enzymatic hydrolysis is one of the important processes in the degradation of lignocellulose component into beneficial products, mostly sugar. Xylanase are able to catalyse the hydrolytic cleavage of complex polysaccharide xylan which major component of hemicellulose into xylose sugar which has wide industrial application. The immobilisation of xylanase could provide an effective use of enzyme in terms of improving its stability and ability to be reused for many cycles. Commonly, the basic principle of enzyme immobilisation involves the immobilisation of initial free enzyme on a mesoporous solid structure. The support selected must offer a high internal surface area accessible to and chemically suitable for the enzyme attachment physically, chemically or both (Mateo et al., 2007).

In order to explore the immobilisation techniques that suit xylanase, different techniques were studied and compared to determine which technique gave the highest immobilisation yield. Combination techniques of entrapment and covalent binding shows the highest immobilisation yield compared to a single technique of entrapment and covalent binding alone as shown in Table 2. The enzyme activity of combined technique retained 79.68 U from the original free xylanase activity of 121.09 U which contributed to 65.81 % of immobilisation yield. Single method of entrapment in beads and covalent binding outside beads contributed to 31.99 % and 48.53 % of immobilisation yield. Lower immobilisation yield obtained in enzyme entrapment might due to the leakage of enzyme from the mesoporous beads structure or poor accessibility of substrate to enzyme entrapped within the beads (Pal and Khanum, 2011). The weak bonding between the enzyme and the beads support is one the reason contributing to lower immobilisation yield resulted in covalent binding technique. Previously study on entrapment of enzyme (inulase and saccharomyces cerevisiae alcohol dehydrogenase, SCAD) on alginate beads reported by Missau et al. (2014) shows lower immobilisation yield obtained which were 39.48 % and 62.76 %. Covalent binding techniques for xylanase was reported by (Ortega et al., 2009) obtained offer about 50 % of immobilisation yield from the original enzyme activity.

Among the numerous way of enzyme immobilisation, entrapment in alginate beads is the most easiest and economic. The enzyme immobilisation on alginate beads is not only inexpensive, but also used in mild conditions (Zhou et al., 2010). Entrapment of enzyme in beads face problem in high probability of leakage from the matrix structure. To overcome the leakage problem, the entrapment beads was covalently immobilised on the surface of the beads by cross-linker glutaraldehyde. The adding of cross-linker glutaraldehyde help to cross-link the entrapped enzymes, form an aggregates and thereby reduce their leakage. Glutaraldehyde also stabilizes the alginate gel, helping in the prevention of leakage of enzymes (Bhushan et al., 2015). A combination of immobilisation technique of entrapment and covalent binding showed a potential in enhancing the efficiency of xylanase immobilisation.

Table 2: Comparison of immobilisation techniques on xylanase

	Free xylanase	Immobilisation technique		
		Entrapment in beads	Covalent binding outside beads	Combined of entrapment and covalent binding
Enzyme activity (U)	121.09 ± 5.04	38.73 ± 0.63	58.76 ± 2.52	79.68 ± 1.89
Immobilisation yield (%)		31.99 ± 0.52	48.53 ± 2.08	65.81 ± 1.56

3.2 Main effects analysis for screening

Preliminary experiments were carried out to screen the parameters that influence the immobilisation of xylanase by entrapment and covalent binding of alginate beads. In this study, five factors were investigated: sodium

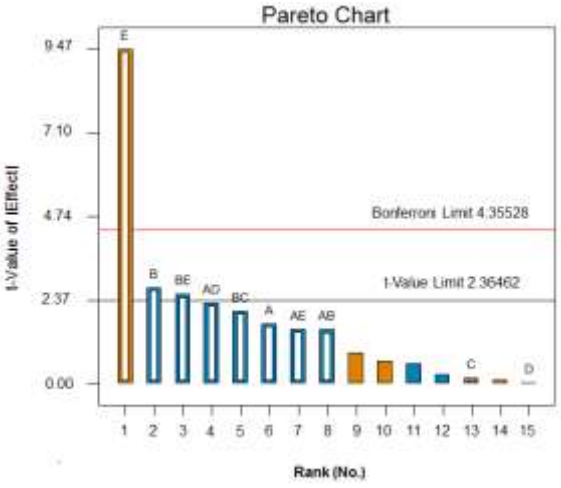
alginate concentration, A (X_1), enzyme loading, B (X_2), agitation rate, C (X_3), calcium chloride concentration, D (X_4) and glutaraldehyde concentration, E (X_5).

From Figure 1, glutaraldehyde concentration showed the most significant factors affecting the xylanase immobilisation which contributes up to 69.01 %. Followed by enzyme loading as a second contributor factor with 5.68 % of contribution and sodium alginate concentration as a third contributor with 2.22 %. The least effective factor for xylanase immobilisation was agitation rate and $CaCl_2$ concentration which only contributes to 0.019 and 0.000465 % and which were insignificant toward the immobilisation process. These findings equivalent to the result obtained by (Pal and Khanum, 2011) where among the variables under parameter study for immobilisation, glutaraldehyde concentration is one of the strongest linear effect on the process yield while the effect of agitation rate was not statistically significant.

Glutaraldehyde concentration showed high impact toward the immobilisation yield of xylanase due to suitable activation agent for xylanase. The same interpretation also indicated by (Pal and Khanum, 2011) and (Ortega et al., 2009) the suitability of glutaraldehyde for immobilisation process. Glutaraldehyde acts as a hardening agent to form compact and very stable beads, contribute to an increase of rigidity and strength of immobilised enzyme. The inclusion of a spacer which is glurataldehyde was vital to improve conformational flexibility (Govardhan, 1999) and improve enzymatic activity and operational activity (Costa et al., 2013) in comparison with the immobilised enzyme without this bifunctional reagent.

Term	Stdized Effects	Sum of Squares	% Contribution
Intercept			
A-Sodium alginate conc.	-5.53	122.32	2.22
B-Enzyme loading	-8.84	312.58	5.68
C-Agitation rate	0.51	1.06	0.019
D-CaCl2 conc.	-0.080	0.026	4.650E-004
E-Glutaraldehyde conc.	30.82	3799.49	69.01
AB	-4.99	99.80	1.81
AC	2.09	17.47	0.32
AD	-7.44	221.41	4.02
AE	-5.01	100.40	1.82
BC	-6.70	179.83	3.27
BD	2.87	32.83	0.60
BE	-8.22	270.27	4.91

(a)



(b)

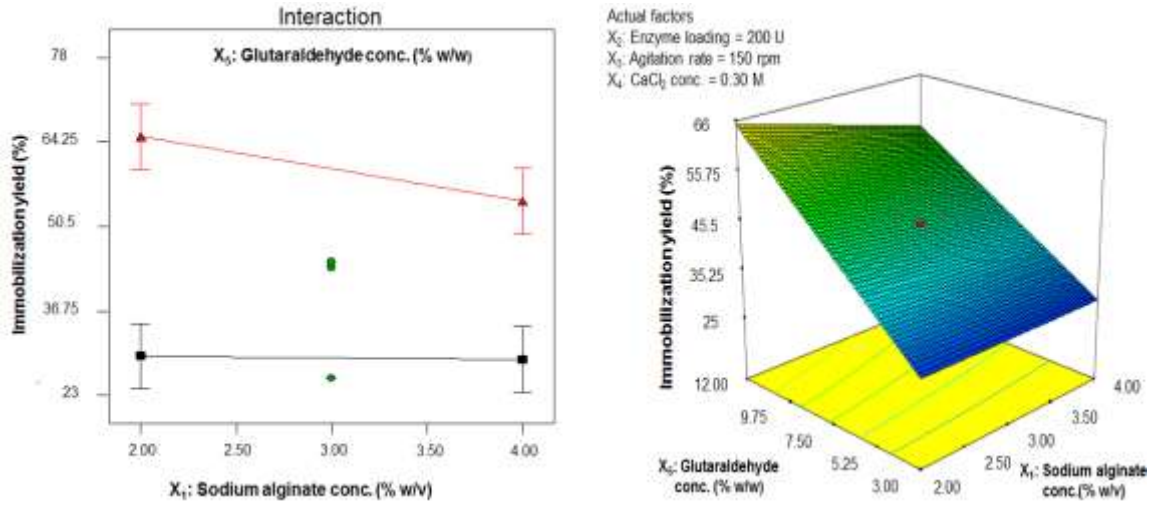
Figure 1: Parameters analysis for (a) the percentage of contribution and, (b) Pareto chart of each main factors and their interaction during the experiment of the immobilisation of xylanase

A factor with a positive coefficient has an improvement impact toward immobilisation yield compared to a negative value which gave the opposite impact. A factorial design model (Eq(2)) in terms of coded variables was obtained from the regression results and insignificant terms were excluded from the equation to form a simpler model. From Eq(2), it can be seen that the main effect of glutaraldehyde concentration has the largest coefficient (X_5; +15.41) followed by agitation rate (X_3; +0.26), $CaCl_2$ concentration (X_4; -0.040), agitation rate (X_1; -2.77) and enzyme loading (X_2; -4.42). These results were closely consistent with the percentage contribution results in Figure 1(a) where glutaraldehyde concentration (X_5) has the highest percentage of contribution toward immobilisation yield.

$$\text{Immobilisation yield } (\%) = 44.51 - 2.77X_1 - 4.42X_2 + 0.26X_3 - 0.0040X_4 + 15.41X_5 - 2.50X_1X_2 - 3.72X_1X_4 - 2.51X_1X_5 - 3.35X_2X_3 - 4.11X_2X_5 \quad (2)$$

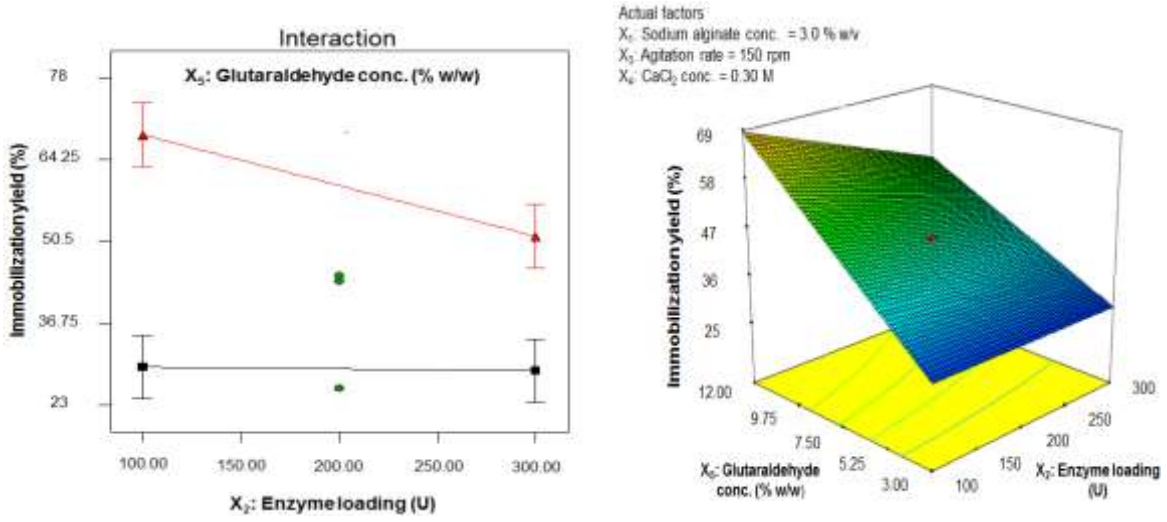
3.3 Interactions between parameters

The interactions among different variables involved in FFD were assessed by plotting the interaction and response surface curves for maximum immobilisation yield. Figure 2 and 3 showed the interaction and surface plots among two factors sodium alginate concentration and glutaraldehyde and enzyme loading and glutaraldehyde by holding others at their center point for the prediction of immobilisation yield.



(a) (b)

Figure 2: Effects between sodium alginate concentration and glutaraldehyde concentration for immobilisation yield: (a) interaction graph, (b) 3D surface



(a) (b)

Figure 3: Effects between enzyme loading and glutaraldehyde concentration for immobilisation yield, (a) interaction graph, (b) 3D surface

It was observed that immobilisation increased when the glutaraldehyde increased to the maximum level (Figure 2 and 3). Both sodium alginate concentration and enzyme loading showed insignificant effect toward the immobilisation yield. The obvious glutaraldehyde concentration effect toward the immobilisation yield must be due to this factor contributes the highest and significant contribution compared to other factors. The improvement in immobilisation yield from 29.36 % to 65.19 % (Figure 2) and 28.84 % to 54.65 % (Figure 2) at 2 % w/v and 4 % w/v of sodium alginate concentration caused by the increase of glutaraldehyde concentration from 3 to 12 % w/v. The same trend of increasing of immobilisation yield also was found in the interaction of enzyme loading and glutaraldehyde concentration. The increasing of enzyme loading gives insignificant effect to immobilisation yield but even worst give a negative effect by declining the immobilisation yield. The decreased of process yield when the enzyme loading added must be due to insufficient attachment point form by glutaraldehyde for enzyme. These findings implied that glutaraldehyde plays an important role in providing support or attachment points for xylanase immobilisation on alginate beads. Same results was found by (Pal

and Khanum, 2011) where higher immobilisation was offered when the alginate beads were activated by high dose of glutaraldehyde. Glutaraldehyde is not only providing attachments points, but it also provides space for conformational flexibility of xylanase which is crucial for catalysis (Govardhan, 1999).

4. Conclusions

In conclusion, xylanase immobilisation could be improved by a combination of entrapment and covalent binding technique on alginate beads. The efficiency of combination technique could achieve up to 65.81 % of immobilisation yield compared to a single technique. FFD was found to be an effective strategy to identify the significant factors toward maximum immobilisation yield of xylanase on alginate beads. From the screening analysis, it was found that glutaraldehyde concentration was the most significant factor influencing the immobilisation efficiency of xylanase with 69.01 % of contribution. The screening results from this work will be further utilized for determination of the optimum condition for xylanase immobilisation.

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