Screening gelatin hydrogel and calcium alginate beads as scaffold approach for immobilization of horseradish peroxidase enzyme: Comparative operational and thermal stability study

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Abstract:

Immobilization of enzymes is a highly beneficial technique where it augments industrial process economics by allowing enzyme re-use and improving process productivity and robustness. Horseradish peroxidase (HRP) is a highly available enzyme of wide industrial applications. Calcium alginate (Ca-Alg-HRP) and gelatin (Gel-HRP) were screened for the immobilization of hydrogen peroxide enzyme for improvement of its industrial applications. The immobilization procedure onto Ca alginate beads was accomplished by gelation method where calcium ions were used as cross-linking agent. For gelatin hydrogel, glutraldehyde was added to enzyme/gelatin mixture to complete cross-linking. The effect of immobilization on the pH and temperature profile, thermal and operational stability and reusability of biocatalyst activity was assessed. Immobilization caused a change in pH/activity profile of HRP with a marginal shift in optimum pH from 7.0 to 7.5 for free and immobilized HRP, respectively. Whereas the temperature/activity profile was improved upon immobilization. Both thermal and operational stability were enhanced as a result of immobilization. The half-life of immobilized HRP at both 50°C and 70°C was found to be higher than the free enzyme with a reported half life of 1034 min and stabilization factor of (61.5%) . Reusability of the immobilized HRP was also studied and the results showed that both methods allowed for consecutive runs with residual catalytic activity of 80% after five successive cycles. The suggested immobilization supports proved to provide protective effect for HRP with improved thermal and operational stability and allowed re-use and recovery of the enzyme.

Keywords: catalase enzyme, gelatin hydrogel, calcium alginate beads, immobilization, stability, half life, entrapment

1. Introduction

Immobilized enzymes are efficient biocatalysts that are confined within a micro-environment whereby they maintain their catalytic efficiency. Hence, immobilization is often employed for preservation of the stability and efficiency of enzymes, rendering them more advantageous for industrial applications. In addition, immobilized biocatalysts are handled more easily and can be recycled for repeated use in batch or continuous operations [1]. Immobilization techniques include chemical and physical methods such as covalent conjugation, adsorption or ionic bindings, cross-linking and encapsulation or entrapment in membranes or sol–gel matrix. Entrapment is one of the physical immobilization techniques where the enzyme mobility is physically restriction by confinement within a carrier or a network that normally allows mass transfer [2]. Immobilized enzymes generally exhibit higher resistance to thermal and chemical denaturation due to improvement of storage and operational stability [2,3]

Immobilization by entrapment offer significant advantages relay in being a fast, low cost and reproducible method where, the process usually is carried out under mild conditions. On the other hand, major draw backs of the entrapment techniques are possibility of enzyme leakage on continuous operation and limited mass transfer, a phenomenon which is usually affected by the matrix or support chosen [3,4]

The employment of naturally occurring polymers has been the subject of interest for researchers during the past decades. Sodium alginate is a sodium salt of alginic acid. It is a natural water soluble biocompatible nontoxic polysaccharide extracted from marine brown algae. Alginates has wide applications in the food and pharmaceutical industries being employed as additives, such as tablet disintegrant, thickening, and suspending agent. Alginates are composed of alternating blocks of 1-4 α-L-guluronic and β-D-mannuronic acid residues where, the addition of divalent cations such as Ca^{2+}, Ba^{2+} and Zn^{2+} results in its transformation into hydrogels [5, 6, 7]. This property has been made use of for preparation of drug loaded beads as well as for immobilization techniques for medical and pharmaceutical applications [4,8]. The gelation process mechanism involves guluronic residues chelation with Ca^{2+} forming the so-called "*egg-box*" structure [1,9]. The alginate gelation offers the advantage of entrapping enzymes under very mild conditions without the need for high temperatures or chemical cross-linking agents [10-12].

Gelatin was one of the first naturally occurring proteins used in the formation of biomaterials. This biopolymer has many advantage points including being biocompatible, biodegradable, abundant material. In addition, it is of low cost and has outstanding functional and film forming properties whereas, it is easily transformed into gel without inactivation of the confined biocatalyst. Gelatin can also be used alone or in combination with other immobilizing agents [11, 12].

The peroxidase obtained from horseradish roots (HRP; hydrogen peroxide oxidoreductase donor, EC 1.11.1.7) is a heme enzyme containing a ferric protoporphyrin IX prosthetic group [13] . Peroxidase enzyme plays significant physiological roles in various important oxido-reductive pathways. Among other peroxidases, HRP has gained researchers interest due to its abundance, ease of purification and wide applications in the pharmaceutical, chemical,

biotechnological industries, including environmental application in the removal of phenols from polluted water and health care fields where it has been investigated in cancer therapy. HRP is regularly employed in bioassays field as a component of hydrogen peroxide biosensors for determination of hydrogen peroxide and other reactive oxygen species (ROS) in oxidative stress [14]. However, utilizing soluble enzyme offers several limitations due to non-reusability, thermal and operational instability, high cost, and possible denaturation. On the other hand, as discussed before utilizing immobilized enzymes lead to improved thermo-stability, operational stability, recyclability and recovery, reusability, high purity, lower cost and high product yields for enzyme industrial applications.

In this context, the aim of this study was to investigate the immobilization of HRP in alginate beads and gelatins hydrogel aiming to investigate and compare between the two immobilization approaches on the thermal and operational stability of catalase. Calculations of rate constant (K_d) and half life($t_{1/2}$) for free and immobilized HRP was carried out, these data has not been previously exploited according to our knowledge. The rationale of this work was linked to the aim of improving HRP stability and effectiveness enforcing its industrial and biotechnological applications.

2. Materials and Methods

2.1. Materials

Horseradish peroxidase (HRP) (250 units/mg solid), pyrogallol , hydrogen peroxide (30% w/w solution), sodium alginate, gelatin, trypsin were purchased from Sigma Aldrich (St. Louis, USA). All other chemicals used were of analytical grade from various suppliers.

2.2. Activity assay and protein determination

HRP activity assay was done using a simple and quantitative spectrophotometric assay **[15, 16]**. Horseradish peroxidase (HRP) activity was determined using H_2O_2 as the oxidizing substrate and pyrogallol as the reducing substrate. The reaction mixture (3 mL) prepared in potassium phosphate buffer (100 mM, pH 7) contained: 320µL of pyrogallol solution (0.5% w/v), 160µL of H_2O_2 (0.5% w/w). The reaction was initiated by addition of aliquots (0.1-0.2 ml) of enzyme containing sample (10 mg/mL prepared in cold phosphate buffer). The rate of formation of the colored product due to oxidation of pyrogallol was followed at wavelength (420 nm) spectrophotometrically (Schimadzu spectrophotometer-UV 2600) at 20 sec intervals. One unit of enzyme activity (U) was defined as the amount of enzyme capable of oxidizing one micromole of pyrogallol per minute under experimental conditions ($\varepsilon = 961\ M\ cm^{-1}$). Activity of HRP was expressed as U/mg protein.

Protein measurements were done according to Bradford Method using bovine serum albumin as a standard **[17]**.

2.3. Screening of Supports for peroxidase Immobilization

2.3.1. Peroxidase immobilization in calcium alginate beads

Peroxidase enzyme was immobilized in calcium alginate as described by Kawaguti et al. [18,19] with applied modifications. Calcium alginate beads were prepared by gelation method where calcium ions were used as cross-linking agent. Briefly, HRP enzyme (200 µg/ml) was added to a solution of sodium alginate (3%). After thorough mixing, the resulting solution was extruded using a syringe to calcium chloride aqueous solution (0.3 M) under constant gentle stirring, at room temperature. After 20 mins, the resulting beads (Ca Alg-HRP) were recovered by filtration, transferred to the calcium chloride solution, and allowed hardening period by incubating at 5°C for at least 4 hours. The beads were thoroughly washed with distilled water to remove any excess calcium and were dried at 40°C overnight.

2.3.2. Peroxidase immobilization in gelatin hydrogel

Immobilization was carried out according to Assis and co-workers [20]. Two grams of gelatin was allowed to swell at room temperature for 30 min in 10 mL of 50 mM potassium phosphate buffer (pH 7.0), and then was dissolved at 60 ºC. After the solution had been cooled to 37 ºC, 2 mL of gelatin solution was transferred to a small beaker, and 200 µL of HRP enzyme solution ((10 mg/mL prepared in cold phosphate buffer) were added, and the mixture was thoroughly mixed under magnetic stirring. 5 mL of a glutaraldehyde solution (10% v/v) were added to the gelatin mixture to complete cross-linking. To enable solidification the mixture was transferred to a Petri dish and stored at 5°C for2 hours. Then, the gelatin hydrogel (Gel-HRP) was cut in cubic shaped particles of similar sizes (roughly 2 mm), they were maintained in 50 mM potassium phosphate buffer (pH 7.0) at 5°C, until further required.

Immobilization yield %:

The immobilization yield was calculated as the enzyme yield % which was immobilized and calculated by the following equation:

Immobilization yield (%) = (Activity of immobilized HRP enzyme / activity of free HRP added) × 100

Catalytic activity was assayed under standard assay conditions.

2.4 Temperature Profile

For determination of optimum temperature, the activities of free and immobilized-HRP Preparations were measured at various designated temperatures (30–80 ºC) under standard assay conditions. The activity of enzyme at zero time was then taken as 100% activity.

2.5 pH Profile

The activities of free and immobilized-HRP beads and hydrogels were measured in different buffers of various pH values (sodium acetate buffer pH (4.0–6.0) and Tris–HCl buffer pH (7.0-7.5).

2.6. Operational and thermal stability

For operational stability, the free or immobilized HRP were tested at different temperatures under stress conditions. Enzyme activity was determined by incubating the enzyme samples at 50 °C and 70 °C under 120 rpm of stirring for 15-45mins. Enzyme activity was determined after equilibrium to 37 °C under standard assay conditions. Following the incubation period; the residual enzyme activity was measured at 37 °C under standard assay conditions. The apparent half-lives were estimated using Eq. (1).

The time where the residual activity reaches 50 % is known as the half-life.

$t_{1/2} = \ln 2/k_d$ (1)

k_d was calculated from reaction rate order equation.

$[A] = [A_0]\, e^{K_d T}$ (2)

Where, A is concentration at time T and A_0 is initial concentration.

The stabilization factor (SF) was calculated as the ratio of the half-life of the immobilized enzyme in relation to that of the free enzyme.

2.7. Reusability

The operational stability of free and immobilized HRP activity against 5 consecutive repeated decomposition cycles was carried out under standard assay conditions as described above. The product was measured at 420nm after 1 min of reaction. Immediately, after each enzymatic cycle, the immobilized biocatalyst was collected, thoroughly washed with potassium phosphate buffer, and used for a consecutive catalytic run. The enzyme activity measured at first run was considered as 100% activity and used calculating of residual percentage activity after each run.

2.8. Effect of trypsin

Free and immobilized HRP were incubated for 15 min with different concentrations of trypsin enzyme (5 25mg trypsin/ml). After incubation period the enzyme activity was assayed under standard assay conditions. The activity without addition of trypsin was calculated as 100%.

3. Results and Discussion

3.1. Immobilization Yield

The experimental design in this study provided an improved biocatalyst prepared through a simple procedure that is generally reproducible. The mild procedure conditions employed preserved the stability of the enzyme immobilized by means of the sol-gel process, while overcoming the lower mechanical stability of these materials. Considering the chosen supports screened in this study, better results regarding immobilization yield were obtained for gelatin hydrogel (75%). Calcium alginate showed lower immobilization yield with 42%. This could be attributed to possible leakage of the enzyme from the matrix during curing time, which has been shown to be particularly notable for calcium alginate supports [21]. It is common to witness a decrease in the enzyme activity upon immobilization; this could be due to limited inactivation due to loss of flexibility of enzyme upon entrapment or due to possible interaction

of reactive groups during immobilization. Alternatively, a measured decrease in activity could be due to external and internal mass transfer limitations [14].

3.2. Temperature and PH Profiles

A detectable change in the optimal pH and temperature profile between free and immobilized enzyme has frequently been reported. This usually occurs as a result of the immobilization methodology, support chosen and conformational change of enzyme structure after entrapment, which might change the accessibility of the catalytic site of the immobilized enzyme to the substrate [22].

The effect of pH on activity of free HRP and immobilized HRP was evaluated by incubating these preparations in different buffers at varying pH values ranging from 4.0 to 7.5(Figure 1). Higher pH values than 7.5 were not included due to autoxidation of the pyrogallol. The pH was shifted from 7.0 for free HRP to 7.5 for the immobilized enzyme. This can be explained as the support carrier can absorb H+ from the reaction solution to its surface, inducing lowering pH in the surrounding environment of the immobilized enzyme than that of bulk medium. Thus, the pH of this region should be raised again to enable proper function of the enzyme. Consequently, the pH of the immobilized HRP enzyme displayed at a higher value than that of the free enzymes [13]. Similar results were reported previously by other authors where the pH was shifted from 7.0 for soluble peroxidase to 7.5 for the immobilized enzyme [22].

The enzymatic temperature-activity profile showed significant differences between the three forms of the biocatalyst (Figure 2). The soluble free HRP exhibited an optimum temperature of 30 ^{o}C, whereas for the immobilized enzyme this temperature was shifted to 40^{o}C for calcium alginate beads and for gelatin (Figure 2). Results show that the activity of free enzyme decreased faster than the immobilized preparation for temperatures in excess of 40°C.The free enzyme retained 30% residual activity at 50 ^{o}C, while the immobilized HRP retained 74 % and 85% of its initial activity at 50 ^{o}C upon immobilization in calcium alginate beads and gelatin, respectively. These results indicated that upon immobilization peroxidase showed higher resistance to denaturation upon increasing temperature, which can be due to the confinement of the enzyme and its restricted mobility [2,23].

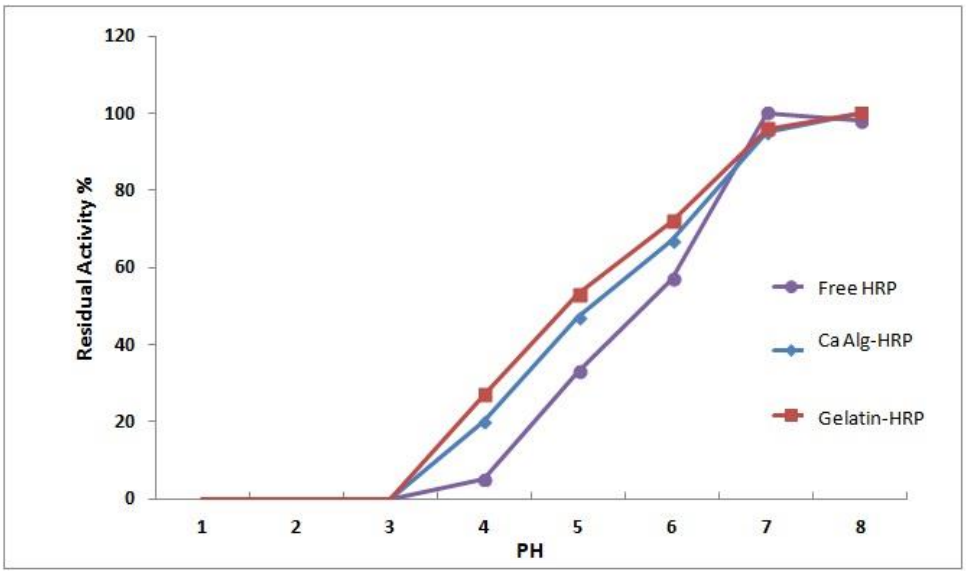

Figure 1: Effect of pH on catalytic activity of free and immobilized peroxidase (HRP) at various pH values pH 4.0-pH 7.5. Each point represents the average of two experiments.

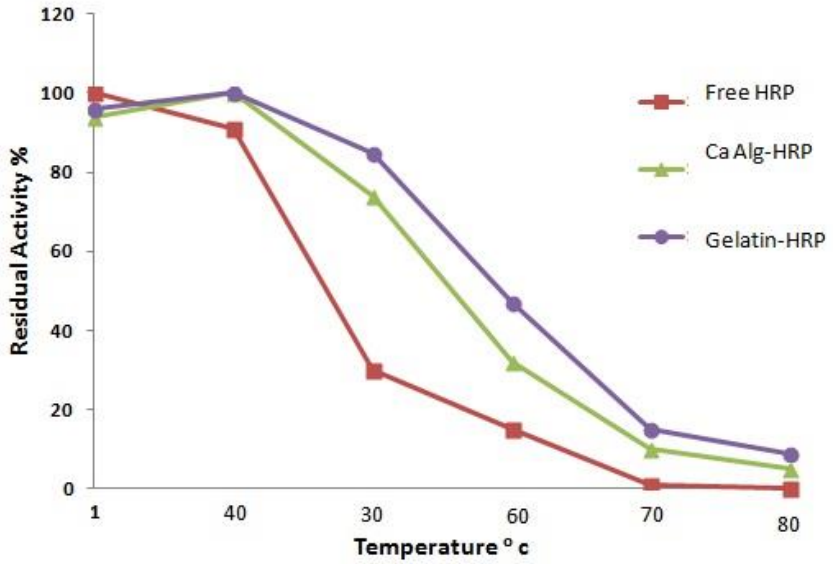

Figure 2: Effect of temperature on catalytic activity of free and immobilized peroxidase (HRP) at various temperatures from 30-80ºC. Each point represents the average of two experiments.

3.3. Operational and thermal Stability

Thermal stability of HRP was improved upon immobilization in both calcium alginate beads and gelatin hydrogel (Figure 3). When free and immobilized HRP were incubated at 50°C

and 70°C, the free enzyme lost completely its activity after 15 min at 70°C, whereas the immobilized one retained about 18% and 22 % residual activity for Ca Alg HRP and Gel HRP, respectively Table 1 presents the operational and thermal stability data of the free and immobilized HRP at 50 °C and 70 °C under 120 rpm of stirring for 15-45mins. Inactivation rate constants (k_d) of free and immobilized HRP at 50°C and70°C were calculated and half-life values were estimated using these constants and Eq. (1), both are presented in Table 1. Results show that there was a significant improvement in the thermal stability of HRP immobilized preparations in comparison to the free HRP with a considerable stabilization factor between a low of 1.28 (12.8%) and a high of 6.15 (61.5%). At 50°C free HRP exhibited less stability with $t_{1/2}$ of 168 mins after 15 min however, immobilized HRP showed better stability with 533 mins and 1034 mins stabilization factor for Ca Alg HRP and Gel HRP, respectively.

The immobilization support usually exhibits a protecting effect that may be due to changes in the conformational flexibility of the enzyme as a result of immobilization. The immobilization entrapment increases the enzyme rigidity, usually observed by an increase in stability towards thermal denaturation [2,24].

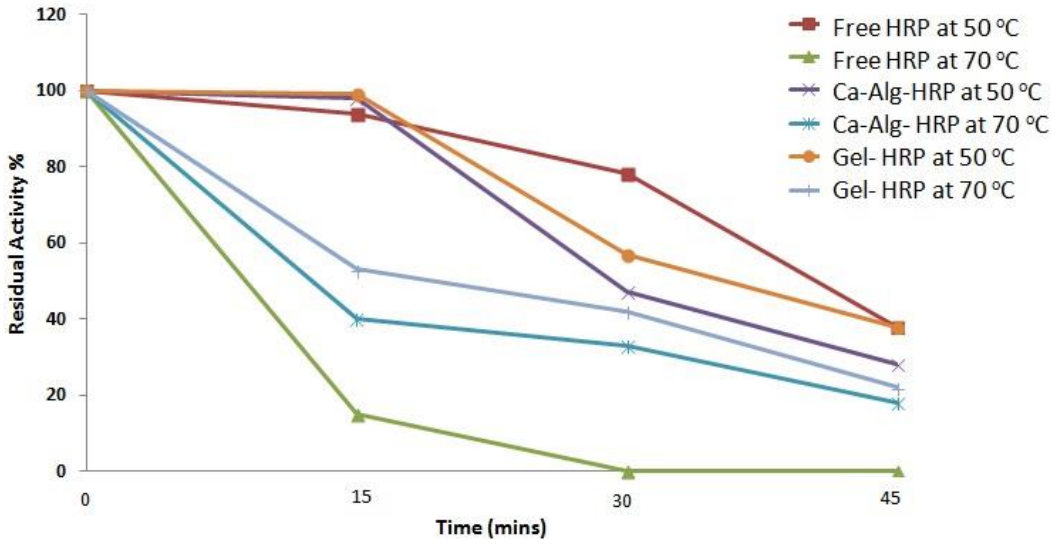

Figure 3: Thermal stability of free and immobilized peroxidase (HRP) measured at different temperatures at 15 mins time intervals. Each point represents the average of two experiments.

Table 1. Half - life ($t_{1/2}$) and Inactivation rate constant (K_d) and stabilization factor (S) of free and immobilized HRP at different temperatures.

Temp (°C)	Time (mins)	Free HRP		Ca Alginate-HRP			Gelatin-HRP		
		kd	$t_{1/2}$	kd	$t_{1/2}$	S	kd	$t_{1/2}$	S
50	15	0.00412	168	0.0013	533.07	3.17	0.00067	1034	6.15
	30	0.0322	21.5	0.0251	27.6	1.28	0.0217	31.935	1.48
	45	0.0381	18.18	0.0282	24.57	1.35	0.0253	27.39	1.5
70	15	0.126	5.5	0.061	11.39	2.07	0.0423	16.38	2.97
	30	------	-----	0.0289	23.97	----------	0.0369	18.78	------
	45	-------	------	0.0381	18.18	----------	0.0336	20.625	-------

Units of half-life were expressed in min and Inactivation rate constant kd were expressed in min^{-1}.

Several authors have reported similar thermal stabilization effect on different enzymes upon immobilization [2,22,25,26]. The improved thermal stability of the immobilized enzyme compared to free enzyme is very crucial for the industrial applications, where some processes are performed at high temperatures.

3.4. Reusability

The reusability of immobilized enzyme preparations is of high significant, as it is considered as a key feature for the economic application of bioprocesses utilizing immobilized enzymes as biocatalysts. The prepared immobilized HRP preparations were reused in consecutive 5 cycles and the residual activity of the immobilized enzyme was calculated throughout the different runs (Figure 4). Results show that Ca Alg-HRP and Gel-HRP retained 80% and 84% residual activity, respectively, suggesting the potential for application in systems with industrial relevance. Several authors have reported improved recyclability of peroxidase upon immobilization in different matrices [13, 27,28].

3.5. Effect of trypsin

To investigate the effect of immobilization on protection of HRP against proteolysis, varying amounts of trypsin enzyme (from 5 to 25 mg) were tested (Figure 5). Ca Alg-HRP showed high resistance to proteolysis by trypsin than that of free enzyme and Gel-HRP. This can be referred to due to protein nature of gelatin. Several authors have reported before the stabilizing effect of immobilization on peroxidase [22,29].

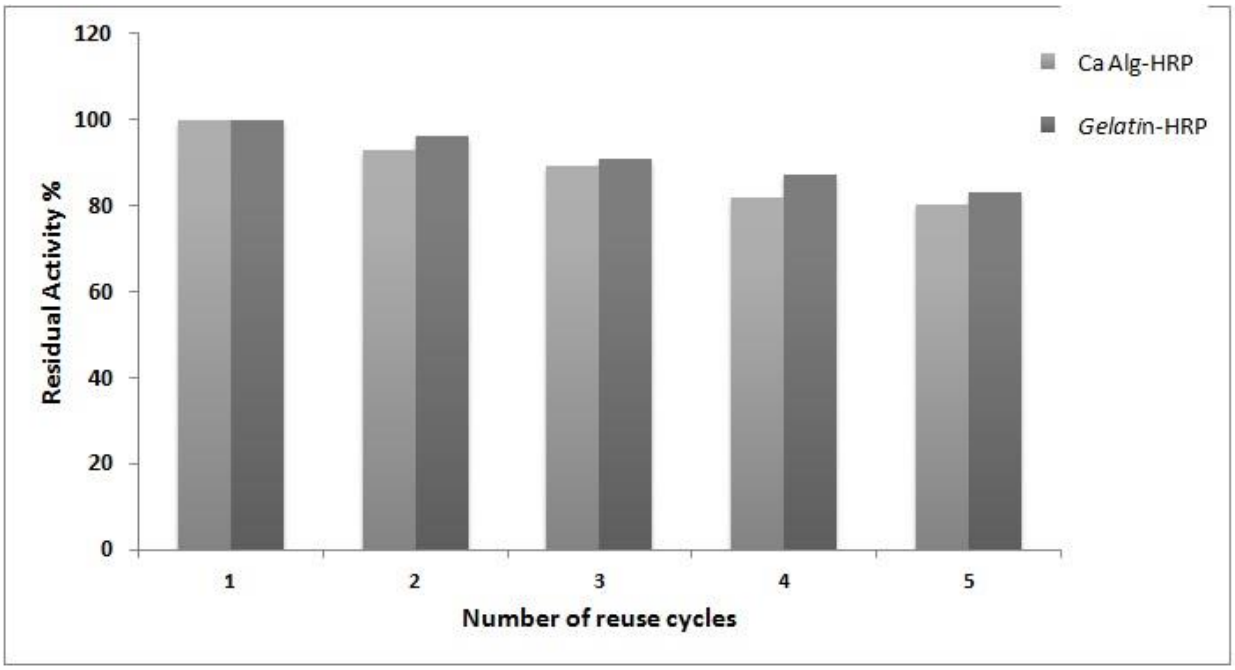

Figure 4: Reusability of immobilized HRP in repeated catalytic cycles. Residual activity of the immobilized HRP (in %) was calculated after each cycle for five cycles of repeated use (initial activity was taken as 100%). Each point represents the average of two experiments.

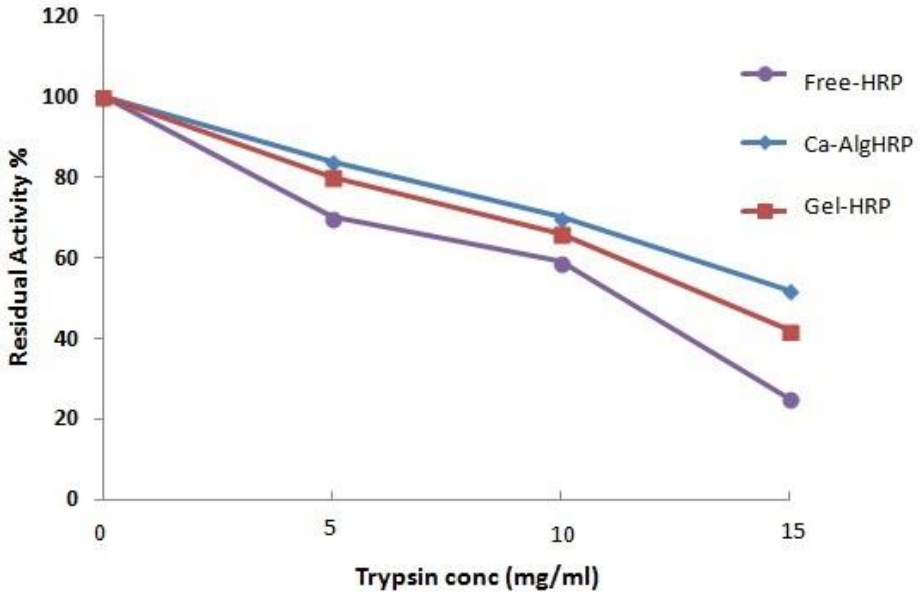

Figure 5: The effect of trypsin concentration on the activity of free HRP and immobilized HRP. Each point represents the average of two experiments.

4. Conclusion

Physical entrapment in sol-gel has drawn much attention as a promising immobilization approach for preparation of stable biocatalysts. Comparative screening of two different commercially available supports and methodologies for the immobilization of HRP has been carried out and has displayed great potential for prospective applications. Neither method caused significant changes in the pH/activity profile. Immobilization of HRP in both calcium alginate beads and gelatin resulted in a higher tolerance to higher temperatures. Both methods enhanced the thermal and operational stability of HRP with a higher calculated stabilization factor than free enzyme, and both supports were tested for reusability in consecutive runs showing high stability. These results suggest that the studied approaches have potential for use of immobilized HRP in industrially relevant processes Accomplishing such goal will require further research in order to evaluate the robustness of these approaches under process conditions.

References:

1 *Kempka A., de Souza S., de Souza A. Lipase immobilisation in matrix comprised of gelatin of different bloom numbers with the addition of hydrophilic plasticisers. Can J Chem Eng. 2014, 92, 989 999.*

2 *AbdeL Mageed H., Radwan R., AbuelEzz N., Nasser h., El Shamy A., Abdelnaby R. , EL Gohary N. Bioconjugation as a smart immobilization approach for α amylase enzyme using stimuli responsive Eudragit-L100 polymer: A robust biocatalyst for applications in pharmaceutical industry (2019). Artificial cells Biomed Nanotech. 2019,47, 2361 2368*

3 *Mateo, C.; Palomo, J.M.; Fernandez Lorente, G.; Guisan, J.M.; Fernandez Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb. Technol. 2007, 40, 1451 1463.*

4 *Homaei AA Sariri R, Vianello F, Stevanato R Enzyme immobilization: an update. J Chem Biol. 2013 Aug 29;6(4):185 205. doi: 10.1007/s12154 013 0102 9*

5 *George M., Abraham T. E. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosana review," Journal of Controlled Release, vol. 114, no. 1, pp. 1 14, 2006.*

6 *Ouwerx C., Velings N., Mestdagh M. M.,. Axelos M. A. V. Physico chemical properties and rheology of alginate gel beads formed with various divalent cations,"Polymer Gels and Networks, vol. 6, no. 5, pp. 393 408, 1998.*

7 *Bajpai S. K. and Sharma S., "Investigation of swelling/degradation behavior of alginate beads cross linked with Ca2+ and Ba2+ions," Reactive and Functional Polymers, vol. 59, no. 2, pp. 129 140, 2004.*

8 *Griffith, L.G. "Polymeric biomaterials,"ActaMaterialia, vol. 48, no. 1, pp. 263 277, 2000.*

9 *Grant G. T.,.Morris E. R.Rees D.A, Smith P. J. C., Thom D., "Biological interactions between polysaccharides and divalent cations: the egg box model," FEBS Letters, vol. 32, no. 1, pp. 195 198, 1973.*

10 *M. J. Fern´andez-Herv´as, M. A. Holgado, A. Fini, and J. T. Fell, "In vitro evaluation of alginate beads of a diclofenac salt," International Journal of Pharmaceutics, vol. 163, no. 1 2, pp. 23 34, 1998.*

11 *Gómez Guillén, M.C., et al., Functional and bioactive properties of collagen and gelatin from alternative sources: A review. Food Hydrocolloids, 2011. 25(8): p. 1813 1827.*

12 *Yasmina R., Shaha M., Khan S., Ali R. Gelatin nanoparticles: a potential candidate for medical applications. Nanotechnol Rev 2017; 6(2): 191 207.*

13 *Chang Q., Tang H. Immobilization of Horseradish Peroxidase on NH2 Modified Magnetic Fe3O4/SiO2 Particles and Its Application in Removal of 2,4 Dichlorophenol. Molecules 2014, 19, 15768 15782; doi:10.3390/molecules191015768*

14 *Suleka F, Fernándezb D, Kneza Z, Habulina M, Sheldon R. Immobilization of horseradish peroxidase as crosslinked enzyme aggregates (CLEAs). Process Biochem. 2011, 46, 765 769.*

15 *Chance B., Maehly A. Methods in enzymology. 1955, 2, 773 775.*

16 *Yuan ZY, Jiang TJ. Horseradish peroxidase. In: Whitaker JR, Voragen A, Wong DWS, editors. Handbook of food enzymology. New York: Marcel Dekker Inc.; 2003. p. 403 11*

17- *Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72: 248 - 254. doi:10.1016/0003-2697(76)90527-3.*
18- *H. Y. Kawaguti, E. Manrich, and H. H. Sato, "Production of isomaltulose using Erwinia sp. D12 cells: culture medium optimization and cell immobilization in alginate," Biochemical Engineering Journal, vol. 29, no. 3, pp. 270–277, 2006.*
19- *H. Y. Kawaguti, M. F. Buzzato, D. C. Orsi, G. T. Suzuki, and H. H. Sato, "Effect of the additives polyethylenimine and glutaraldehyde on the immobilization of Erwinia sp. D12 cells in calcium alginate for isomaltulose production," Process Biochemistry, vol. 41, no. 9, pp. 2035–2040, 2006.*
20- *Assis S., Ferreira B., Fernandes P., Guaglianoni D., Cabral J., Oliveira O. Gelatin-immobilized pectinmethylesterase for production of low methoxyl pectin. Food Chemistry, 2004, 86, 333–337.*
21- *Fernandes P., Enzymes in food processing: a condensed overview on strategies for better biocatalysts. Enzyme Research, vol. 2010, pp. 1–19.*
22- *Alshawafi W., Aldhahri M., Almulaiky Y., Salah N., Moselhy S., Ibrahim I.,El-Shishtawy R., Mohamed S. Immobilization of horseradish peroxidase on PMMA nanofibers incorporated with nanodiamond, Artif Cells, Nanomed Biotechn, 2018, 46, S973-S981, DOI: 10.1080/21691401.2018.1522321*
23- *Mohamed S., Abdel-Mageed M., Tayel S., El-Nabrawi, Fahmy. Characterization of Mucor racemosus lipase with potential application for the treatment of cellulite. Process Biochem 2011,46; 642–648.*
24- *Figueira J., Gonc F.¸ Dias A., Sato H., Fernandes P. Screening of Supports for the Immobilization of β-Glucosidase. Enzyme Research 2011, Article ID 642460, 8 pages doi:10.4061/2011/642460.*
25- *Abdel-Mageed, H.; El-Laithy H., Mahran L., Fahmy A., Mäder K., Mohamed, S. Development of novel flexible sugar ester vesicles as carrier systems for the antioxidant enzyme catalase for wound healing applications. Process Biochem. 2012, 47, 1155–1162.*
26- *Abdel-Mageed, H.; Fahmy, A.; Dalia, S.; Mohamed, S. Development of novel delivery system for nanoencapsulation of catalase: formulation, characterization, and in vivo evaluation using oxidative skin injury model, Artif Cells Nanomed Biotechnol, 2018, 24, 362-371. DOI: 10.1080/21691401.2018.1425213*

27- *El-Sayed A., Rabie G., El-Gazzar N., Ali G. Immobilization and Characterization of Purified Aspergillus flavus Peroxidase Mediated Silver Nanoparticle Synthesis: Peroxidase Surface Reactive Residues are Implemented for Reduction of Silver Ions, More than Its Active Sites. J Nanomed Nanotechnol 2017, 8: 420-428. doi: 10.4172/2157-7439.1000420*

28- *Pandey VP, Awasthi M, Singh S, Tiwari S, Dwivedi UN (2017) A comprehensive Review on Function and Application of Plant Peroxidases. Biochem Anal Biochem 6: 308. doi: 10.4172/2161-1009.1000308*

29- *Kulshrestha Y, Husain Q. Direct immobilization of peroxidase on DEAE cellulose from ammonium sulphate fractionated proteins of bitter gourd (Momordica charantia). Enzyme Microb Technol. 2006;38:470–477.*