International Journal for Research in Engineering Application & Management (IJREAM)
ISSN : 2454-9150 Vol-07, Issue-02, MAY 2021

Immobilization and Characterization Of Protease And Peroxidase From Crude And Purified Extracts Of *Carica papaya* Seeds And *Brassica oleracea* Wastes

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ABSTRACT - The organic solid wastes of high biodegradability contributing to pollution can be used as a source of bio-valuable products since they are rich in bio-active compounds. One of the important biomolecules of diverse use is the enzymes and these enzymes could be made stable, resistant, and efficient by immobilization. In this study, we isolated enzymes; protease and peroxidase from the wastes of papaya (seeds) and cabbage (leaves). The crude and purified extracts were immobilized by different methods and tested for their reusability and storage activity. Their residual activity was studied and the samples were characterized based on pH and temperature. The comparative study of pure and immobilized samples and as well as the effective kind of immobilization could be helpful for the proper selection of samples to be used for applications.

KEYWORDS - ***Alginate- Chitosan, Agar- Agar, Immobilization, Fruit and Vegetable wastes, Protease, Peroxidase and Sodium-Alginate.***

I. INTRODUCTION

Our world is facing a major environmental catastrophe due to improper solid waste management (SWM) [11]. In India, approximately 0.4 million tons of solid waste is generated annually. The enormous waste production is triggered by the surge in industrialization and urbanization as a result of the exponential population growth [21]. SWM faces a downside in fulfilling the criteria of environmentally effective, socially acceptable, and affordable management [15]. More than 1.3 billion tons of food wastes are generated per year according to FAO (Food and agricultural organization of the United Nations). There are two main categories of food wastes: animal wastes (diary, fisheries, and meat processing industries) and plant wastes (roots, cereals, fruits, and vegetables). Food waste reduction and utilization can reduce negative environmental and socioeconomic impacts [6]. The Vegetable and Fruit wastes (VFW) are categorized based on their stages of production, distribution and transportation, processing, retailing/supply, and consumer level [16]. VFW cannot be subjected to incineration due to its high moisture content, and when disposed of as landfills they can produce a large amount of leachate due to their high organic content [22].

These residual agricultural wastes are rich in bio-active compounds like phenolic compounds and secondary metabolites; hence its re-utilization does not limit its application to food\feed additives, nutraceuticals, and cosmeceuticals but also helps in the production of other value-added products as well as a beneficial measure for the environment [7]. Researchers used microbial methods to successfully develop products like enzymes, bio-ethanol, flavouring agents, etc from VFW [16]. The high substrate specificity, easier production, and green chemistry became why the process of bio-catalysis is applied to every sector [20].

Proteases are one of the most common degradative enzymes found in plants and animals [8]. The seeds of *Carica papaya* that contribute to VFW are a rich source of proteases. These are cysteine proteases among which are the most abundant Papain-like cysteine proteases (PLCPs) [4]. The papaya proteases have four types of cysteine proteases; papain (<10%), glycyl endopeptidases III and IV (23-28 %), chymopapain A and B (26-30 %), and caricain (14-26 %) forming a total protein content of 70-90%. The proteases have broad thermo-stability and specificity. The papain belongs to the hydrolases group. They have an isoelectric point at pH 8.75, a molecular weight of 23,406, and

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optimum catalytic activity at the range of pH 5-8 [5]. The papain has applications in the medical field; against cancer, inflammation, diabetes, etc. The papain from seeds is found to have anthelmintic properties [4]. It is also used in the clarification of beverages, as meat tenderizing agents, and as a digestive aid [13].

Peroxidases are hemoproteins that are widely found in nature. They play an important role in catalyzing the reduction of H202 which is an end product of oxidative metabolism [2]. The cabbage heads and leaves which form the part of the VFW are rich in peroxidases. The cabbage peroxidases are known to be a heterogenous heme protein that possesses high heat stability, optimum activity in the range of 6-8 pH, and are a good choice for effective immobilization [2]. The Cabbage peroxidases have quite versatile applications in different fields. They are efficient for the construction of stable bio-electrocatalytic systems for oxygen reduction, blanching procedures in the food industry due to their heat stability [5]. and in the removal of pollutants (phenol and dye) [14].

The extensive commercialization of enzymes has its limit due to the expense, low reusability, and other dependent parameters. These challenging factors can be tackled by efficient immobilization; the confinement of enzymes to a suitable solid matrix/support. The ideal matrix is chosen based on the characteristics like inertness, physical strength, stability, reusability, ability to enhance enzyme activity, reduce product inhibition, prevent contamination, and as well as be economical. Entrapment, adsorption, covalent binding, and membrane confinement are the different methods of immobilization [20].

II. MATERIALS and METHODS

1. Preparation of crude and purified homogenate

The papaya and cabbage wastes were collected from the local market and cleaned.154 g of papaya seeds was homogenized with 0.1 M phosphate buffer, pH 7.2, and centrifuged at 4 ºC for 20 minutes at 5000 rpm and filtered through Whatman No 1 filter paper. 200 g of cabbage wastes were homogenized with 0.1M Tris HCl, pH 7.5. The solution obtained was centrifuged at 4 ºC 20 minutes at 5000 rpm and filtered through Whatman No 1 filter paper to obtain a clear solution. This filtrate was subjected to thermal treatment at 65 ºC for 5 minutes, followed by cooling on ice for 25 minutes to selectively remove contaminate traces of other enzymes. These crude extracts were subjected to Ammonium Sulphate precipitation (0-100% concentration), followed by dialysis of the specific aliquots having a high concentration of enzyme. Both the samples containing enzymes were then used for immobilization.

2. Immobilization

2.1 Sodium-Alginate

This is one of the common methods of immobilization by entrapment by the formation of Calcium-Alginate beads. The sodium alginate mixture (2%) is mixed with enzyme solution in the ratio of 1:1. Using a 10 ml syringe, this mixture was added dropwise into 0.2M $CaCl_2$. Left the beads formed to harden for 20 minutes and washed with deionized water for a couple of times followed by respective buffers and stored at 10 ºC.

2.2 Alginate-Chitosan

200 mg of alginate was dissolved in 10 ml of the respective buffers at 40 ºC for 30 minutes and brought to room temperature to which liquid enzyme (0.5 mg/ml) was added and stirred till a complete homogenate was obtained. Another solution was prepared by mixing 500 mg of chitosan in acetic acid (v/v, 2%) and heated at 50 ºC. 0.7 M $CaCl_2$ was added into this clear solution and stirred. In order to form the beads, the Enzyme-Alginate mixture was added to Chitosan-$CaCl_2$ using a syringe and these beads were washed using the buffer and stored at 10 ºC.

2.3 Agar-Agar

2 % agar was prepared in respective buffers by heating at 50 ºC. When the solution reaches room temperature equal volume of enzyme solution was added and poured into plates to solidify. The gels formed were cut into cubed pieces, washed with buffer, and stored at 10 ºC.

3. Protease assay

The reaction was carried out in triplicates.1 ml of the enzyme and 0.5 ml of casein was incubated for 10 minutes and to which 5 ml of TCA followed by 30 minutes of centrifugation at 5000 rpm was done. 2 ml of the supernatant was mixed with 5 ml of Sodium Carbonate and 1 ml of Folin-ciocalteau and incubated for 10 minutes. The colour developed was measured colorimetrically at 670 nm. Enzyme activity was calculated from the formula,

Units/ml enzyme =

µmol tyrosine equivalents released x total vol. of the assay (ml)

vol. of enzyme used (ml) x time of assay in minutes x vol. used in colorimetric determination (ml)

4. Peroxidase Assay

The 100 µl of the enzyme was mixed with 3 ml buffer followed by 50 µl guaiacol and 30 µl H_2O_2 and the colour developed was read at 450 nm from time to time for 10 minutes.

Enzyme activity (Units/L) = (ΔAbs ×Total assay vol)/Δt×€×l× enzyme sample volume

5. Determination of reusability and storage

For the reusability test, the assays were repeated for alternate days for up to 9 and 7 days for crude and purified samples. For the storage test, the stored immobilized samples were assayed on a period of 5 or 7 days. The residual activity was recorded after each assay.

6. Characterisation based on pH and Temperature

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For pH, the reaction was carried out in triplicates at 5-9 pH and for temperature, 10,30,50,70 C were taken. At those particular conditions, the enzyme activity was recorded and studied.

III. RESULTS

1. Immobilized Crude Protease

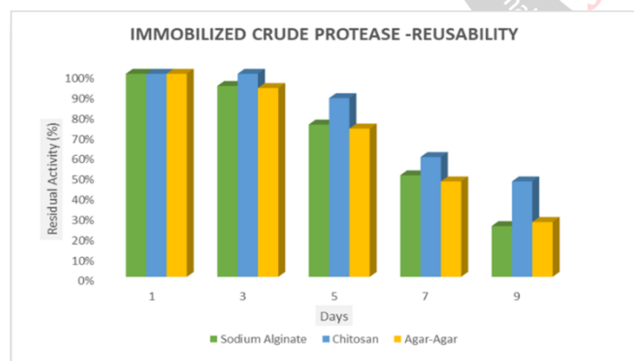
50 ml of crude was used to produce 43.12 g Sodium-Alginate beads and 64 g of Agar-Agar gels. 23 g of Alginate-Chitosan beads were made from 10 ml of the extract. The beads are shown in figure 1.

Figure 1: a) Sodium-Alginate b) Alginate-Chitosan and c) Agar-Agar

1.1 Determination of reusability

The reusability of all the beads was checked during the period of alternate 9 days. Figure 2 shows the gradual decrease in the immobilized crude protease activity. The enzymes entrapped in Sodium-Alginate beads showed a 100 % activity for the first day and 94, 75, 50, and 25 % respectively for the 3rd, 5th, 7th and 9th day. This shows that from the 3rd day, it showed a decline in activity by 25% till the 9th day. For Alginate-Chitosan beads, the enzyme activity showed a stable 100% activity till the 3rd day, then it showed a gradual decline by 12-50 %, whereas in the case of Agar-Agar gels the enzyme activity recorded were 100, 93, 73, 47 and 27 % till the 9th day.

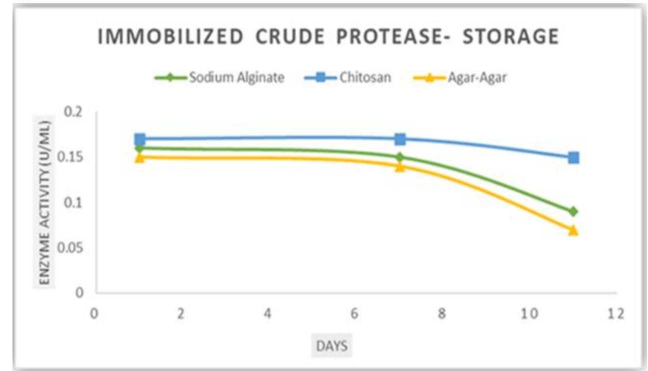
Figure 2: Determination of reusability of immobilized crude protease



1.2 Determination of storage capacity

The enzyme activity of the stored beads and gels is shown in figure 3. The activity was checked for the 1st ,7th, and 11th day. The stored beads of Alginate-Chitosan showed a constant 100% activity for 7 days and the curve declined by 12% by the 11th day. The enzyme activity was a 100, 94 and 56 % from the Sodium-Alginate beads. The Agar- Agar gels had 100, 94 and 47 % enzyme activity. There-fore the Sodium-Alginate beads and Agar-Agar gels showed a 6 % decline by the 7th day and 44 % and 53 % respectively by the 11th day.

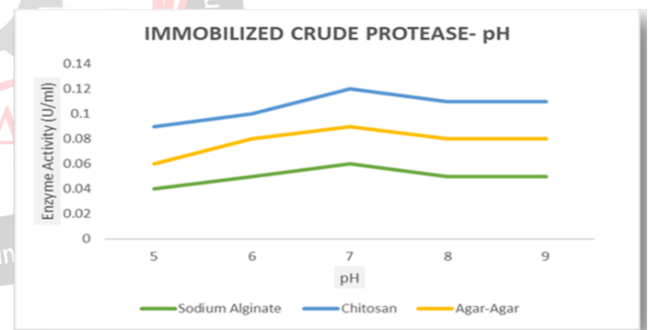
Figure 3: Determination of storage capacity of immobilized crude protease



1.3 Effect of pH

The pH stability was monitored using phosphate buffers of varying pH 5, 6, 7, 8, and 9. Figure 4 represent the findings of the pH stability. The Sodium- Alginate samples had enzyme activity of 66, 83, 100, 83 and 83 % at pH 5, 6, 7, 8 and 9. Whereas it was 75, 83, 100, 92 and 92 % for the Alginate- Chitosan samples and a 67, 89, 100, 89 and 89 % for the Agar- Agar samples for the respective pHs. The enzyme activity showed a peak in pH 7 compared to the other ranges in all the three types of immobilized samples. There was a slight decrease in pH 5 and 6 and the activity remained constant through pH 8-9 for the Sodium-Alginate, Alginate-Chitosan and Agar-Agar samples.

Figure 4: Effect of pH on immobilized crude protease



1.4 Effect of temperature

The temperature variation is depicted in figure 5. The enzyme activity from all the beads and gels was tested at temperatures 10 °C, 30 °C, 50 °C, and 70 °C. The enzyme activity was dependent on the temperature. There was a gradual increase in activity with the temperature in case of all samples. The enzyme activity was 50, 67, 100, and 100 % at the respective temperatures for the Sodium-Alginate samples and it was a 76, 82, 94 and 100 % for the Alginate-Chitosan samples. The Agar- Agar samples showed an enzyme activity of 50, 60, 90 and 100 % at temperatures 10 °C, 30 °C, 50 °C and 70 °C. The lowest activity was shown at 10 °C and a stable increase was recorded through 30 °C – 50 °C. The optimum activity was shown at 70 °C for all kinds of immobilization.

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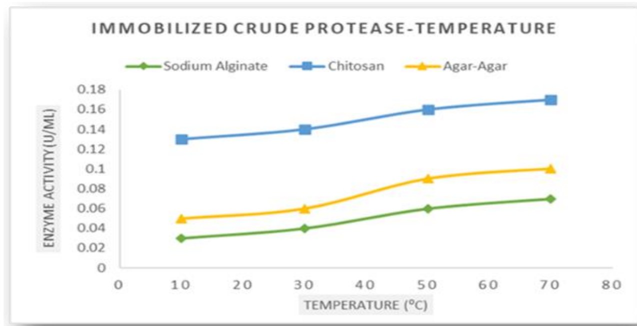
Figure 5: Effect of temperature on immobilized crude protease



2. Immobilization of Purified Protease

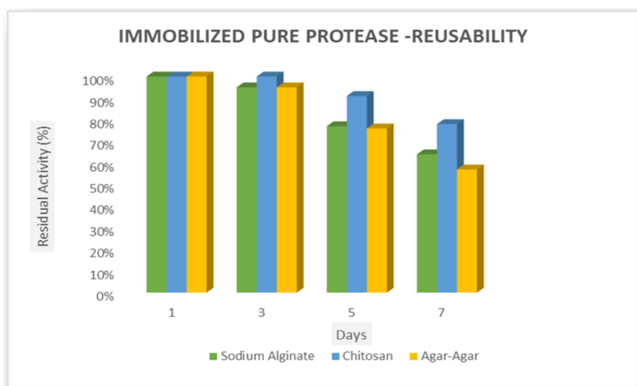
50 ml of the purified sample yielded 44 g and 60 g of Sodium-Alginate beads and Agar-Agar gels and 10 ml yielded 32g of Alginate-Chitosan beads as shown in figure 6.

Figure 6: a) Sodium-Alginate b) Alginate-Chitosan and c) Agar-Agar

2.1 Determination of reusability

The reusability data studied for all three types of immobilization is given in figure 7. The enzyme activity was checked for a period of alternate 7 days. For the first 3 days, the Alginate-chitosan beads released enzyme in a constant amount and it showed 100% activity. The rate decreased by 9% and 12% by the next 4 days. The enzyme activity recorded for Sodium-Alginate samples were 100, 95, 77, and 64 % for the 1st, 3rd, 5th, and 7th day. The Agar-Agar samples had 100, 95, 76, and 57 % of enzyme activity for the respective alternate days. Hence by the 7th day the enzyme activity decreased by around 30 and 40 % for the Sodium-Alginate and Agar-Agar samples.

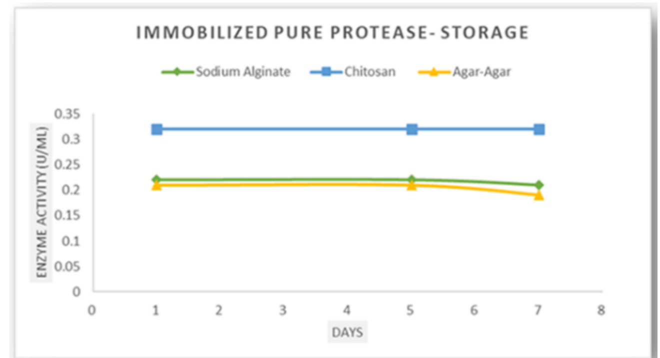
Figure 7: Determination of reusability of immobilized purified protease



2.2 Determination of storage capacity

The storage capacity of the immobilized beads and gels is depicted in figure 8. The enzyme activity remained linear showing 100% of its effect for the stored Alginate-Chitosan beads when checked for the 5th and 7th day. In the case of Sodium-Alginate and Agar-Agar, the activity was 100% when checked on the 5th day and decreased by 5% and 14% respectively when checked on the 7th day since it showed a residual activity of 95 and 90 %.

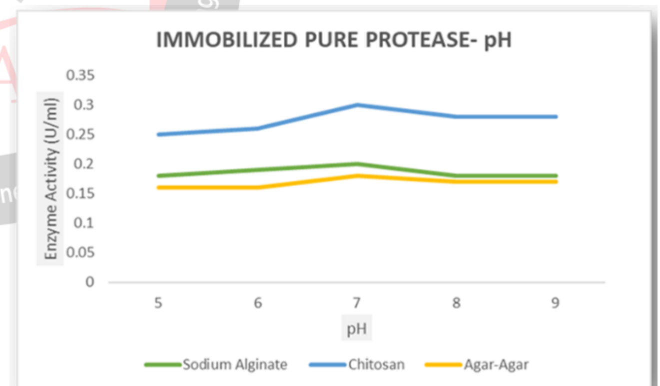
Figure 8: Determination of storage capacity of immobilized purified protease



2.3 Effect of pH

The pH of the varying range tested is depicted in figure 9. Phosphate buffers of pH 5,6,7,8, and 9 were used to record the stability of the immobilized enzyme. There was a gradual increase in the activity from pH 5-7, with the highest activity at 7. After that, the activity started to decline from pH 8-9. The enzyme activity was 90, 95, 100, 90 and 90 % for the Sodium-Alginate samples and 89, 89, 100, 94 and 94 % for Agar-Agar samples. The Alginate-Chitosan samples had an enzyme activity of 87, 93, 100, 93 and 93 % at pH 5, 6,7,8, and 9.

Figure 9: Effect of pH on immobilized purified protease



2.4 Effect of temperature

The thermal activity was recorded at temperatures 10 ºC, 30 ºC, 50 ºC, and 70 ºC as shown in figure 10. There was a stable linear increase in the activity for Alginate- Chitosan, Sodium-Alginate and Agar-Agar samples. The optimum activity was observed at temperature 70 ºC for all the samples. The samples of Sodium-Alginate showed an activity of 71, 86, 96 and 100 % and those of Agar-Agar had 83, 89, 92 and 100 % activity at those respective temperatures. The enzyme activity was 81, 90, 96 and 100 % for the Alginate- Chitosan samples at 10 ºC, 30 ºC, 50 ºC and 70 ºC.

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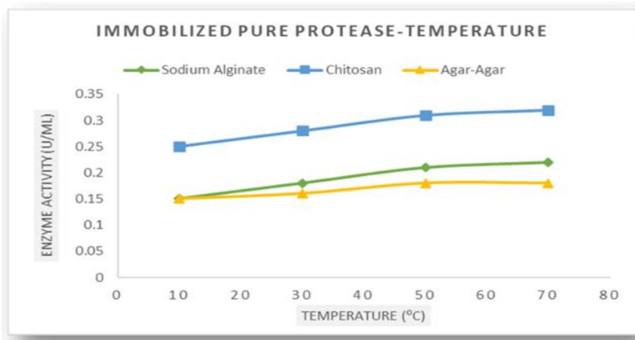
ISSN : 2454-9150 Vol-07, Issue-02, MAY 2021

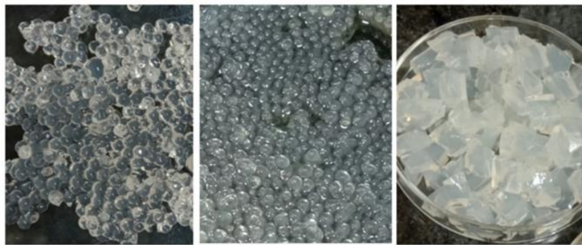
Figure 10: Effect of temperature on immobilized purified protease



3. Immobilization of Crude Peroxidase

The 50 ml of sample yielded 51 g and 62 g of Sodium-Alginate beads and Agar-Agar gels respectively. 10 ml of the sample yielded 22 g of Alginate-Chitosan beads (Figure 12).

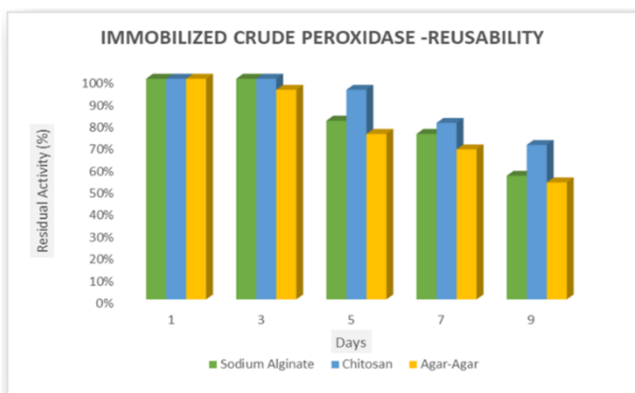
Figure 12: a) Sodium-Alginate b) Alginate-Chitosan and c) Agar-Agar



3.1 Determination of reusability

The reusability of immobilized crude peroxidase was tested alternatively for 9 days as shown in figure 13. The Alginate-Chitosan and Sodium-Alginate samples showed 100% activity for the first 3 days and the former showed a decline by 5% whereas the latter by 18% by the 5th day. The enzyme activity was 82, 75, and 56 %; 95, 80 and 70 % during the 5th, 7th, and 9th day for Sodium-Alginate and Alginate-Chitosan samples. The enzyme activity observed for Agar-Agar was 100, 95, 79, 68, and 53 % for the alternate 9 days. The Agar-Agar samples showed a decline by 50 % by the 9th day.

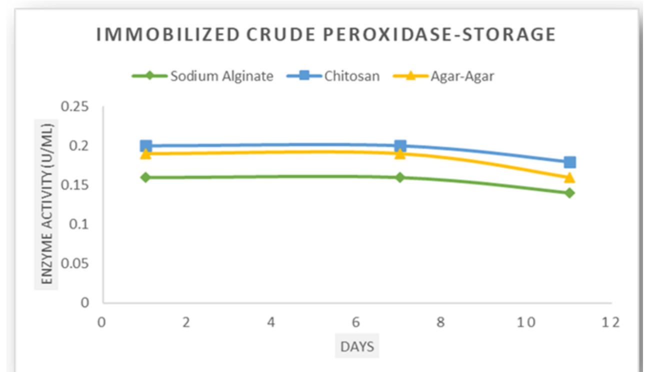
Figure 13: Determination of reusability of immobilized crude peroxidase



3.2 Determination of storage capacity

Figure 14 shows the storage capacity of the immobilized crude samples for 11 days. The Alginate-Chitosan sample showed a 100% activity for 7 days and declined by 5% on the 11th day since it recorded a 95 % of enzyme activity. The Sodium-Alginate and Agar-Agar samples showed 100% activity when checked on the 7th day and decreased by 12% and 15% respectively for the 11th day. On the 11th day the Sodium-Alginate and Alginate-Chitosan showed an enzyme activity of 87 and 84 %.

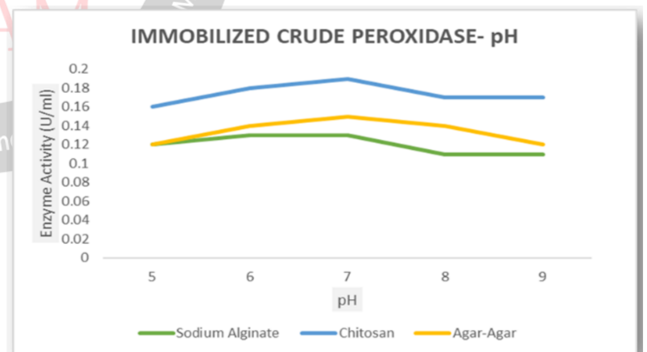
Figure 14: Determination of storage capacity of immobilized crude peroxidase



3.3 Effect of pH

The pH ranges of 5-9 were checked as shown in figure 15. The tris HCl buffer of varying pH was used to check for stability. The samples showed increased stability from 5-7 pH with its peak at pH 7. The activity declined for the pH range 8-9. The Sodium-Alginate showed enzyme activity of 100 % at pH 7 and 85 % for both pH 8 and 9. At an acidic pH of 5 it showed 92 %. The Alginate-Chitosan samples showed an increasing activity of 89, 95 and 100 % from pH 5-7 and a 89 % of activity at 8 and 9 pH. The Agar-Agar gels showed an enzyme activity of 80, 93, 100, 93 and 80 % at the respective pHs.

Figure 15: Effect of pH on immobilized crude peroxidase



3.4 Effect of temperature

The thermal stability was tested at temperatures at 10 ºC, 30 ºC, 50 ºC, and 70 ºC is shown in figure 16. The activity increased gradually from 10 ºC-50 ºC with its peak at 50 ºC. At 70 ºC, the activity started decreasing by 20-40 % in the case of all the samples. The Sodium-Alginate samples showed 86, 93, and 100 % activity and the Alginate-Chitosan showed 84, 95, and 100 % activity at temperatures 10 ºC, 30 ºC, and 50 ºC. The Agar- Agar samples had an activity of 87, 94, and 100 % till 50 ºC. At 70 ºC, the activity recorded was 71, 65, and 81 % for the Sodium-Alginate, Alginate-Chitosan and Agar- Agar samples.

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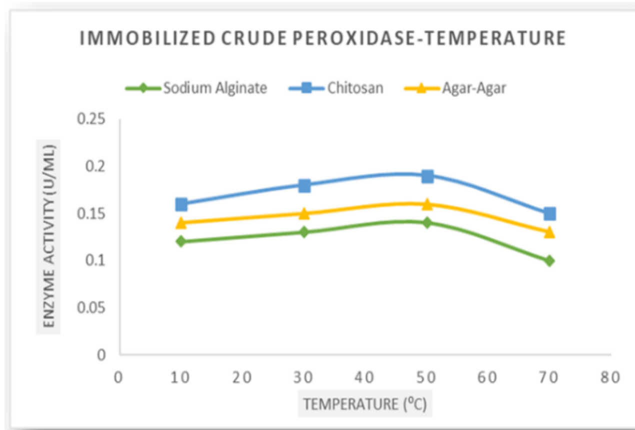
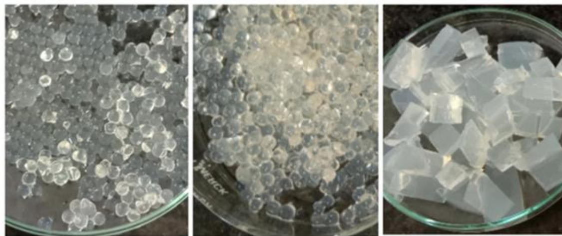
Figure 16: Effect of temperature on immobilized crude peroxidase



4. Immobilization of Purified Peroxidase

50 ml of the purified sample yielded 50 g and 58 g of Sodium-Alginate beads and Agar-Agar gels and 10 ml yielded 30 g of Alginate-Chitosan beads as shown in figure 17

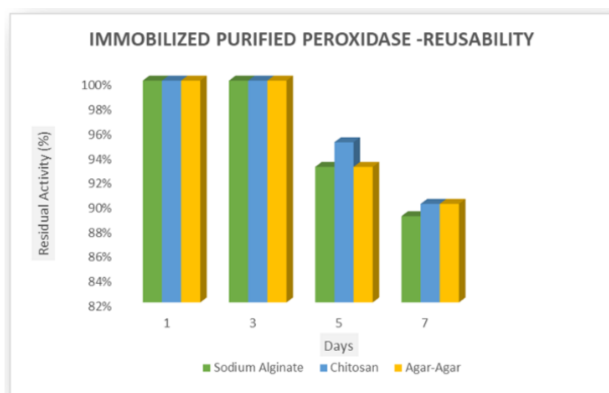
Figure 17: a) Sodium-Alginate b) Alginate-Chitosan and c) Agar-Agar



4.1 Determination of reusability

The reusability of the immobilized pure peroxidase was tested for 7 days as shown in figure 18. From the graph, it is evident that all the samples showed maximum activity till the 3rd day. For the next 4 days the activity showed a gradual decline of 8-11%. The enzyme activity for Sodium-Alginate samples was 92 and 89 % and for Agar-Agar samples it was 93 and 90 % for the 5th and 7th day. The Alginate-Chitosan samples displayed an enzyme activity of 93 and 90 % on the last two alternate days.

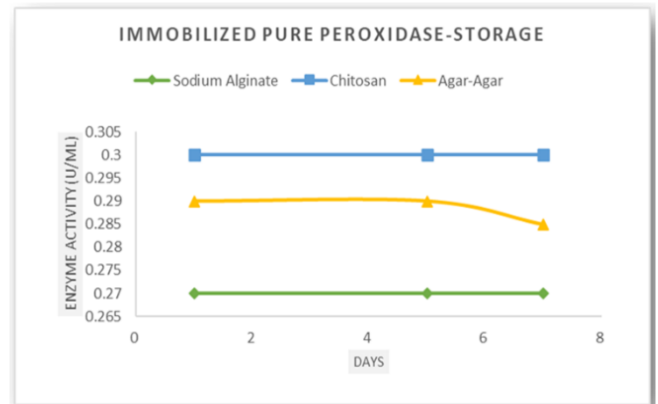
Figure 18: Determination of reusability of immobilized purified peroxidase



4.2 Determination of storage capacity

The storage capacity of the stored samples of immobilized pure peroxidase is depicted in figure 19. It shows a stable and uniform enzyme activity of 100 % for 7 days in the case of the Alginate-Chitosan samples. For the Agar-Agar samples, it showed a 100 % activity for the 5th day and declined by 4% on the 7th day since it recorded an enzyme activity of 96 %. The Sodium-Alginate samples also showed a 100 and 96 % activity on the 5th and 7th day.

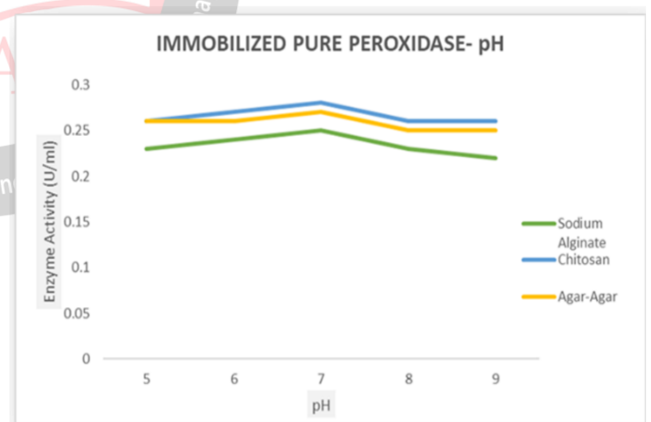
Figure 19: Determination of storage capacity of immobilized purified peroxidase



4.3 Effect of pH

The pH range of 5-9 was tested as shown in figure 20. The samples show a gradual increase from pH 5-7 with peak activity at pH 7 and a decline from 7-9 pH. The Sodium-Alginate showed an enzyme activity of 92, 96, 100, 92 and 88 % at the respective pHs (5, 6, 7, 8 and 9). The Agar-Agar samples showed a 96 % activity at pH 5 and 6; 92 % at pH 8 and 9; and a 100 % at pH 7. The enzyme activity recorded for the Alginate-Chitosan samples were 93, 96, 100, 93, and 93 %. This shows the peak activity at pH 7.

Figure 20: Effect of pH on immobilized purified peroxidase



4.4 Effect of temperature

The thermal stability was recorded at temperatures 10 ºC, 30 ºC, 50 ºC, and 70 ºC as shown in figure 21. The activity showed a constant increase from 10-50 ºC and steeps down at 70 ºC. The enzyme activity was 88 and 96 %; 86 and 96 %; 89 and 96% at temperatures 10 ºC and 30 ºC for the Sodium-Alginate, Alginate-Chitosan and Agar-Agar samples. All the beads and gels recorded a 100 % activity at 50 ºC. At 70 ºC, the activity recorded was 81, 79, and 74 % for the Sodium-Alginate, Alginate-Chitosan and Agar-Agar samples.

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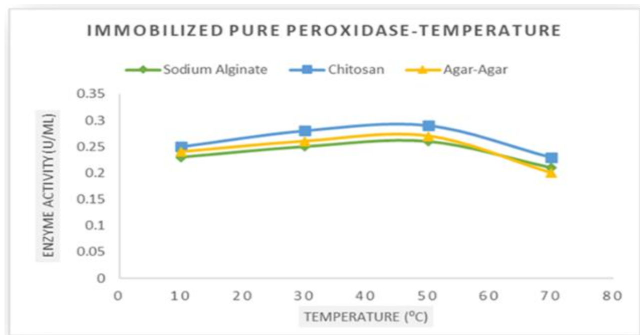
Figure 21: Effect of temperature on immobilized perified peroxidase



IV. DISCUSSION

The immobilized crude protease of the Alginate-Chitosan method retained 47% of residual activity by the 9th day. The Sodium-Alginate and Agar-Agar immobilization left a residual activity of 25 and 27% of crude protease. This shows the reusability of the enzyme was effective for Alginate-Chitosan immobilization compared to the other two methods. The decrease in enzyme activity might be due to the frequent thawing and change of buffers. [19] stated that the residual activity was 50 % up to 10th use when protease immobilized on Glutaraldehyde-Activated Chitosan matrix was used. For the storage capacity, the enzyme activity was 88 % on the 11th day for Alginate-Chitosan immobilized protease and 56 and 47 % respectively for Sodium-Alginate and Agar-Agar methods. This proves the storage time of much effective with minimal loss of activity is possible with Alginate-Chitosan immobilization of crude protease. The activity remained constant through pH 8-9. The optimum pH was 7 for immobilized plant protease [17]. The stability of pH is achieved by all the types of immobilization since the difference between the optimum pH and the consecutive ones is not more than 30 %. The pH stability is high for immobilized proteases compared to soluble proteases [19]. The optimum temperature range of immobilized proteases is around 70 ºC [9]. The broad range of thermal stability shows that immobilization could be an effective and ideal method to obtain high yield in industrial applications.

The residual activity of immobilized purified protease was much higher compared to the immobilized crude protease. They showed a 78, 64 and 57 % activity by the 7th day for Alginate-Chitosan, Sodium-Alginate, and Agar-Agar respectively. In another study, the activity of immobilized protease and purified protease was 70 % for 10th reuse and 65 % for the 6th cycle [10] and [12]. This shows that purified protease could be effectively reused when immobilized on the Alginate-Chitosan matrix followed by the other two methods. In my study, the residual activity was 100,95 and 90% on the 7th day when the pure protease was immobilized on Alginate-Chitosan, Sodium-Alginate, and Agar-Agar for the storage capacity assay. This activity is twice greater than that was recorded for the crude protease making the sample ideal for storage for further use. The chances of decline in the activity after the tested time period could be only by 4 % or less if stored under proper conditions. According to [12], the storage activity decreased by 12 % after 10 days. The immobilization protected the enzyme enabling a broad range of pH stability. The temperature of 70 ºC being optimum is supported by [9]. The immobilized pure protease shows a broad range of thermal stability.

The immobilized crude peroxidase showed residual activity of 70,56, and 53% for the 9th day for Alginate-Chitosan, Sodium-Alginate, and Agar-Agar methods. The chitosan method of immobilization has high reusability compared to others. Immobilization of peroxidase from a different source showed 75 % reusability in another study [1]. The stored samples showed a residual activity of 95,87 and 84% for Alginate-Chitosan, Sodium-Alginate, and Agar-Agar respectively when checked for the 11th day. It is evident that the Chitosan matrixes retained the enzyme activity effectively compared to the other two. [1] stated the storage stability of immobilized peroxidase dropped to 63 % after 8 weeks. The optimum activity between pH 6-7 is supported by [3]. This shows the immobilized crude peroxidase showed activity at a broad with only a 5-10 % difference compared to the optimum pH. The broad range of thermal stability is an advantage for industrial applications.

The residual activity of immobilized purified peroxidase was higher than immobilized crude peroxidase as it showed. The enzyme activity was 90,90 and 89% for Alginate-Chitosan, Agar-Agar, and Sodium-Alginate methods. This shows both Chitosan and agar matrixes were efficient in maintaining the enzyme activity [18]. The storage capacity of immobilized purified peroxidase is higher than immobilized crude samples. They showed a residual activity of 100,96 and 96 % on the 7th day for Alginate-Chitosan, Sodium-Alginate, and Agar-Agar samples. The Chitosan matrixes retained the enzyme activity efficiently, followed by the other two types. [18] stated that the storage capacity of immobilized peroxidases relatively higher than peroxidases. The optimum pH activity was observed in the range of 6-7 [1]. The activity was quite higher than the immobilized crude peroxidase. Even though it shows a decline from the optimum activity, there is a broad range of stability. The high activity of cabbage peroxidase at 50 ºC is supported by [3]. The immobilized enzyme shows significant stability over a range of temperatures. At very high temperatures the enzyme behaves inversely proportional to the change.

The methods of immobilization of protease and peroxidase were compared as shown in figures 22 & 23. Among the three types (Sodium-Alginate, Alginate-Chitosan, and Agar-Agar), the Alginate-Chitosan immobilized crude and purified samples showed promising results when compared to the Sodium-Alginate and Agar-Agar immobilization. The enzyme activity was high (as evident in the given figures) when the reusability and storage capacity were checked using the same making it the ideal form of immobilization

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to opt. When compared to the Alginate-Chitosan (100 %) the activity for Sodium-Alginate and Agar-Agar for immobilized crude and purified protease is 94 and 88 %; 68 and 65 % whereas for the immobilized crude and purified peroxidase is 80 and 95 %; 90 and 97 % respectively. The Sodium-Alginate showed the second highest activity for immobilized protease samples whereas it is the Agar-Agar method for the immobilized peroxidase samples. It could be the stronger bonding resulting in greater stability compared to the latter. The Agar-Agar showed relatively less activity and stability for protease. It was expected of it because the gels started to break down or dissolve after a few experiments. Chitosan mixed matrixes were much stable and efficient since they showed promising results when tested under varying pH and temperatures. Future experimentations can be carried out using different concentrations of the all these matrices to increase the efficiency furthermore.

Figure 22: Comparison of Immobilized crude and purified Protease based on its activity

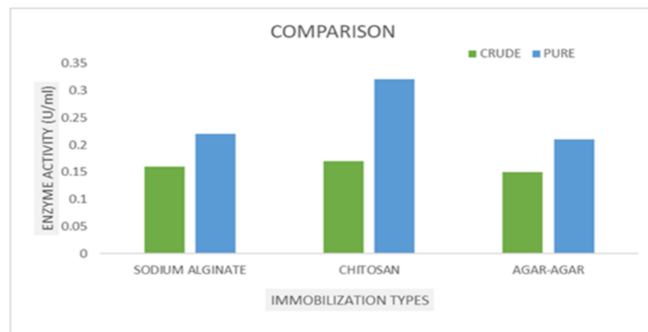
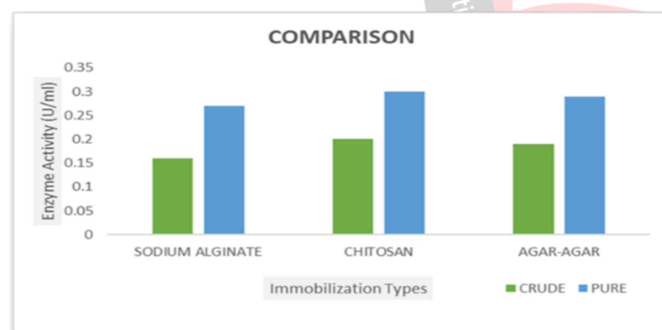


Figure 23: Comparison of Immobilized Crude and Purified Peroxidase based on its activity



V. CONCLUSION

Immobilization by different methods helps to understand the interaction between enzymes and the matrices. The immobilized purified samples exhibited high enzyme activity, reusability, and storage with a thermal and pH stability compared to the immobilized crude samples. The reusability assay of the immobilized crude and purified protease showed that the enzyme activity was 53 % by the 9th day and 78 % by the 7th day for the immobilized crude and purified samples of Alginate-Chitosan methods which was the highest compared to the Sodium-Alginate and Agar-Agar methods. The stored samples of immobilized crude and purified protease had 88 and 100 % activity on the 11th and 7th day. The stored Sodium-Alginate and Agar-Agar samples of immobilized purified protease also showed high level of activity of 95 and 90 % on the 7th day. Both the immobilized crude and purified protease had optimum pH of 7 and a temperature of 70 ºC. This activity showed only little variation at other ranges of the chosen parameters proving the stability range of the immobilized samples. In case of peroxidase, the reusability assay showed 70 % and 90 % activity for the Alginate-Chitosan samples of immobilized crude and purified enzyme on the 9th and 7th day. The immobilized purified samples of peroxidase showed 89 and 90 % activity for the Sodium-Alginate and Agar-Agar samples on the 7th day. The stored immobilized crude and purified peroxidase of Alginate-Chitosan method had 95 and 100 % activity when checked on the 11th and 7th day. All the peroxidase samples had an optimum pH of 7 and temperature of 50 ºC. These findings conclude the effective method of immobilization as well as the source needed to be used. The immobilized forms of these enzymes can reduce the cost and increase the efficiency of product formation with minimal waste since both protease and peroxidase finds application in wide variety of fields. The immobilized purified protease can be applied for detergent industry, to remove different types of stain, since the free enzymes are susceptible to denaturation in the presence of other chemicals like oxidizing agents. These immobilized proteases are well protected within a porous matrix hence they will not be destroyed by other cells of the body hence can also be used to study and treat cancer and diabetes. The immobilized peroxidases also showed a promising activity at different ranges of pH and temperature hence these, especially the purified ones will be able to remove effluents and dyes from the polluted waters since it can withstand the harsh conditions of wastewater treatment.

ACKNOWLEDGEMENT

I owe a special tribute to God Almighty for the opportunity given to take up to complete my work successfully. I express my deep sense of gratitude to all my higher authorities, Dr. (Thiru) S.P. Thiyagarajan, chancellor, Former Chancellor Dr. (Thiru) P.R. Krishna Kumar, (Late), Dr. Premavathy Vijayan, Vice Chancellor, Dr. S. Kowsalya, Registrar, Dr. A. Vijayalakshmi, Dean, School of Biosciences, Professor and Head, Department of Botany, Dr. P.R. Padma (Late), former Dean and Head, Department of Biochemistry, Biotechnology and Bioinformatics, and Dr. G.P. Jeyanthi, Director, Research and Consultancy. I express my gratitude to my co-authors Dr. A. Shobana, Assistant Professor, Department of Biochemistry, Biotechnology and Bioinformatics, Dr. Sujatha, Assistant Professor, Department of Biochemistry, Biotechnology and Bioinformatics, and Dr. Anitha Subash, Professor and Head, Department of Biochemistry, Biotechnology and Bioinformatics, of Avinashilingam Institute for Home Science and Higher Education for Women for their immense support. I submit my sincere thanks to all The Staff Members of the Department. I express my sincere

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gratitude to my parents Nancy.J and Jayan.J for their moral support & guidance throughout my study. I express my sincere heart bound thanks to my friends and seniors, Department of Biochemistry, Biotechnology and Bioinformatics, for giving an affectionate advice, unconditional love and incredible supports for the completion of my project work.I acknowledge the contribution of all other unseen hands during the course of the study for help rendered in the successful completion of the study.

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