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Characterization of Immobilized Thermostable Alkaline Protease 50a using Calcium Alginate

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ABSTRACT: Thermostable alkaline protease 50a (TAP50a) enzyme has been immobilized by calcium alginate due to maintain its characterization. It is done to get better potential in its use when applied and mixed with other ingredients. Alkaline protease immobilization play an important role in detergent industry to facilitate the discharge of super molecule materials in stains like those of milk and blood in the better ways. TAP50a were immobilized with 2%-4% (w/w) of sodium alginate solution to get the best sodium alginate concentration. The optimal immobilization conditions of TAP50a in calcium alginate beads were investigated and obtained with 3% (w/w) alginate using TAP50a (10 U/ml) and 0.1 M $CaCl_2$ solutions. The hydrolytic properties of free TAP50a and immobilized TAP50a in terms of stability as well as the effects of the temperatures and metal ions were determined. The optimum activity of both the free TAP50a and the immobilized TAP50a was at 90°C. Hence, we propose that, this method can be used to produce immobilized TAP50a which can be used in various areas such as detergent additives and dehairing hides.

Keywords: Protease; immobilization; thermostability, calcium alginate

1. Introduction

Enzymes are thought to be abundant in microorganisms. Among 3000 enzymes, most of them have been extracted from mesophyll microorganisms [1]. However, thermophilic microorganisms have potential to produce thermophilic enzymes which have capability to produce functional enzyme to withstand high temperature, and capacity to maintain their native state in extreme conditions. At the same time the thermostable enzyme opens new opportunities for their biotechnological applications [2]. Proteases are the most important enzyme used in biotechnology applications. Proteases are a class of enzymes that have the catalytic function of hydrolysing protein peptide bonds. They are also known as peptidases or proteinases. They are categorized as acid, neutral, and alkaline proteases [3]. Plants, animals, and microorganisms can produce these enzymes under a variety of circumstances, as well as high salt concentrations. Certain protease enzymes have possible uses in detergents, pharmaceutical industry, bioremediation processes and food productions [4][5].

However, enzyme stabilisation is critical issues when they have been used in related formulation. Then to overcome this issue, immobilization to related enzymes have been used because it able to restrain area of enzyme space without disrupt catalytic activity of enzyme. Immobilization of enzymes is a common practise and normally to reduce the impact of enzyme costs by allowing the enzyme to be reused multiple times. The enzyme is physically confined, often in the form of beads or membranes in a polymer matrix, thus it cannot escape into solution [6].There are various methods of immobilization including adsorption, crosslinking and entrapment of enzyme within polymeric matrix [7][8]. Adsorption immobilization is a technique for immobilising enzymes by attaching the enzyme to the carrier surface using pathetic forces such as van der Walls force, ionic bonding, electrostatic force, hydrophobic interaction, and hydrogen bonding [7]. The method can be useful to enzymes of numerous types that catalyse various types of reactions, for example protease from *Bacillus thuringiensis* strain-MA8 was successfully immobilized onto activated alginate/dextrose (Alg/dex) beads as a new carrier with immobilization yield 77.6 % [6][9].

Meanwhile, enzyme entrapment immobilization is the capture of enzymes inside a polymeric network or microcapsules of polymers that enables substrate and products to move through whereas keeping the enzyme in place. This might be a practical approach for escaping the aggregation of enzymes. Entrapment can be achieved using a variety of matrices, including chitosan, calcium alginate, collagen, and cellulose triacetate. Enzymes are immobilized by gel or fiber entrapment or microencapsulation in this process. Enzymes are maintained in the 10 linkages as substrates and products move through, resulting in less enzyme leaching, improved stability, and the ability to generate enzymatic reactions. Aside from occlusion, there is occlusion in polymeric networks. Enzymes have recently been embedded in metal–organic frameworks (MOF). The zeolitic imidazolate system (ZIF-8) is frequently used to entrap enzymes in this mode [9]. This study will go through the entrapment method of

immobilization using different concentrations of calcium alginate beads. The immobilized enzyme is then characterized for comparison between free and immobilized enzymes.

2. Experimental

2.1 Immobilization of thermostable alkaline protease 50a (TAP50a) using calcium alginate

The TAP50a was used in five ml (10 U/mL) and was mixed well with 20 ml of (2 %, 3 % and 4% w/v) sodium alginate solution then the mixture was mix thoroughly to ensure complete mixing. The solution was transferred into the syringe and dripped it into 30 ml of calcium chloride solution in a beaker. The formed beads were allowed to continue hardening for 20 minutes. Then, the filtered immobilized TAP50a beads obtained were counted and washed with Tris HCl buffer (0.2M, pH 7.5).

2.2 Characterisation of immobilized thermostable alkaline protease 50a (TAP50a)

2.2.1 Effect of Temperature

The TAP50a was tested at temperatures 50°C, 60°C, 70°C, 80°C, 90°C and 100°C for 30 minutes. Azocasein with 0.5% (w/v) of concentration was dissolved in 0.1 M Tris-HCl and 2 mM of $CaCl_2$ buffer at pH 9. Then, 100 µL of immobilized TAP50a was added to 1 ml pre-incubated substrate and incubated. The protease activity was assayed as in Section 2.3. Free enzymes is used as a positive control.

2.2.2 Thermostability test

The optimum temperature for TAP50a got from section 3.3.1 then was screened for its half-life. Azocasein was dissolved in 0.1 M Tris-HCl and 2 mM of $CaCl_2$ buffer at pH 9. Then, 100 µL of immobilized TAP50a was added to 1ml pre-incubated substrate and incubated at 90 °C (±1°C) from 0 to 140 minutes' intervals. The protease activity was assayed as in section 2.3. Free enzyme is used as a positive control.

2.2.3 Effect of metal ions

To investigate the effects of metal ions on the TAP50a stability, 2 mM of different monovalent (KCl, NaCl) and divalent ($CaCl_2$, $MgCl_2$, $MnCl_2$) metal ions were added in the buffer solution with pH 9. Then, azocasein was dissolved at a concentration of 0.5% (w/v) in the buffers containing different metals ion. 100 µL of immobilized TAP50a was added in 1ml pre-incubated substrate and incubated at 90°C(±1°C) for 30 min. The protease activity was assayed as in section 2.3. Free enzyme is used as a positive control.

2.3 Protease Assay

Protease activity was determined using sulphanilamide azocasein as a substrate. 0.5 % of azocasein was dissolved in 0.1 M Tris-HCl and 2 mM of $CaCl_2$ buffer at pH 9. The azocasein was pre-incubated for 5 minutes at 90°C (±1°C). Then, the TAP50a and azocasein were mixed and incubated for 30 minutes at 90°C (±1°C) with 1 ml azocasein solution and 100 µl enzyme solution. After the incubation, and additional 1 ml of 10% (w/v) trichloroacetic acid (TCA) was added to the inactivate the reaction and allowed the mixture to stand at room temperature for 30 minutes before being centrifuged at 10,000 x g for 10 minutes. Then, the supernatant obtained was neutralised by using 1 ml of 1 M sodium hydroxide (NaOH) solution. Then the absorbance of that supernatant was determined at 450nm [10]. One unit (U) of azocaseinase activity was defined as the amount of enzyme activity that produces a change of absorbance (0.001 per min) at 450nm under the standard assay conditions.

3. Results and Discussion

3.1 Selection of Sodium Alginate Concentration

There are numerous limitations to employing the protease, and the general consensus is that protease are sensitive, unstable, ineffective as catalysts, and undesirable in most synthesis and reaction processes [11]. When compared to other approaches, entrapment within insoluble calcium alginate microspheres has proven to be the most successful way due to biocompatibility, low cost, availability, well tolerated in human and resistance to microbial contamination [12], [13]. The findings suggest that the immobilized amylase from Isolate MW2 using alginate gave maximum immobilization yield of 72.18% compared to chitosan and agar as a matrix of immobilization [14]. Meanwhile, dextranase became more stable after immobilized using alginate which it able retained more than 50% of its original activity at elevated temperature after exposure for about two hours [13].

To get the best immobilization yield, different sodium alginate concentrations were tested. The maximum yield was reported in several studies ranging from 2% to 4% (w/v) of calcium alginate [13][14][15]. Therefore, in this study 2% to 4% (w/v) of sodium alginate were used to obtain the most suitable concentration and high

immobilization rate. **Fig. 1** showed that the highest rate of protease activity is using 3% (w/v) of calcium alginate compared to 2% and 4% (w/v) of calcium alginate, meaning 3% (w/v) of calcium alginate is selected to be applied in the next parameter. It is similar report when applying 3% concentration of sodium alginate to immobilise amylase from isolate MW2 which able gave maximum activity of 87.78 IU/g with highest yield of 72.18% [14].

In a 3% solution, calcium alginate and TAP50a will interact and improve the conformational stability of the immobilized enzyme compared to the 2% sodium alginate concentrations. Due to the soft, unstable, and fragile nature of the beads at low sodium alginate concentrations, enzyme may leak out during immobilization [16]. **Fig. 2** shows the beads produced are different in size when the concentration of calcium alginate is different. It was proved that the higher the percentage rate of alginate used results in beads of larger diameter size.

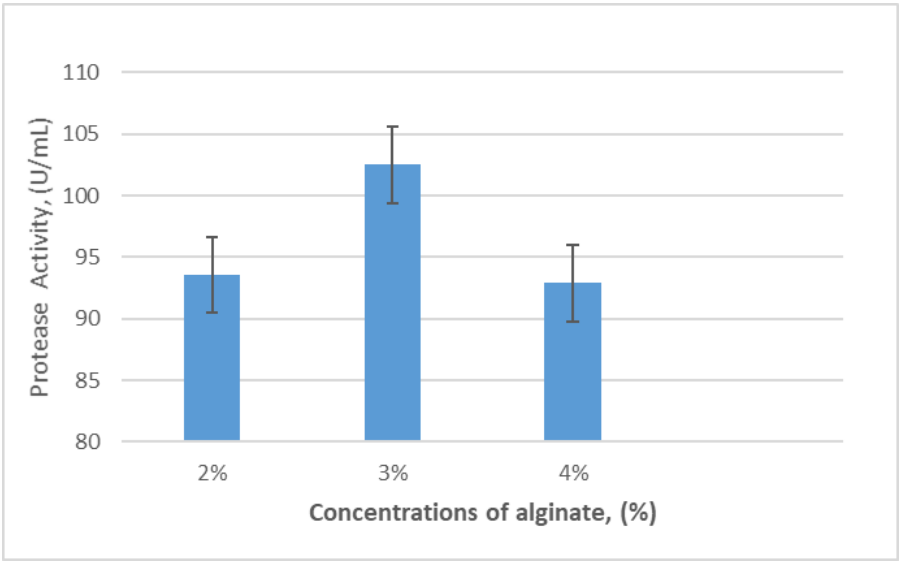

Figure 1: Protease activity TAP50a with difference sodium alginate concentrations.

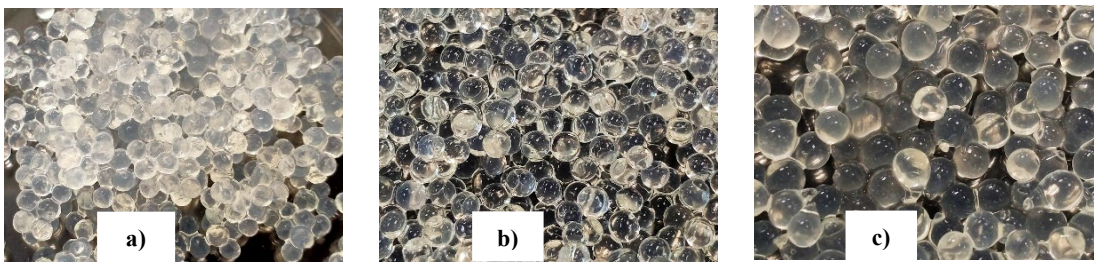

Figure 2: Immobilizations TAP50a with a) 2% (w/w), b) 3% (w/w) and 4% (w/w) of sodium alginate. Size of each approximately 2mm, 3mm and 5mm respectively

2.4 Characterisation of immobilization thermostable alkaline protease 50a (TAP50a)

2.4.1 Temperatures

Temperature optimization is a critical step in achieving optimal enzyme activity in both free and immobilized enzymes. The optimum activity of TAP50a from wild type, *Bacillus subtilis* 50a was at 70°C [10]. Thus, the effect of temperature on activity was determined by assaying enzyme activity which at 50°C to 100°C for 30 minutes. As a result, both free TAP50a and immobilized TAP50a have shown the same range of optimum temperatures which 80°C – 90°C and started to denature at temperature 100°C (**Fig. 3**). From temperature 50°C to 90°C, both free TAP50a and immobilized TAP50a have shown the activity (U/ml) increased evenly. Despite that, the immobilized TAP50a showed better and higher activity at each temperature compared to free TAP50a. Mostly, when compared to the free enzyme, immobilization results in a broadening of the functional temperature range of enzyme activity [17][18]. These results are due to the possibility of the formation of covalent bonds between the enzyme and the agarose which caused the increase of enzyme rigidity and in consequence, the activity

of the immobilized enzyme was higher than the free enzyme. A similar effect was observed for immobilized protocatechuate 3,4-dioxygenase [19].

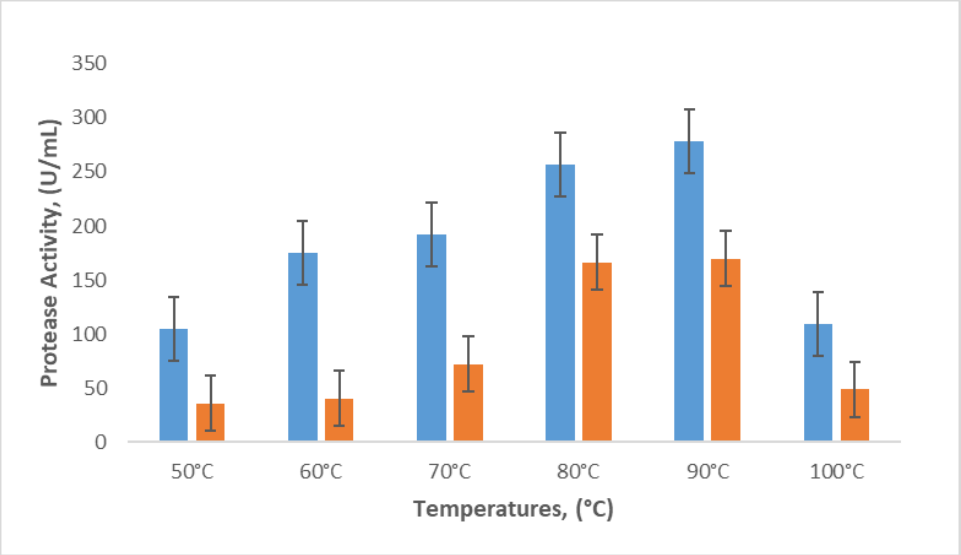

Figure 3: Effect of temperatures on ( ) immobilized TAP50a and ( ) free TAP50a.

2.4.2 Thermostability

Thermostability of enzymes is critical for industrial applications, and it can be improved through immobilization. By incubating free and calcium alginate entrapped TAP50a at 90°C for 0-140 minutes, the thermostability of free and calcium alginate entrapped TAP50a was determined. According to **Fig. 3**, the optimum temperature for both free TAP50a and immobilized TAP50a were same at 90°C, Thus, the half-life was tested at 90°C. As a result, in **Fig. 4**, free TAP50a has reached its optimum level of activity at 90°C for 60 minutes with the range of time from 40 minutes to 80 minutes. The activity drastically decreased at 140 minutes. Compared to the immobilized TAP50a, it has reached its optimum level of activity at 90°C for 120 minutes with the range of time from 100 minutes to 120 minutes. The activity slowly decreased to 140 minutes. It has been proven that immobilized TAP50a has a higher level of resistance at its optimum temperature compared to free TAP50a. Then, alginate matrix preserved the structure of the TAP50a after immobilization and protected it from conformational changes caused by temperature effects. The stabilizing impact of the support matrix, which limits the conformational alterations of thermal denaturation, has been credited with improved thermostability [20]. Polysaccharides like alginate have been successfully used as carriers for ionic binding in immobilization. This method causes little or no change of conformation in the structure of the active site of the enzyme protein and thus in many cases, the immobilized enzymes using polysaccharides gave high enzyme activity [21]. Both free TAP50a and immobilized TAP50a lost their complete activity after 140 minutes of incubation at 90°C due to protein denaturation.

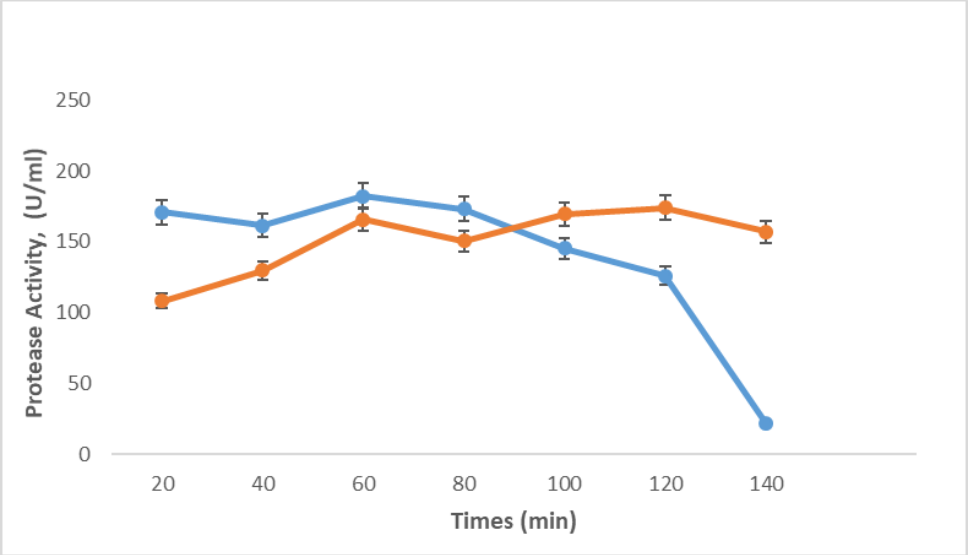

Figure 4: Thermostability test on ( ) immobilized TAP50a and ( ) free TAP50a activity.

2.4.3 Metal Ions

Five different types of metal ions were used which $CaCl_2$, $MgCl_2$, KCl, $MnCl_2$ and NaCl. Based on **Fig. 5**, protease activity for metal ion type $CaCl_2$ is the most suitable and effective metal ion to increase protease activity and followed by $MnCl_2$, $MgCl_2$, KCl and NaCl with an average difference was 11% - 39% meant for free TAP50a. For immobilized TAP50a, it was shown that the protease activity was also higher by using $CaCl_2$ and followed by $MnCl_2$, $MgCl_2$, KCl and NaCl with an average difference was 8%-40%. These indicate that the enzyme requires metal ions as cofactors. There are also researchers who inform that Na^+, K^+ and Mg^{2+}increased *S. xylosus* A2 protease activity [22]. A similar effect was observed between free and immobilized xylanase, which increased its residual activity from 84% to 87% respectively [23]. Overall protease activity of immobilized TAP50a was higher than free TAP50a.

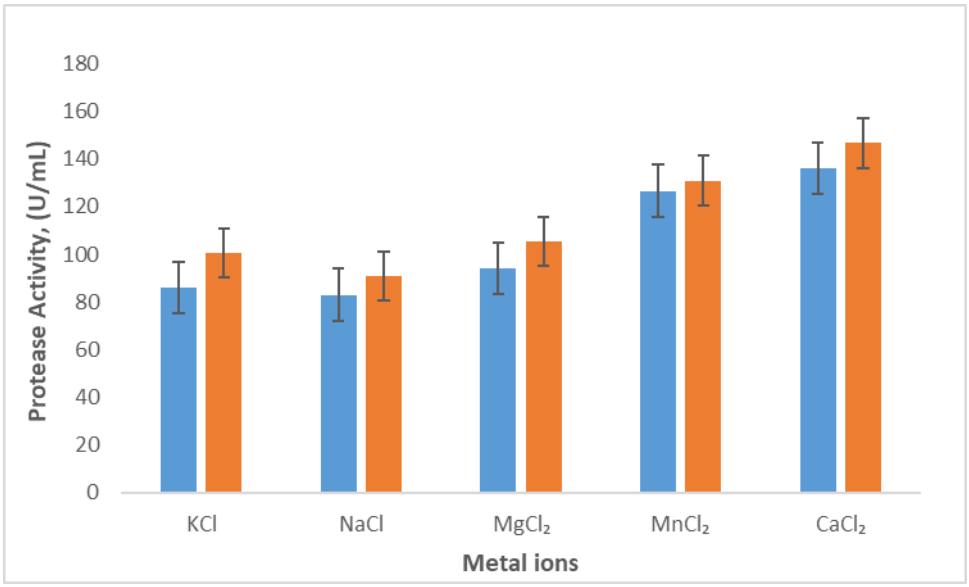

Fig. 5: Effect of metal ions on ( ) immobilized TAP50a and ( ) free TAP50a activity.

4. Conclusions

In conclusion, thermostable alkaline protease (TAP50a) has been successfully immobilized using the entrapment immobilization method using the calcium alginate. The optimum activity of the immobilized TAP50a

was observed at 90°C and it was able to maintain its activity for 120 minutes at the same temperature. It was also proven that immobilized TAP50a has more resistance at high temperatures than free TAP50a. The results achieved showed that the immobilized TAP50a can be used for further study due to its characteristics.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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