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Evaluation of Different Entrapment Strategies for the Immobilization of Protease from *Bacillus subtilis* KIBGE HAS

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Abstract:-Enzyme immobilization is industrially valuable as it reduces production costs and enhances enzyme reusability. In this study, partially purified protease from *Bacillus subtilis* KIBGE HAS was immobilized using three entrapment matrices: 2% calcium alginate, 7.5% polyacrylamide, and 2% agar-agar. All supports successfully maintained the enzyme's optimum pH (7.5) and temperature (50°C), indicating that the entrapment process did not alter these essential parameters.

Immobilization increased the Km values compared to the free enzyme (1.3 mg/mL), reflecting reduced substrate affinity. The Km values were 2.7 mg/mL for calcium alginate, 4.1 mg/mL for polyacrylamide, and 10 mg/mL for agar-agar. Notably, the agar-agar–entrapped protease exhibited a higher Vmax (159.3 U/mL) than the free enzyme, suggesting improved catalytic efficiency and potential industrial relevance.

Agar-agar also demonstrated superior operational stability, allowing four reuse cycles, while calcium alginate and polyacrylamide supported three and two cycles, respectively. In storage studies, agar-agar–entrapped protease retained activity for 15 days at 4°C and 7 days at 30°C, outperforming calcium alginate (10 days) and polyacrylamide (2 days). Overall, agar-agar proved to be the most effective matrix for protease immobilization.

Keywords: protease, *Bacillus subtilis*, immobilization, entrapment, agar-agar

INTRODUCTION

Industrial enzymes offer significant advantages in various biotechnological processes; however, their large-scale utilization faces several challenges. The bulk demand for enzymes increases the overall cost of isolation and purification, while their proteinaceous nature makes them susceptible to denaturation under extreme process conditions such as temperature, pH, and chemical exposure. In their soluble form, enzymes form homogeneous catalytic systems, making recovery and reuse difficult and increasing the risk of product contamination, which necessitates additional downstream processing (Abolpour et al., 2013).

Although hydrolytic enzymes exhibit high catalytic efficiency in solution, their high cost and low operational stability limit industrial application. Enzyme immobilization has therefore emerged as a promising strategy, offering advantages such as reusability, improved process control, enhanced mechanical stability, and, in some cases, increased catalytic efficiency (Kulkarni et al., 2016). The choice of immobilization method depends on parameters such as process efficiency, cost, reagent toxicity, and desired properties of the final biocatalyst (Alnoch et al., 2020). Among the conventional techniques—physical adsorption, covalent binding, bioconjugation, and entrapment—the entrapment method is particularly advantageous, as it minimizes adverse effects on enzyme activity and improves stability (Abolpour et al., 2013). Common entrapment supports include agar-agar, polyacrylamide gel, and calcium alginate.

Proteases represent approximately 60% of the global enzyme market (Matkawala et al., 2021) and are utilized in detergent, textile, pharmaceutical, medical, food, and leather industries (Morellon-Sterling et al., 2022; Calzoni et al., 2021; Cesaretti et al., 2021). The present study evaluates the entrapment of partially purified protease from *Bacillus subtilis* KIBGE HAS (accession number EU819145) using calcium alginate, polyacrylamide, and agar-agar matrices to determine the most efficient and cost-effective support for industrial applications.

MATERIALS AND METHODS

Protease production and partial purification

Bacillus subtilis KIBGE HAS was grown in a liquid medium containing 0.1 % glucose, 2.5 % peptone, 0.01% $CaCl_2$, 0.02 % yeast, 0.01 % $MgSO_4$ with pH adjusted to 7.5. After 24 hours of incubation, the fermented broth was centrifuged at 10,000 rpm for 10 minutes at 4°C. For partial purification, the crude enzyme was poured into the Amicon ultrafiltration assembly and allowed to pass through Ultrafiltration membranes (NMWL-30,000) with the cut-off size of 30,000 Dalton and NMWL-50,000with cut off size of 50,000 Dalton). The aliquots greater than or equal to 30 KDa and less than 50 KDa obtained through these membranes were precipitated with 40% ammonium sulfate, then dissolved in minimal volume of Tris-HCl buffer of pH 7.5 and left for overnight dialysis against the same buffer at 4°C.

Entrapment of partially purified enzyme in calcium-alginate beads

The partially purified enzyme was immobilized via the enzyme entrapment method in calcium-alginate beads, agar gel, and polyacrylamide gel for the comparative immobilization analysis. The entrapment methods of calcium-alginate with %percent immobilization of 45% are clearly described by Anwar et al, 2009.

Entrapment of partially purified enzyme in agar-agar

The partially purified enzyme was incorporated with agar in 1:1 ratio, solidified in a plate 12 × 80 nm. The plate was kept in a refrigerator at 4°C for 1 hour, then cut into square of 3 × 3 mm. 0.5 g of gel cubes are used for the proteaseassay.

Entrapment of partially purified enzyme in polyacrylamide gel

For entrapment in polyacrylamide gel, 2.5 ml polyacrylamide-Bisacrylamide solution in a final concentration of 7.5% was taken in a Petri plate; 2.5 ml of the partially purified enzyme was added with 4.95 ml deionized water and mixed gently. The gel was solidified by adding 50ul APS, 5.0 ul TEMED and was allowed to solidify for 30 minutes. 2.5 ml of buffer was added in case of control. The gel was cut into squares of 0.5 cm. For protease activity, 0.5gm of gel was taken.

Enzyme assay

The protease activity was monitored using 1% casein as a substrate with slight modifications of the method described by Kembhavi et al, 1993.

"*One unit of protease hydrolyzed casein to produce color equivalent to 1.0 µmol (181µg) of tyrosine per minute at pH 7.5 at 50°C.*

The activity of the immobilized enzyme was derived in units/0.5 g of support and was compared with that of the partially purified enzyme and all three supports.

Substrate maxima

Substrate maxima of immobilized protease was performed usingcasein as a substrate rangingfrom 0.25%to 2% in 50 mM Tris-HCl buffer. The Km and Vmax of entrapped protease was measured using the Michaelis Menton equation and a line weaver Burk plot, respectively.

Temperature, storage stability and pH maxima

The entrapped enzyme was assayed at different temperatures, various pHs, ranging from 20°C to 70°C and pH 6.0 to 8.5, respectively. Thepercent activity of the entrapped enzyme was compared with the percent activity of the free enzyme.The entrapped enzyme was stored at 4°C and 30°C, and protease activity was determined at different time of interval.

Operational stability of the entrapped enzyme

The activity of the entrapped enzyme was assayed for up to 04 cycles to determineits reuse or operational stability. After each cycle, the beads were rinsed with deionized water to remove the blockage hindering the reaction, which might have occurred due to the substrate or product deposition on the pores of beads, and the beads were recycled by adding fresh substrate.

RESULTS AND DISCUSSION

Immobilization is a technique used to attach an enzyme to a solid support, allowing easy separation from reactants and products, while enhancing its reusability and stability.

Comparision of different support on Immobilization of Protease

Different supports were used for the immobilization of the protease enzyme. The highest immobilization efficiency (80%) was achieved with 7.5% polyacrylamide gel, compared to calcium alginate, which showed 45% immobilization. When agar-agar was used as the support, 61% immobilization efficiency was observed.

Effect of temperature on enzyme activity before and after entrapment

The optimum temperature of the entrapped enzyme in all three supports was found to be 50°C, which was identical to that of the free enzyme. A marked decline in enzyme activity was observed when the temperature increased from 50°C to 60°C in the agar-agar and polyacrylamide gel–entrapped enzymes. In contrast, the Ca-alginate–entrapped enzyme retained 52% of its total activity even when the temperature was raised from 50°C to 70°C, whereas the free enzyme maintained only 39% of its activity under the same conditions. These results suggest that all three supports provided thermal stability and helped preserve the enzyme's tertiary structure at elevated temperatures (Fig. 1).

Effect of reaction time on enzyme activity

The effect of reaction time on the activity of immobilized enzymes was evaluated using different support matrices. Maximum enzyme activity was observed after 20 minutes of enzyme–substrate incubation for the Ca-alginate–entrapped protease, and after 15 minutes for the polyacrylamide- and agar-agar–entrapped enzymes.

The Ca-alginate–entrapped protease required a longer reaction time (20 minutes) to reach maximum activity compared to the polyacrylamide- and agar-agar–entrapped enzymes (15 minutes). This difference is primarily due to the structural characteristics and diffusion properties of the support matrices. Calcium alginate forms a denser, more compact gel network with smaller pore sizes, which slows the diffusion of substrate molecules into the bead interior. As a result, the substrate takes longer to reach the entrapped enzymes, delaying the attainment of peak catalytic activity.

In contrast, polyacrylamide and agar-agar matrices generally have larger pores and lower mass-transfer resistance, allowing faster substrate penetration and quicker enzyme–substrate interaction. Therefore, enzymes immobilized in these matrices achieve their maximum activity in a shorter time.

A similar observation was reported by Mahajan *et al.* (2010), who found that the reaction time of amylase entrapped in agar-agarose and polyacrylamide gels increased compared with that of the free enzyme.

Effect of pH onProtease Activity

The protease exhibited maximum activity at pH 7.5, with only a slight, non-significant decrease at pH 8. The optimum pH of the immobilized enzyme remained the same as that of the free enzyme (pH 7.5). However, when the pH was increased or decreased from this optimum, a sharp decline in enzyme activity was observed for all three support matrices (Fig. 2).

At pH 7.5, all supports—including agar-agar, which is slightly acidic—behaved as neutral carriers, allowing optimal enzyme–substrate interactions. Deviations from this pH likely caused changes in the ionization state of both the enzyme and the support matrices, leading to altered charge distribution and reduced catalytic efficiency.

These findings align with previous reports. Arya and Srivastava (2006) observed no change in the optimum temperature or pH of CGTase before and after entrapment in Ca-alginate beads. Similarly, other studies have reported no shift in the pH or temperature optima of proteases immobilized in polyacrylamide matrices.

Optimization of substrate concentration

The K_m values for alginate-, polyacrylamide-, and agar-agar-entrapped protease were found to be 2.7 mg/mL, 4.1 mg/mL, and 10 mg/mL, respectively, while the highest K_m observed for agar-agar-entrapped protease. Although more substrate was required for the activity of the agar-entrapped enzyme, it exhibited the highest Vmax of 159.3 U/mL compared to 113.3 U/mL for the free enzyme. In contrast, Ca-alginate- and polyacrylamide-entrapped enzymes showed Vmax values of 56.1 U/mL and 104.6 U/mL, respectively (Fig. 3). These results indicate that agar-agar-entrapped protease exhibited approximately 40% higher activity than the soluble enzyme.

Typically, immobilized enzymes display lower Vmax than their soluble counterparts, often due to conformational changes during immobilization (Zhang et al., 2006; Hota et al., 2007). However, several studies have reported increased Vmax following immobilization. For example,

Flores-Maltos et al. (2011) observed a significant increase in both Km and Vmax of Ca-alginate-entrapped tannase. Similarly, the Km and Vmax of invertase increased threefold and 5.7-fold, respectively, after immobilization on crystalline cellulose and Nylon-6 (Angels et al., 2008). Beta-galactosidase also exhibited increased Km and Vmax when covalently immobilized on thermostable biopolymers (Zhang et al., 2006). Flores-Maltos et al. suggested that interactions between the substrate (tannic acid) and the polymer matrix could explain the increased Vmax in immobilized systems.

In the present study, the enhanced Vmax of agar-entrapped protease may similarly result from interactions between casein and agar-agar, potentially exposing scissile bonds to the enzyme. This property makes agar-entrapped protease particularly promising for industrial applications. In contrast, Ca-alginate-entrapped enzyme exhibited the lowest Km (2.7 mg/mL) and Vmax (29.69 U/mL) among the immobilized enzymes, consistent with previous reports of reduced activity for invertase in alginate gel (Vu and Le, 2008).

For polyacrylamide-entrapped protease, the Km was 4.136 mg/mL, higher than the soluble enzyme (Km=1.3 mg/mL), while its Vmax (104 U/mL) was slightly lower than that of the free enzyme (113 U/mL). Despite this, the immobilized enzyme retained 94% of its activity, indicating minimal activity loss. Comparable results have been reported for other immobilized proteases, which retained 90% of the native enzyme activity (Najafi et al., 2005). Similarly, polyacrylamide-entrapped cellobiose exhibited higher Km than the native enzyme (Sciences et al., 2008).

Storage Stability

The stability of entrapped protease is an important factor in determining their utility and effectiveness in various applications. Due to the fact that entrapped enzyme was more stable when stored at different temperatures.Therefore, the maximum storage stability ofagar-entrapped enzyme was obtained at 4 °C and 30 °C, showing 12% activity even after 15 days at 4 °C and 6% activity after 7 days at 30 °C (Figure 4).However, Ca-alginate entrapped protease depicted 35% loss in activity after two days and 89% loss in activity after ten days when stored at 4°C. (Figure).Whereas,polyacrylamide gel entrapped enzyme showed 50% activity loss after two days and

95% loss in activity on the 3rd day when stored at 4°C, while the enzyme activity at 30°C showed 90% loss in activity at 2nd day and no activity was found atthird day. This loss in immobilized enzyme activity might be due to the autolysis of the enzyme within the carrier (Figure 6).

Operational Stability

The reusability of immobilized enzymes is of great importance for their industrial applications, as it reflects the economic viability and operational stability of the biocatalyst. Among the different supports used for protease entrapment, agar-agar and calcium alginate retained approximately 80% of their initial activity when assayed for the second time, compared to polyacrylamide-entrapped enzyme, which retained only 60% activity during the second cycle. However, in the third cycle, agar-agar retained 50% of its activity, while calcium alginate retained 30%. The agar-agar-entrapped enzyme further exhibited 20% residual activity during the fourth cycle, demonstrating the highest operational stability among the tested supports. In contrast, no activity was observed in the fourth cycle of the alginate-entrapped enzyme, and complete activity loss occurred by the third cycle, indicating poor reusability (Figure 8).

The reduced reusability observed in some matrices may be attributed to enzyme leakage from the beads or gels during washing and reuse. Similar findings were reported by Mahajan et al. (2010), where immobilized α-amylase in a polyacrylamide gel exhibited no significant operational stability after repeated use.

CONCLUSION

Agar-agar was found to be the most effective entrapment support compared to polyacrylamide gel and calcium alginate for enzyme immobilization. It exhibited the highest V_{max}, along with superior operational stability and reusability, indicating a favorable interaction between agar-agar and the entrapped enzyme. Moreover, agar-agar demonstrated excellent storage stability, further emphasizing its suitability for practical enzyme applications.

In conclusion, agar-agar proved to be a promising support matrix for enzyme entrapment, offering enhanced stability, high catalytic efficiency, and good reusability. However, the

selection of the optimal support material ultimately depends on the specific requirements of the intended application and the functional characteristics of the immobilized enzyme.

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CONFLICTS OF INTEREST
The authors declare that there are no conflicts of interest regarding the publication of this paper.
AUTHOR' S CONTRIBUTION

This research was designed and supervised by **Dr. Shah Ali ul Qader**. **Dr. Abida Anwar** conducted the analysis, interpreted the results, and prepared the first draft of the manuscript. **Dr. Saeeda Bano** edited the initial draft and contributed to its critical revision. **Dr. Samina Iqbal** performed proofreading, formatting, and prepared the final draft of the manuscript. All authors reviewed and approved the final version for publication.

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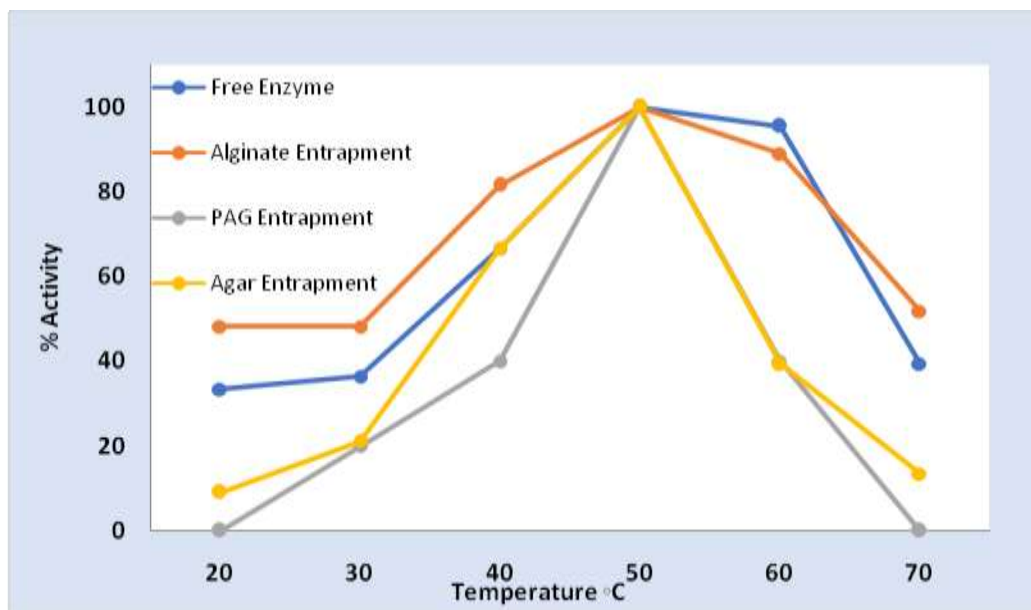


Figure.1: *Optimum temperature for free and entrapped protease activity*

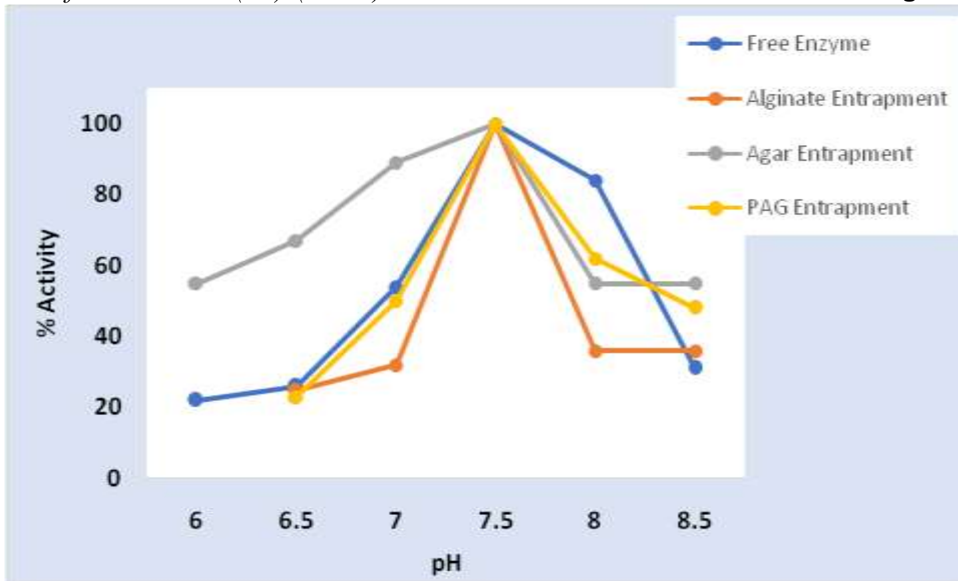


Figure.2*: Optimum pH for the free and entrapped protease activity*

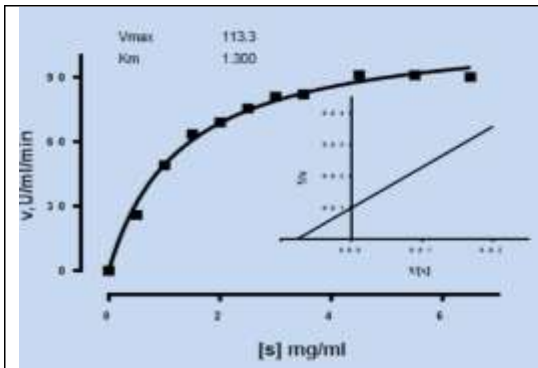


Fig. 3a)Michalis-Menten and Lineweaver-Burk plot of the partially purified free protease.

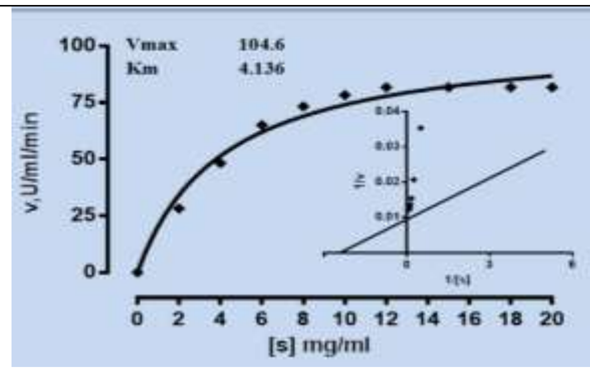
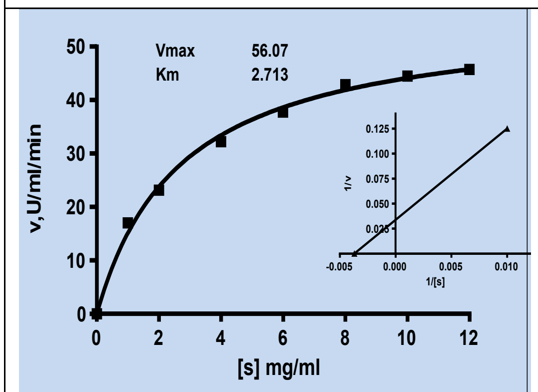


Fig. 3b)Michalis- Menten and Lineweaver-Burk plot of polyacrylamide get entrapped protease.



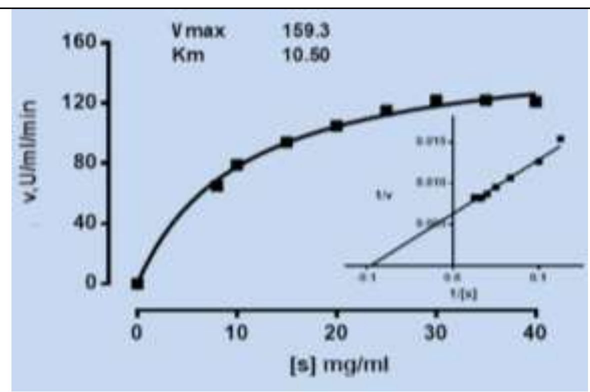


Fig. 3c) Michaelis -Menten and Lineweaver-Burk plot of calcium alginate entrapped protease.	*Fig. 3d) Michaelis-Menten and Lineweaver-Burk plot of agar-agar entrapped protease.*

Figure 3*: Michalis-Menten and Lineweaver-Burk plot of the free and immobilized protease on different supports*

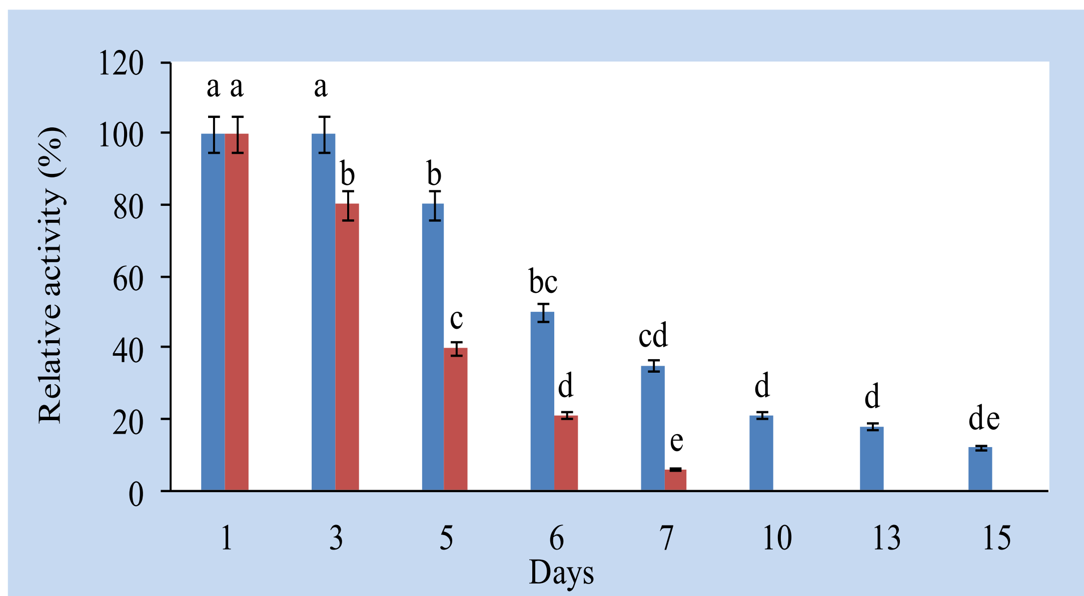


Figure 4*: Storage stability of immobilized proteases in agar-agar at 4 °C (****blue****) and 30 °C (****red****). Symbols (means± S.E., n = 6) having similar letters are not significantly different from each other (Bonferroni test, P < 0.05).*

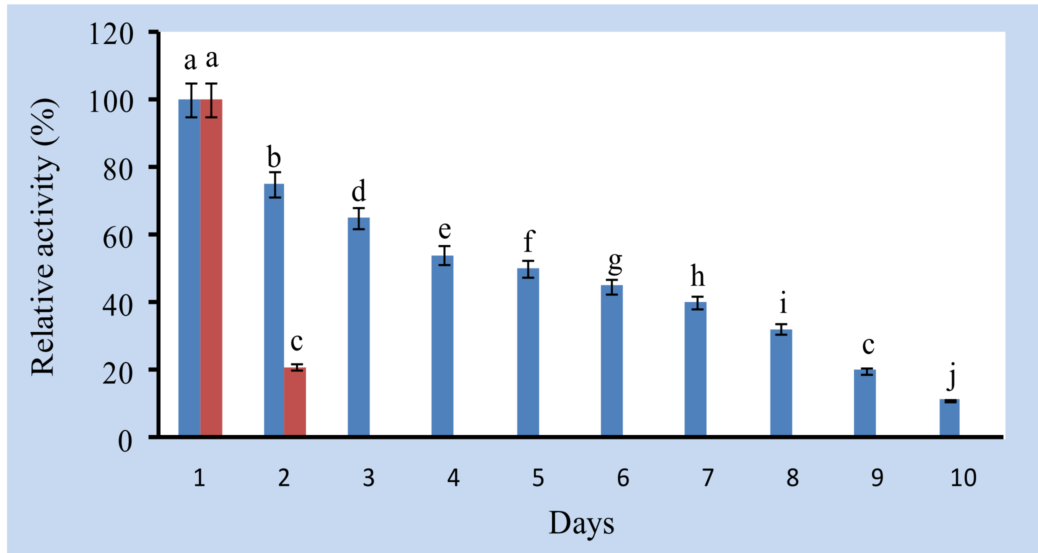


Figure 5: *Storage stability of immobilized proteases in calcium alginate at 4 °C (blue) and 30 °C (red). Symbols (means± S.E., n = 6) having similar letters are not significantly different from each other (Bonferroni test, P < 0.05).*

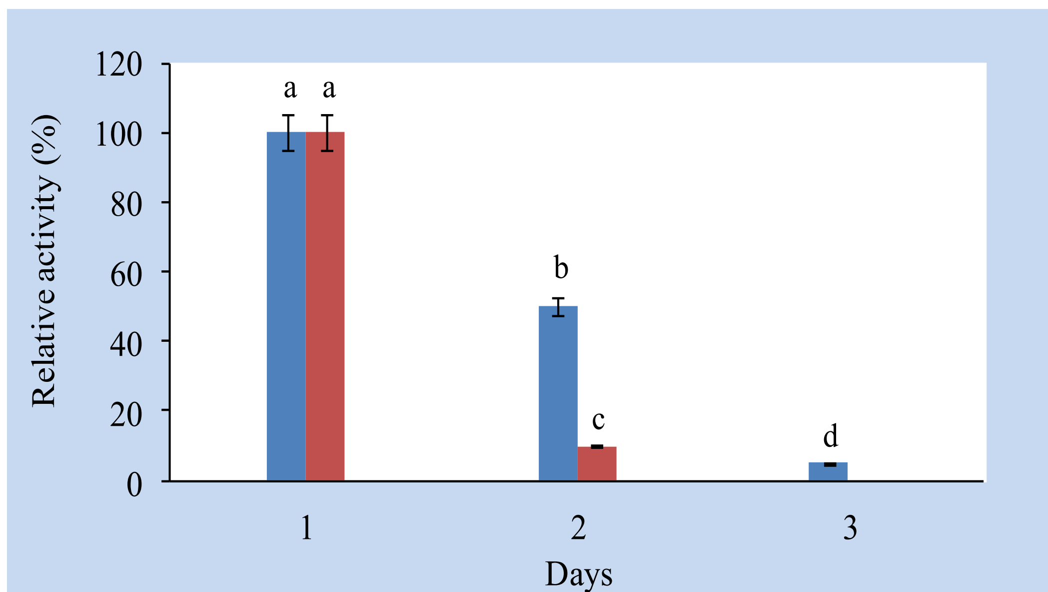


Figure 6: *Storage stability of immobilized proteases in polyacrylamide gel at 4 °C (blue) and 30 °C (red). Symbols (means± S.E., n = 6) having similar letters are not significantly different from each other (Bonferroni test, P < 0.05).*

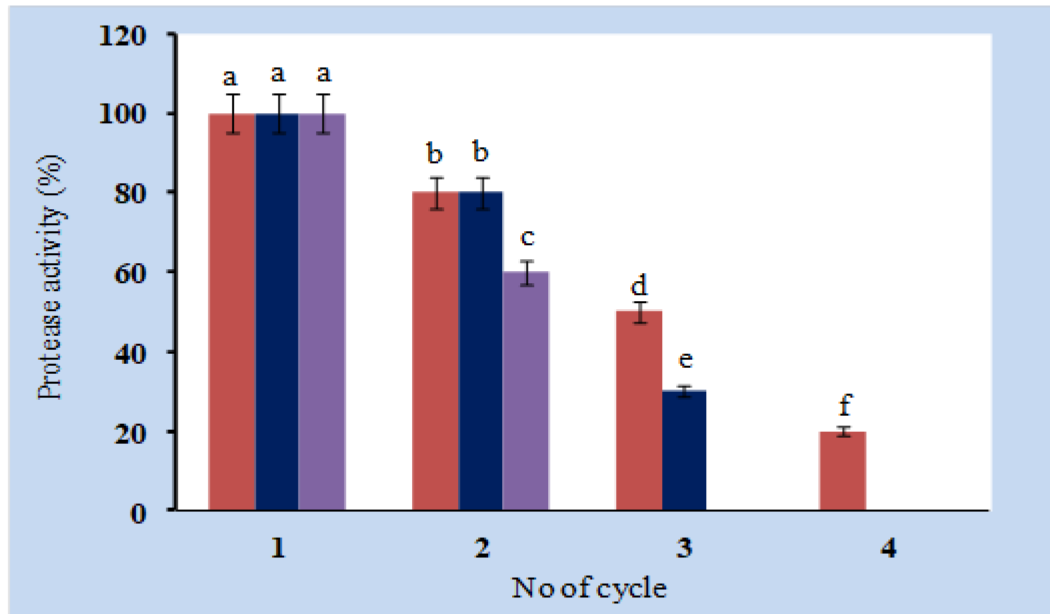


Figure 7: *Operational stability of proteases on different supports. Agar-agar (red), Calcium alginate (dark blue), Polyacrylamide gel (purple). Symbols (means± S.E., n = 6) having similar letters are not significantly different from each other (Bonferroni test, P < 0.05).*