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Effects of Support Matrix for Xylanase Immobilisation on Alginate Hydrogel Beads

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Abstract–Enzymes serving as biocatalysts and play an important roles in many industrial field. However, the limitation of enzyme usage due to its high cost and unstable conditions of soluble enzyme to harsh conditions lead to findings an alternative to enhance the enzyme efficiency by immobilisation (insoluble enzyme). The present work reported a combination of immobilisation technique of xylanase by entrapment and covalent binding on alginate hydrogel beads. Xylanase enzyme was effectively immobilised within the support matrix, alginate hydrogel beads by entrapment and covalent binding on the surface of beads using glutaraldehyde as a cross-linked agent. The effects of support matrix comprised of sodium alginate concentration (% w/v) and calcium chloride, $CaCl_2$ (M) were studied in order to obtain a better immobilisation yield. The suitable concentration of sodium alginate and $CaCl_2$ to ensure a robust and stable hydrogel beads with higher immobilisation yield were formed as a support matrix for xylanase immobilisation. The analysis of xylanase activity was determined using dinitrosalicyclic (DNS) acid reagent method. Maximal enzyme immobilisation yield (>80 %) was achieved at 3.0 % w/v of sodium alginate concentration and 0.3 M of $CaCl_2$. The study shows the support matrix of hydrogel beads gave a significant impact towards the immobilisation yield of xylanase.

Index Terms – Xylanase; Immobilisation; Sodium alginate; Calcium Chloride

I. INTRODUCTION

Hemicelluloses are the second abundant polysaccharides in nature after cellulose. Xylan, a polymer having primarily of β-1,4-linked xylose residue is the main component of hemicelluloses [1]. Hydrolysis of xylan occurs by the action of several hydrolytic enzymes such as endoxylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase [2]. Xylanase is one of the most important enzymes that hydrolyse the xylan structure into beneficial products like xylose and xylooligosaccharides. 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, animal feeds, paper, pulp, and textiles industries [3]. Due to huge potential of xylanase application in many industries, immobilisation technologies of enzyme become crucial in reducing the cost and for a continuous process. Enzyme immobilisation offer a significant advantages with the possibility of continuous processing, recycle of enzyme, high stability of enzyme and reduction of auto-digestion [4,5] compare to its original state of enzyme which is soluble that unable to be recycle and has poor stability.

Numerous immobilisation techniques offer for xylanase such as entrapment, covalent binding, adsorption and cross-linking. Different techniques have their own properties in terms of their advantages and weaknesses. Entrapment on alginate beads is not expensive, very easy to carry out and provides mild condition which considerably potential for industry application. 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

techniques [6–8]. However, this technique has limitation in mass transfer and problem in enzyme leaking from the beads structure. In order to overcome the limitation of entrapment, the beads were introduced with covalent binding of enzyme on the surface of the bead by cross-linking agent. The use of cross-linking agent has earlier been seen in activating the alginate beads for covalent immobilisation of neutrase [9]. Many reports are available on xylanase immobilisation [1,5,10], but studies reporting the combination of entrapment and covalent binding within and onto alginate bead are very limited.

In the current investigation, xylanase was immobilised within and onto alginate hydrogel bead by combination of entrapment and covalent binding technique. The effects of support matrix, calcium alginate and $CaCl_2$ concentration on xylanase immobilisation yield were studied. The optimum sodium alginate and $CaCl_2$ concentration for xylanase immobilisation were determined to obtain higher immobilisation yield.

II. METHODOLOGY

A. Materials

The enzyme used was xylanase from *Thermomyces Lanuginosus* purchased from Sigma-Aldrich Chemical Co., USA. Sodium alginate, calcium chloride ($CaCl_2$), and glutaraldehyde were supplied by Merck, Germany. All other chemicals used were of analytical grade.

B. Enzyme Assay

Xylanase activity was assayed by the method [11] using xylan from beechwood as a substrate. The activity of xylanase was determined based on the reducing sugar production by dinitrosalycyclic acid (DNS) method described by [12]. The absorbance with wavelength 575 nm was read using UV-VIS spectrophotometer (Hitachi, U1800) and the amounts of xylose as a reducing sugar were determined. The activity of xylanase was calculated based on the determination of enzyme amount that released 1 µmol xylose per minute. All the experiments were carried out in triplicate and the average value with standard deviation were recorded throughout the study.

C. Preparation of immobilised xylanase by a combination of entrapment and covalent binding

The immobilisation of xylanase by a combination technique of entrapment and covalent binding were prepared by the method proposed by Sukri and Munaim [13]. Xylanase stock solution was prepared by diluting xylanase in powder form in sodium citrate buffer (0.05 M, pH 4.8). Xylanase dilution with specific activity was mixed in equal volume (1:1) of (1, 2, 3, 4, and 5 % w/v) sodium alginate. This mixture then were added drop wise into calcium chloride solution (0.2, 0.3, 0.4, and 0.5 M) with continuous stirring and let it hardened in this solution for 30 min. Xylanase immobilised beads was formed as insoluble beads. These beads were washed with distilled water before store for further used. Sodium alginate beads were activated by dipping in (6 % w/w) glutaraldehyde solutions in citrate buffer (0.05 M, pH 4.8). The activation process was carried out at room temperature under orbital stirring (200 rpm) for 3 h. A ratio of 1:10 (w/v) was kept between beads and glutaraldehyde solution. The beads were filtered and washed with distilled water to remove the unbound glutaraldehyde. The resulting beads were coupled with xylanase by covalent binding on the surface of beads. Xylanase dilution with specific activities was added to the activated beads and immobilization was carried out at room temperature under orbital stirring range of 50-250 rpm for 100 min. During the coupling

reaction, a ratio of 1:1 (w/v) was kept between activated beads and enzyme dilution. After defined time, the beads were washed with distilled water until no enzyme activity was detected in washings. The immobilisation yield was calculated according to the following equation [14].

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

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

III. RESULTS AND DISCUSSION

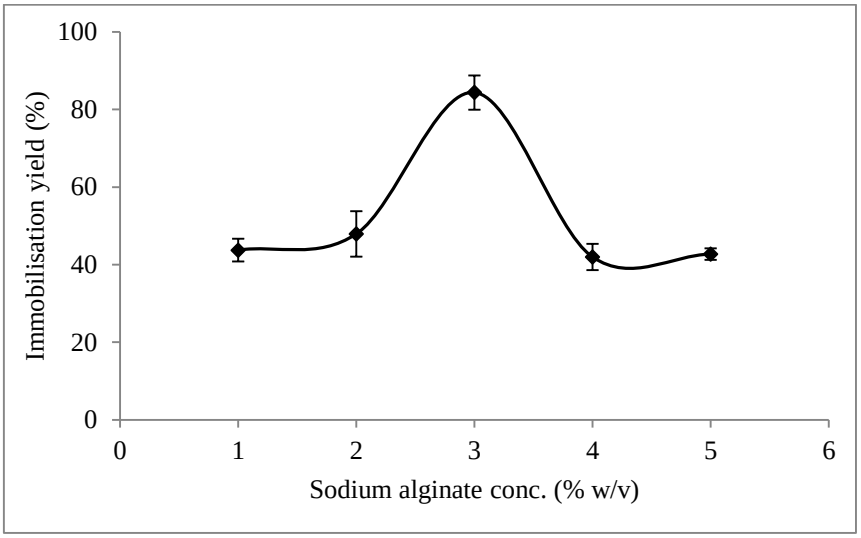
Xylanase catalyse the hydrolytic cleavage of complex polysaccharides xylan into xylose and others sugar which has wide industrial utilisation and immobilisation provide an effective mean for its repeated use and costing. Since xylanase immobilisation by entrapment in alginate beads offers an inexpensive material and also used in mild condition [6], it has been considered for the entrapment of enzymes. Alginate is an anionic polymer composed of α-L-glucuronic acid and β-D-mannuronic acid. Entrapment of enzyme in alginate beads are produced by the cross-linking between α-L-glucuronic acid and divalent cations such as calcium ions [15,16]. The addition of alginate mixture to the $CaCl_2$ solution, instantaneous interfacial cross-linking takes place with precipitation of Ca-alginate, to form a three-dimensional hydrogel. Enzyme entrapment in beads depends on the concentration of sodium alginate and calcium ions. Therefore, the effects of these parameters were investigated on affecting the immobilisation efficiency.

In the preliminary work, the immobilisation conditions (sodium alginate concentration and calcium chloride concentration) for enzyme entrapment and covalent binding in alginate beads that would affect the immobilisation yield were studied. The optimal concentration of sodium alginate as a support matrix is very important for enzyme immobilisation and substrate penetration in the porous structure of alginate beads. Besides, it also plays a vital role for enzyme immobilisation due to the porosity of the hydrogel beads depends on the concentration of the matrix. Different concentrations of sodium alginate range from 1–5 % w/v were used to form robust and stable immobilised beads. Fig. 1 showed the effect of sodium alginate concentration on enzyme activity and immobilisation yield at $CaCl_2$ concentration of 0.3 M, agitation rate of 200 rpm and glutaraldehyde concentration of 6 % w/w. The maximum immobilisation yield (84.38 %) was achieved at 3.0 % w/v sodium alginate concentration as shown in Fig. 1 (a). The effectiveness of immobilised xylanase gradually decline to 41.96 % and 42.71 % when the concentration of sodium alginate increases to 4 % and 5 %. The highest xylanase activity (168.27 U) was observed in Fig. 1 (b) at 3 % of sodium alginate and started to decrease to 83.69 U when the concentration of sodium alginate was raised beyond this concentration.

These trends proved that the concentration of sodium alginate affects the porosity of the alginate beads, hence affect the immobilisation yield. On the contrary, at low concentration of sodium alginate below 3 %, more fragile hydrogel beads was formed resulting in lower immobilisation yield < 50 %. A fragile bead also results in less enzyme entrapment within the porous structure of beads and large pore size of the beads also contribute to enzyme leaking especially during repeated washing of the beads. Different results obtained by Missau et al. [17]

where the best conditions for inulase immobilisation yield (39.48 %) was found at lower sodium alginate concentration at 1 % w/v. Meanwhile, the maximum entrapment yield of immobilised endo-β-1,4-xylanase studied by Bibi et al. [5] was obtained when the sodium alginate was 4 % w/v. Similar trend occur when increased or decreased in sodium alginate concentration declined the enzyme entrapment efficiency. This is due to the fact that lower concentration of sodium alginate produced a fragile hydrogel bead with large pore size, hence causes the leaching of enzymes from beads. At higher concentration, it led towards lower penetration of substrate into the beads for the reaction process to form products [15,18]. The suitable concentration of sodium alginate for beads forming is important for the enzyme entrapment since it allows the substrate penetration to the entrapped enzyme within the matrix structure.

a)



b)

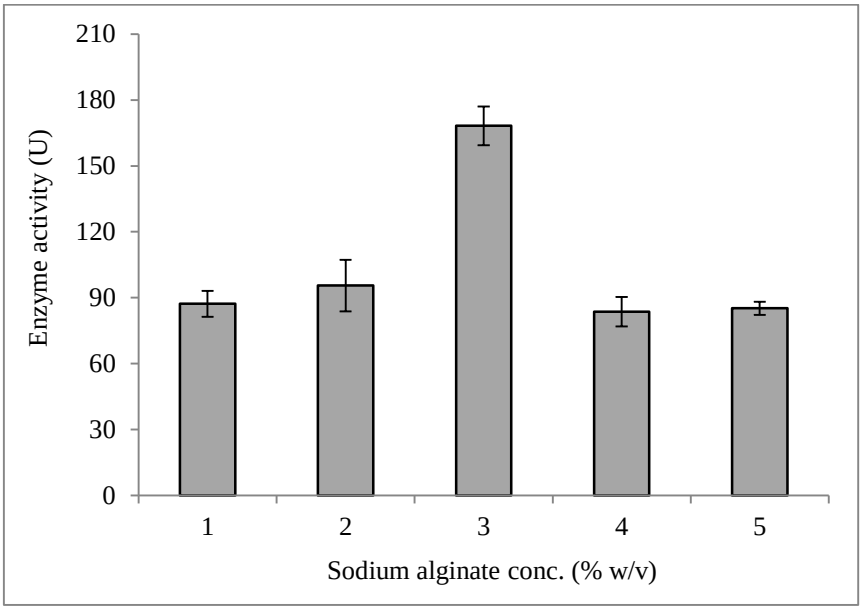
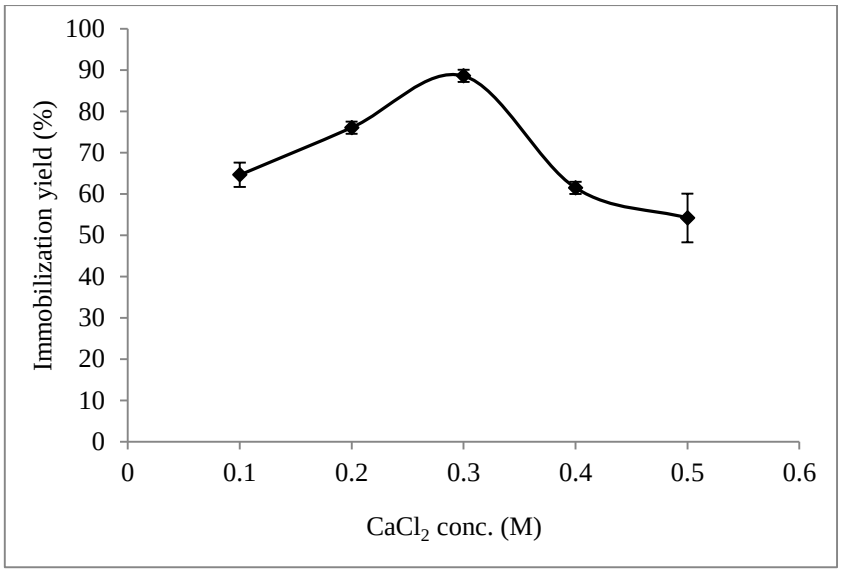


Figure 1: Effect of different concentrations of sodium alginate on the a) immobilisation yield and b) enzyme activity of immobilised xylanase

Concentrations of calcium chloride ($CaCl_2$) were also varied to determine the suitable condition in obtaining a stable bead structure. In Fig. 2 (a) and (b), the maximum immobilisation yield obtained was 88.54 % (176.58 U) at 0.3 M $CaCl_2$ concentration. Increased of $CaCl_2$ concentration up to 0.4 M resulting in lower immobilisation yield obtained with only

54.17 % from the original xylanase activity offers before immobilisation. Decreased and increased of $CaCl_2$ concentration lower or higher than 0.3 M decreased the immobilisation yield of immobilised xylanase. This is due to unstable hydrogel beads were formed when sodium alginate was drop into too low or too high $CaCl_2$ concentration. Increase of $CaCl_2$ higher than 0.3 M caused the immobilisation yield dropped to 61 % and 51 % at 0.4 M and 0.5 M of $CaCl_2$ concentration, respectively. Increase of $CaCl_2$ concentration caused change in pH of the solution and this might be one of the reasons affecting the declined in activity of the enzyme [19]. In agreement with the finding reported by Anwar et al. [20] that the relative activity of entrapped enzyme declined with increment of $CaCl_2$ concentration.

a)



b)

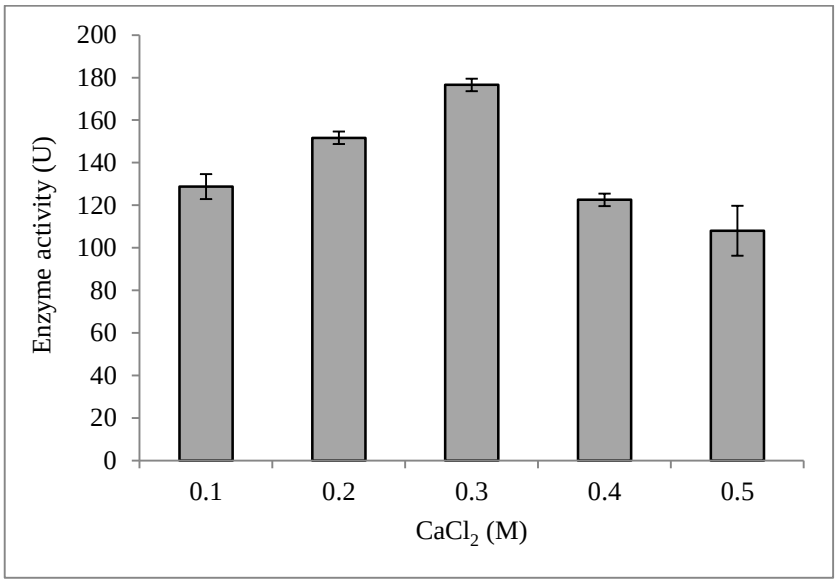


Figure 2: Effect of different concentrations of $CaCl_2$ on the a) immobilisation yield and b) enzyme activity of immobilised xylanase

Both parameters sodium alginate and $CaCl_2$ concentration play an important role in obtaining a stable hydrogel bead of immobilised xylanase. Similar conclusion was reported by Missau et al. [17] that sodium alginate and calcium chloride had a significant effect at 90 % of

confidence on the inulase immobilisation yield using experimental design by Plackett and Burman for optimisation of inulase immobilisation.

Generally, the activity of immobilisation enzyme presented lower value than activity of enzyme offered by free enzyme. The reduction of enzyme activity of the immobilised xylanase caused by diffusional limits, steric effects, and structural changes in the enzymes after the reaction of immobilisation process or also might due to lower accessibility of substrate to the active site of the immobilised enzyme [9].

Sodium alginate has been considered since a long time for the entrapment of enzymes due to its biocompatibility and processibility. However, the major disadvantages of alginate beads are the sensitivity to calcium chelators and larger matrix pores [15]. The combination technique of immobilisation by entrapment and covalent binding are able to cover the deficiency of entrapment properties of larger matrix pores, caused an enzyme leakage problems throughout the beads. Therefore, adding a cross-linker agent like glutaraldehyde could cross-link the entrapped enzymes so as to make their aggregates and thereby reduce their leakage. Other than providing attachments points, glutaraldehyde provides a space for conformational flexibility of xylanase for catalysis [21] and as a hardening agent to form compact and very stable beads, leading to an increase in the rigidity and mechanical strength of the immobilised enzymes.

IV. CONCLUSION

To overcome the problem of enzyme leakage within hydrogel beads, the entrapment immobilised xylanase was covalently binding with xylanase on the outer surface of hydrogel beads using glutaraldehyde as a cross-linker. The immobilisation yield could achieve higher than 80 % from the total activity of free xylanase offers before immobilisation. The support matrix plays an important role in forming a robust and stable alginate hydrogel bead as a support carrier for xylanase immobilisation. Accessibility of substrates toward the entrapped and attached enzymes within and onto the support matrix demonstrates the efficiency of the enzyme immobilisation. The stability and characterisation of immobilised xylanase should be further study before could be utilised in the enzymatic hydrolysis for targeted products.

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