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Effect of Xylanase Immobilisation Conditions by Combination of Entrapment and Covalent Binding on Alginate Beads

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Abstract. The immobilisation of enzymes offer improvement in enzyme stability and characteristics as well as overcome the limitations of free enzyme systems for commercial purposes. In the current study, xylanase was immobilised using a combination technique of entrapment and covalent binding within and onto calcium alginate beads. The sodium alginate and calcium chloride ($CaCl_2$) concentration used for the preparation of alginate beads which is the support matrix for xylanase immobilisation were fixed at 3% (w/v) and 0.3 M, respectively. The effect of immobilisation conditions (agitation rate, enzyme loading, and glutaraldehyde concentration) were studied using One-Factor-At-a-Time (OFAT) approach. The best condition for optimum immobilisation yield (83.93%) was found to be made up of the following parameter combination: agitation rate, 200 rpm; xylanase loading, 200 U; and glutaraldehyde concentration, 12% (w/w). The study shows the immobilisation conditions play a significant role towards the immobilisation yield of xylanase.

1. Introduction

Xylan is the major constituent of hemicellulose which is abundantly available in nature. Biodegradation of xylan into valuable monosaccharides requires the enzymatic action of xylanases to hydrolyse the complex xylan structure. The utilisation of xylanase for industrial application has increased the demand for its research and attention owing to their industrial potential in wide range of fields covering food and functional food ingredient (XOS), animal feeds, paper, pulp, and textiles industries [1–2]. Generally, the original state of an enzyme which is free and soluble is unable to fulfil the industrial requirements because of its lower operational stability, difficulty in recovery from the reaction mixture, and inability to be recycled [3]. Hence, enzyme immobilisation is an effective way to overcome these problems, enhancing the catalytic property of the enzyme, and ensuring the possibility of recycling for a number of times without much loss of activity for continuous economic industrial operation [4].

Numerous techniques for enzyme immobilisation are available such as entrapment, covalent binding, physical adsorption, and aggregation using various supports such as alginate, chitosan, polyacrylamide, and agarose [5] and simple method of carrier-free immobilisation [6]. In recent years, researchers actively involved in manipulating diverse immobilisation techniques for different enzyme types and entrapment technique have proved it to be the convenient way especially for scale up

purposes because it offers diverse optimised parameters using different methods and conditions [7]. Entrapment of enzyme within the alginate beads is an effective approach due to its biocompatibility (nontoxic), low cost, effective particle size, and availability compared to other methods [8]. However, entrapment technique sometimes physically restricts the entrapped enzyme or if entrapped within the support, it could lead to enzyme leakage from the matrix polymer [3], thus causing negligible effect on enzyme catalytic properties. Immobilisation by covalent binding using glutaraldehyde as a crosslinking agent proved also to be an effective technique for xylanase and cellulase immobilisation [9]. Therefore, a combination technique of entrapment and covalent binding could overcome the weaknesses of each technique and improve the efficiency of the immobilised xylanase.

From our previous study [10], the preparation of the support matrix which is the alginate bead with higher efficiency has been obtained. So, this present study aims to evaluate the efficiency of immobilised xylanase by combination technique by determining the effects and the best conditions for higher immobilisation yield.

2. Methodology

2.1. Raw Material
The commercial xylanase from *Thermomyces lanuginosus* was purchased from Sigma-Aldrich Co. (St. Louis, USA). Commercial xylan from beechwood (Sigma-Aldrich) was used as a substrate for the enzymatic reaction of xylanase. Xylose, which is the standard reducing sugar, was supplied by Merck Sdn Bhd (Selangor, Malaysia). All other chemicals used in the experiment were analytically pure grade.

2.2. Enzyme Assay
The catalytic activity of the free and immobilised xylanase was determine by estimating the amount of reducing sugar produced within a specified period using the 3,5-dinitrosalicyclic acid (DNS) method described by Miller [11]. Xylan from beechwood was used as the substrate and xylose as the standard reducing sugar for the generation of the calibration curve. One unit of xylanase was defined as the amount of enzyme required to release or produce 1.0 µmol of xylose per minute under standard assay conditions. The optical density was measured using UV-VIS spectrophotometer (Hitachi U1800, United Kingdom) at 575 nm. All the experiments were performed in triplicates and the results presented as the mean value of the triplicated with the standard deviation.

2.3. Preparation of the Immobilised Xylanase
The preparation of the immobilised xylanase was carried out in two steps of modified entrapment [4], followed by the covalent binding [12]. For the entrapment process, the xylanase was entrapped within the sodium alginate beads, followed by attachment on the outer surface of the beads through covalent binding. A xylanase solution with specific activity was prepared by mixing it in sodium citrate buffer (0.05 M, pH 4.8). The entrapment of the xylanase was initiated by mixing an equal amount of xylanase solution with 3% (w/v) sodium alginate solution at a ratio of 1:1. The solution was then added drop wise in 0.3 M $CaCl_2$ solution with continuous stirring which led to the formation of insoluble alginate beads. The beads were left in the $CaCl_2$ solution for 30 min to harden. After 30 min, the beads were washed three times with buffer (sodium citrate buffer, pH 4.8, 0.05 M) to remove the unbound or loosely bound xylanase. The entrapped beads were stored at 4 ºC until further used.

The entrapped beads were further activated for covalent binding using glutaraldehyde as a cross linker to attach the xylanase on the outer surface of beads. The beads were dipped in 6% (w/w) glutaraldehyde solution in citrate buffer (0.05 M, pH 4.8). A ratio of 1:10 (w/v) was maintained between the beads and glutaraldehyde solution. The attachment process was carried out at room temperature with an orbital stirring at 150 rpm for 3 h. The beads were filtered and washed with distilled water to remove the unattached glutaraldehyde. The formed beads were considered activated beads and stored at 4 ºC for further use. For covalent binding attachment, xylanase solution was added

to the activated beads at room temperature under orbital stirring (200 rpm) for 100 min. During the reaction, a ratio of 1:1 (w/v) was kept between the activated beads and xylanase solution. Finally, the beads were washed with distilled water until no enzyme activity was detected in washing. The resulting beads were considered as a product of the combined entrapment and covalent binding immobilisation technique. The immobilisation yield was estimated based on the following equation [13].

$$\text{Immobilisation yield (\%)} = \left(\frac{A_o - A_u}{A_o} \right) \times 100$$

A_o = the total activity offered for immobilisation

A_u = the unbound enzyme activity   (1)

$A_o - A_u$ = the theoretical immobilised enzyme activity

2.4. Screening of Xylanase Immobilisation Conditions using OFAT

The effect of agitation rate, enzyme loading, and glutaraldehyde concentration on xylanase immobilisation was examined using OFAT. The variation of each process parameter during the study is shown in table 1. The range of condition for the fixing of the parameters was based on previous literatures on enzyme immobilisation through entrapment and covalent binding [12], [14]. For the initial OFAT study, the level of two parameters out of three were fixed (enzyme loading at 100 U and glutaraldehyde concentration at 6.0% w/w). The first parameter was then varied until the best condition was achieved. To study the other parameters, the screened parameters were fixed while the parameter of interest was varied.

Table 1. Variation of process parameters for xylanase immobilisation using OFAT.

Parameters	Variations of factors		
	Agitation rate (rpm)	Enzyme loading (U)	Glutaraldehyde conc. (% w/w)
Effect of agitation rate (rpm)	50.0 – 250.0	100.0	6.0
Effect of enzyme loading (U)	200.0	50.0 – 250.0	6.0
Effect of glutaraldehyde conc. (% w/w)	200.0	200.0	4.0 – 20.0

3. Results and Discussions

3.1. Effect of Agitation Rate

Xylanase immobilisation was carried out at different agitation rates ranging from 50 to 250 rpm to determine the agitation rate at which the maximum immobilisation yield will be achieved. From the graph shown in figure 1, the maximum immobilisation yield of 81.25% was achieved at the agitation rate of 200 rpm. The immobilisation yield did not increase with further increase in the agitation rate (figure 1). The influence of agitation on the immobilisation yield of xylanase was less significant compared to the effect of other factors due to the homogeneity of the reaction. In agreement with the study on xylanase immobilisation by Pal and Khanum [12], the effect of agitation rate was not statistically significant compared to other variables.
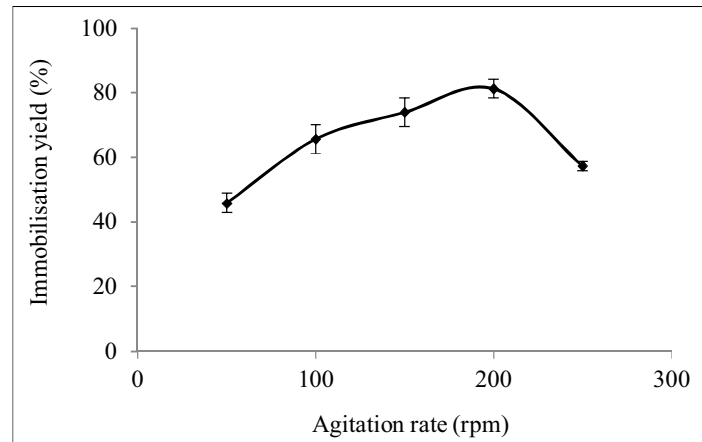
Figure 1. Effect of agitation rate on the immobilization yield of xylanase. The error bars indicate the standard deviation of the mean values.

3.2. Effect of Enzyme Loading

The impact of enzyme loading on the immobilisation yield of xylanase was investigated at the enzyme loading range of 50–250 U. The effect of enzyme loading on the immobilisation yield is shown in figure 2. At low enzyme concentration (50 U) low immobilisation yield of 58.74% was achieved, but as the xylanase loading was increased to 200 U, the immobilisation yield increased to 75.78%. Further increase in the enzyme loading (> 200 U) caused decrease in the immobilisation yield due to the limiting effect of the substrate on the enzymes at higher concentration, resulting to a decrease of the corresponding products. These results point out that the immobilisation yield of xylanase was not favoured by high enzyme loading. According to Pal and Khanum [12], increment of enzyme loading has negative effect on the immobilisation yield probably due to the limited number of attachment sites offered by glutaraldehyde and the resulting washing away of the enzyme.
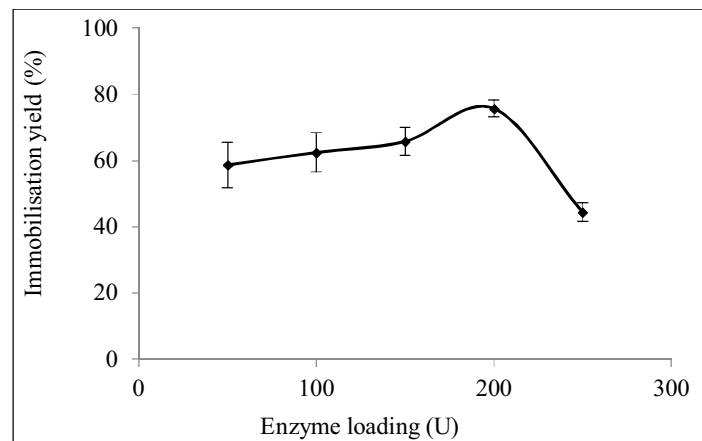

Figure 2. Effect of enzyme loading, on the immobilisation yield of xylanase. The error bars indicate the standard deviation of the mean values.

3.3. Effect of Glutaraldehyde Concentration

For the covalent binding reaction on the hydrogel beads, glutaraldehyde was used as a cross linker for xylanase attachment on the surface of the beads. Besides, glutaraldehyde is a cheap and efficient cross linker suitable for crosslinking reactions although other reagent and newer method are available [15]. Glutaraldehyde concentrations of 4–20% (w/w) were used for the covalent binding of xylanase immobilisation. The concentration of glutaraldehyde affected the immobilisation yield of xylanase as can be seen in figure 3. The immobilisation yield increased from 33.93% to 83.93% which was the maximum immobilisation yield achieved when glutraldehyde concentration was increased from 4 to 12% (w/w). As the glutaraldehyde concentration was increases higher than 12% (w/w), the immobilisation yield dropped to 55.36% (w/w). The low immobilisation yield at low glutaraldehyde concentration might be due to insufficient attachment sites for xylanase on the surface of the beads.
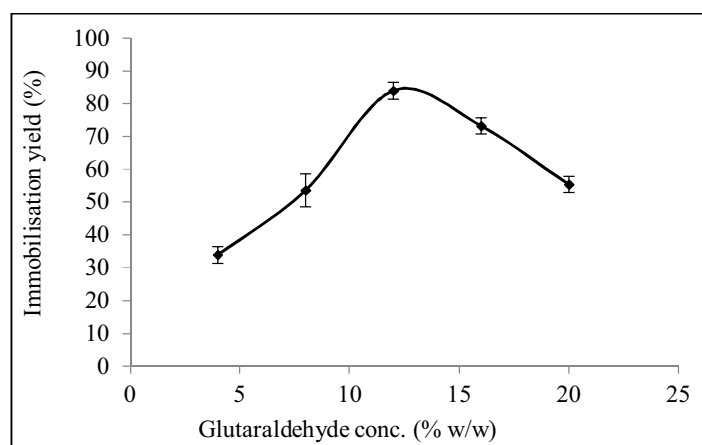

Figure 3. Effect of glutaraldehyde concentration on the immobilisation yield of xylanase. The error bars indicate the standard deviation of the mean values.

Glutaraldehyde was not only used to stabilise the alginate beads, but also helped in preventing enzyme leakage from the beads [16]. Moreover, glutaraldehyde acts as a hardening agent to form compact and stable beads, contribute to an increase in the rigidity and mechanical strength of the immobilised enzymes [17]. According to Pal and Khanum [12], adding glutaraldeyhyde to the immobilised bead activated the immobilisation reaction by providing support/ attachment points for xylanase immobilisation over alginate beads. This is contrary to the outcome of immobilised beads without glutaraldeyhde which showed a weak response due to surface adsorption of the enzyme. Besides providing attachment points, glutaraldehyde provides a space for conformational flexibility of xylanase which is critical for catalysis [18].

4. Conclusions

By varying the parameter of this immobilisation using OFAT approach, an improvement of immobilisation yield (83.93%) could be found at their best conditions of immobilisation process at agitation rate (200 rpm), xylanase loading (200 U) and glutaraldehyde concentration (12% (w/w)). The immobilisation conditions play an important role in forming a stable alginate beads with higher accessibility of substrates toward the entrapped and attached enzymes within and onto the support matrix. The combination of entrapment and covalent binding for xylanase immobilisation has potential for further utilisation in the enzymatic hydrolysis for value-added products conversion.

5. Acknowledgements

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