Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2582-2845.9025

ISSN: 2582 – 2845

Ind. J. Pure App. Biosci. (2023) *11*(5), 22-31

Indian Journal of Pure & Applied Biosciences

Peer-Reviewed, Refereed, Open Access Journal

Research Article

Immobilization Strategies for L-Asparaginase from *Ganoderma australe* GPC191: Impact on Enzyme Activity, Stability, and Reusability

Meghna Chakraborty[1] and Srividya Shivakumar[2*]

[1]Department of Microbiology, School of Sciences, [2]School of Allied Healthcare and Sciences (SAHS), JAIN (Deemed-to-be University), Bangalore, Karnataka-560066, India

*Corresponding Author E-mail: sk.srividya@jainuniversity.ac.in

Received: 25.04.2023 | Revised: 22.06.2023 | Accepted: 08.07.2023

ABSTRACT

L-asparaginase has garnered significant attention for its potential therapeutic applications in cancer treatment, as well as its role in mitigating acrylamide in the food industry. However, the widespread implementation of free L-asparaginase in industrial processes has faced substantial obstacles, primarily attributed to issues such as reduced stability, high production costs, and limited recyclability. In order to address these challenges and fully unleash the capabilities of L-asparaginase, the current investigation was centered on the exploration of L-asparaginase immobilization derived from Ganoderma australe GPC191. The various immobilization matrices were assessed, including gelatin, agarose, agar, and combinations thereof with sodium alginate, carboxymethyl cellulose, and calcium phosphate. These matrices were evaluated based on critical parameters such as enzyme activity after immobilization, loading efficiency, recyclability, and storage stability. Among the diverse matrices considered, the alginate-gelatin-calcium phosphate capsules emerged as the best, exhibiting remarkable characteristics such as an enzyme activity of immobilized L-asparaginase at 60.43 U/mL, an impressive loading efficiency of 94.82%, sustained recyclability with 51% stability even after 16 cycles, and a storage efficiency only reducing to 27% at six months. These outstanding attributes position them as a promising choice for industrial applications. The encouraging outcomes of this investigation could be further refined through research into the effects of altering the concentrations of matrix components, shedding light on how these adjustments influence enzyme properties and their interactions with the external environment.

Keywords: *Ganoderma australe, L-asparaginase, Immobilization, Sodium alginate-gelatin-CaP capsules*

INTRODUCTION

Enzymes are veritable catalysts that play indispensable roles across a spectrum of industries, spanning from medicine to food production. Among these enzymes, L-asparaginase has garnered considerable attention for its therapeutic potential in cancer treatment and its role as an acrylamide mitigation agent in the food industry.

Cite this article: Chakraborty, M., & Shivakumar, S. (2023). Immobilization Strategies for L-Asparaginase from *Ganoderma australe* GPC191: Impact on Enzyme Activity, Stability, and Reusability, *Ind. J. Pure App. Biosci. 11*(5), 22-31. doi: http://dx.doi.org/10.18782/2582-2845.9025

However, the comprehensive adoption of free L-asparaginase within industrial operations has encountered substantial impediments. Reports of low stability under extreme industrial conditions, high production costs, and limited recyclability have not only elevated production expenses but have also acted as significant barriers to its large-scale applicability (Xie et al., 2022).

To overcome these challenges and unlock the full potential of L-asparaginase, researchers have explored immobilization as it is a widely used technique to eliminate the drawbacks of free enzymes in industrial applications. This transformative process can improve the catalytic and stability attributes of enzymes, encompassing factors such as selectivity, specificity, pH tolerance, and thermal stability (Xie et al., 2022).

An array of materials—ranging from organic to inorganic and hybrid compositions—has been employed for the immobilization of L-asparaginase. However, the choice of support material and immobilization method has a profound impact on the properties of the immobilized enzymes. An important step is the careful selection of carriers boasting attributes like a substantial specific surface area, resilience during immobilization and catalysis, mechanical robustness to endure multiple usage cycles, inertness to prevent interference with the enzyme's active sites and compliance with safety standards. Another significant consideration for the selection of immobilization matrix and method is safety, especially in the food industry, necessitating the use of natural, non-toxic carriers and cross-linking agents with compatibility, strength, porosity, hydrophilicity, and easy separation properties suitable for enzyme applications (Alam et al., 2021; & Li et al., 2020).

Keeping these into focus, the present investigation is the first report on exploring the immobilization of L-asparaginase from *Ganoderma australe* GPC191 by evaluating the diverse immobilization matrices.

MATERIALS AND METHODS

Chemicals and Reagents

All the chemicals and reagents were of analytical grade and procured from HiMedia, Mumbai, India.

Source of fungal L-asparaginase

G. australe GPC191, previously isolated and identified (GenBank accession number MN809333) from our earlier investigation, was used in this study to produce L-asparaginase. Modified Czapek Dox medium was used for L-asparaginase production, after incubation for 264 h (Chakraborty & Shivakumar, 2021). The L-asparaginase obtained was purified and used further in the present study.

Preparation of Immobilization Systems

Based on the literature, various support matrices were evaluated for L-asparaginase immobilization.

Immobilization of L-asparaginase in Gelatin Matrix

For immobilization of L-asparaginase using gelatin, the purified L-asparaginase (249.03 U/mg) was added to sterile gelatin (10% w/v), poured into a sterile glass petri plate, and allowed to solidify at room temperature. Following this, small size cubes of (1 cm^3) were sliced and kept in cold storage (4°C) after submerging in 50 mM of Tris-HCl buffer (pH 8) until further use (Amena et al., 2010).

Immobilization of L-asparaginase in Agarose Blocks

To immobilize the purified L-asparaginase in agarose blocks, the purified L-asparaginase (249.03 U/mg) was added to a sterile solution of agarose (1.5 %) prepared in Tris-acetate buffer (25 mM, pH 7.2) containing $CaCl_2$ (2 mM). This was further poured into a sterile glass petri plate and allowed to solidify at room temperature. Following this, small size cubes of (1 cm^3) were sliced and kept in cold storage (4°C) after submerging in 50 mM of Tris-HCl buffer (pH 8) until further use (Kumar et al., 2013).

Immobilization of L-asparaginase in Agar Blocks

For immobilization of the purified L-asparaginase in agar blocks, the purified L-asparaginase (249.03 U/mg) was added to a sterile solution of agar (4 %). This was further poured into a sterile glass petri plate and allowed to solidify at room temperature. Following this, small size cubes of (1 cm^3) were sliced and kept in cold storage (4°C) after submerging in 50 mM of Tris-HCl buffer (pH 8) until further use (Kumar et al., 2013).

Immobilization of L-asparaginase in Sodium Alginate Beads

The technique for sodium alginate immobilization was adopted with a minor adjustment based on the method by Amena et al. The purified L-asparaginase (249.03 U/mg) was introduced into a sterile sodium alginate solution (3 %, w/v). The resulting mixture was gently mixed at room temperature. It was gradually extruded as droplets from a dropper (0.9 mm diameter) placed at a 15-20 cm height into a cooled solution of $CaCl_2$ (0.2 M), forming alginate beads. These beads were subsequently transferred into a fresh solution of $CaCl_2$ (0.2 M) and preserved under cold conditions (4°C) for 2 h, facilitating the solidification of the beads. After this hardening process, the beads underwent a thorough cleansing with sterile distilled water. Subsequently, they were stored in 50 mM Tris-HCl buffer (pH 8) at 4°C, ensuring their preservation until their intended future use (Amena et al., 2010).

Immobilization of L-asparaginase in Sodium Alginate-CMC beads

This is a variant of the method detailed in the previous section, wherein the enzyme-alginate mixture was added dropwise into a cooled solution of $CaCl_2$ (0.2 M), forming alginate beads. These beads were left for curing under cold storage (4°C) for 2 h, following which they were rinsed with sterile distilled water and transferred to CMC solution (1 % solution, w/v). The beads were kept in this solution for 30 min before rinsing them with sterile distilled water. The resulting multilayer beads were stored in 50 mM Tris-HCl buffer (pH 8) at 4°C, ensuring their preservation until their intended future use (Bannikova et al., 2017).

Immobilization of L-asparaginase in Alginate-Gelatin Fibers

To immobilize the purified L-asparaginase in alginate-gelatin fibers, the purified L-asparaginase (249.03 U/mg) was added into the sterile solution of the matrix prepared with 3 % gelatin in 5 % sodium alginate solution. Glutaraldehyde (2.5 %) was added to this mixture to facilitate cross-linking and homogenization. The resulting mixture was then extruded as droplets from a dropper (0.9 mm diameter) placed at a 15-20 cm height into a cooled solution of $CaCl_2$ (0.2 M), forming alginate-gelatin fibers. These generated fibers were subsequently transferred into a fresh solution of $CaCl_2$ (0.2 M) and preserved for curing under cold conditions (4°C) for 2 h. After this hardening process, the fibres were sliced into 3 mm pieces and cleansed thoroughly with sterile distilled water. Subsequently, they were stored in 50 mM Tris-HCl buffer (pH 8) at 4°C, ensuring their preservation until their intended future use (Amena et al., 2010).

Immobilization of L-asparaginase in Alginate-Gelatin-Calcium Phosphate Capsules

Purified L-asparaginase (249.03 U/mg) was added to sodium alginate (2 %, w/v), which was prepared in potassium phosphate buffer (0.1 M, pH 7.5) to prepare the enzyme mix (mixture 1) for the capsule preparation. Further, 75 mM $CaCl_2$ solution was mixed with gelatin to achieve 1 % concentration (mixture 2). Once these mixtures were ready, the capsules were formed by

dropwise addition of mixture 1 in mixture 2 using a dropper (0.9 mm diameter) placed 15-20 cm height above. The resultant capsules were kept aside for 1 h for hardening, followed by filtration and distilled water wash (to remove any unbound enzymes). A final wash was done with potassium phosphate buffer, after which the capsules were stored in phosphate buffer at 4°C, ensuring their preservation until their intended future use (Shen et al., 2011).

Evaluation Parameters of Matrices for L-asparaginase Immobilization

Each immobilized system was evaluated for enzyme activity, loading efficiency, recyclability, and storage stabilities as per Gur et al., 2018 and Shen et al. 2011 to determine the most efficient system for the present investigation.

Enzyme Activity of Immobilized L-asparaginase

The enzyme activity of the immobilized L-asparaginase was evaluated by incubating with L-asparagine in the presence of Tris-HCL, and the L-asparaginase activity was assessed as per Nesslerization reaction as detailed by Imada et al., after incubation at 37 ± 2 °C for 30 min.

Loading Efficiency of Immobilization Matrices

The following equation calculated the loading efficiency of each matrix:

$$Loading\ efficiency = 100 - \frac{C_i V_i - C_f V_f}{C_i V_i} \times 100$$

C_i and V_i are the protein concentration and initial volume of solution, respectively, when the enzyme was added into the immobilization matrix. The C_f and V_f are the final protein concentration and filtrate volume obtained when the immobilized matrices are disintegrated in Tris-HCl (pH 8.0) of equal volume as the initial solution and filtered.

Recycling Stability of Immobilized L-asparaginase System

To determine and track the activity of L-asparaginase post-immobilization, the percentage of activity at each stage was calculated as per the following equation:

$$Recycling\ stability = \frac{Immobilized\ enzyme\ activity}{Free\ enzyme\ acitivty} \times 100$$

The recycling stability of the immobilized L-asparaginase was determined by calculating the percentage of its activity after each system after every cycle until the enzyme activity was negligible. The activity determined for the first batch of immobilized L-asparaginase for each matrix was considered 100 % for the calculations.

Storage Efficiency of Immobilization Matrices

The efficiency of storage of immobilized L-asparaginase for long periods, especially at cold temperatures, is a crucial factor for selecting the best matrix. This is evaluated by calculating storage efficiency as per the following equation:

$$Storage\ efficiency = \frac{Immobilized\ enzyme\ activity\ after\ storage}{Immobilized\ enzyme\ acitivty\ at\ initial\ stage} \times 100$$

The immobilization matrix ensuring maximal L-asparaginase activity with better loading efficiency and prolonged storage and recycling stabilities was selected for further processes.

Statistical Analysis

The experimental data was collected at each stage in triplicates and recorded as mean ± SD using Microsoft Excel software and analyzed using GraphPad Prism 9. The comparison between the immobilization matrices as per parameters mentioned in the above sections was done using one-way analysis of variance (ANOVA), followed by post-hoc analysis using Dunett's multiple comparison test when free enzyme activity was compared to the enzyme activity of the

immobilized L-asparaginase in respective matrices. Tukey's multiple comparison tests were performed when each immobilized L-asparaginase system was compared with each other in the case of enzyme activity and loading efficiency comparisons. The *p*-value > 0.05 was considered as the minimum level of significance for this selection process, post-statistical analysis. For the recyclability and storage assessments of the immobilization matrices, mean ± SD was integrated into the graphs.

RESULTS AND DISCUSSION

The various immobilization techniques evaluated in the present study are based on principles such as entrapment (e.g., alginate, gelatin), and cross-linking (e.g., calcium alginate-gelatin). The use of natural polymers such as gelatin, agar, and agarose in the present study is due to their status as generally recognized as safe (GRAS) and application as food additives (Zucca et al., 2016; & Scardi, 1987). Further, natural materials such as cellulose have been used as a matrix component along with gelatin, agar, and its derivatives, as these are inexpensive, easily accessible, biodegradable, non-toxic, and biocompatible. Lastly, some matrices used in the present investigation also involved multilayered encapsulation of these natural polymers to understand their superiority in terms of efficiency and stability. However, the best matrix among those evaluated was selected based on the matrix demonstrating the highest enzyme activity recorded for L-asparaginase post-immobilization, loading efficiency of the matrix, reusability, and storage stability.

Enzyme Activity of Immobilized L-asparaginase

After assessing L-asparaginase activity post-immobilization, it became evident that immobilized L-asparaginase exhibited significantly higher activity compared to the free enzyme (*p*-value < 0.0001). This heightened activity could be attributed to several factors. First, immobilization potentially increased the interaction between the substrate and the entrapped enzyme, reducing interferences from L-asparaginase's microenvironment during the catalysis process. Additionally, the enhanced activity might result from effects such as the partition effect on substrate concentration, restricted mobility of the immobilized L-asparaginase, alterations in molecular orientation favoring increased interaction with L-asparagine, and greater flexibility for conformational changes within the matrix (Ulu & Ates, 2017; & Chaudhari et al., 2015).

However, among the various immobilized systems, the gelatin and agar block matrices yielded significantly low enzyme activity (*p*-value < 0.0001; as shown in Figure 1). This outcome may be attributed to the cumulative effects of L-asparaginase denaturation due to latent heat generated during matrix solidification and leaching of L-asparaginase during storage, potentially caused by variations in pore sizes within the beads (Amena et al., 2010; & de Alteriis et al., 1987).

Conversely, matrices composed of agarose, sodium alginate, and alginate-gelatin demonstrated significantly higher L-asparaginase activity than gelatin and agar block matrices (*p*-value < 0.0001), likely due to the presence of an additional stabilizing layer of calcium chloride. However, significantly highest L-asparaginase activity was achieved with matrices featuring a triple-layer configuration, i.e., an additional layer of CMC or calcium phosphate (as depicted in Figure 1). This superior activity may be attributed to the mechanical stability provided by these additional layers and their ability to prevent the chelation of calcium chloride as a result of phosphate in the buffer system (Shen et al., 2011).

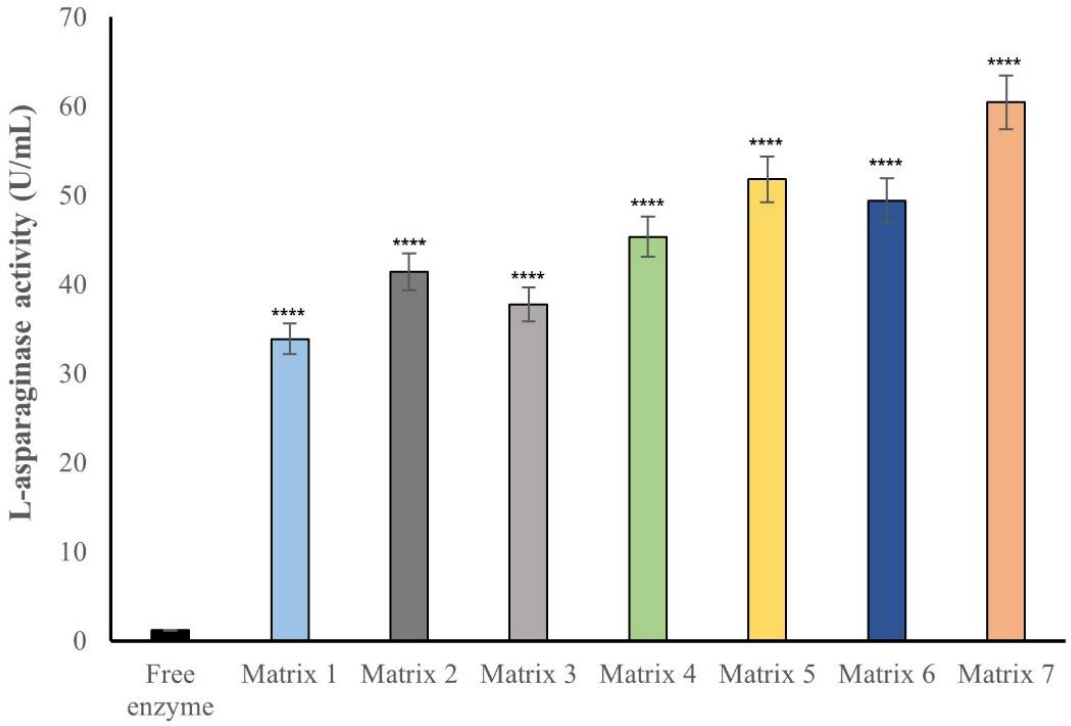

Figure1. Effect of immobilization on enzyme activity of L-asparaginase from *Ganoderma australe* GPC191 [Matrix 1: gelatin; Matrix 2: agarose blocks; Matrix 3: agar blocks; Matrix 4: sodium alginate beads; Matrix 5: sodium alginate-CMC beads; Matrix 6: alginate-gelatin fibers; Matrix 7: alginate-gelatin-calcium phosphate capsules; ** = *p*-value < 0.0001 wherein each matrix was compared to each other]**

Loading Efficiency of Immobilization Matrices

The loading efficiency, which signifies the ability of immobilization matrices to retain the enzyme within them, is a crucial parameter in determining the most suitable support for immobilization. In our current study, agar blocks exhibited significantly low loading efficiency compared to the other immobilization supports, as depicted in Figure 2. This finding, coupled with the previously observed low enzyme activity, suggests that the matrix formed by agar is inadequate in preventing the leakage of L-asparaginase. Furthermore, the substantial disparity in loading efficiencies between agar and gelatin matrices reinforces the notion that the reduced activity in the gelatin system may be attributed to the denaturation of L-asparaginase.

Matrices comprised of agarose, sodium alginate, and alginate-gelatin also exhibited poor efficiency in enzyme loading, as shown in Figure 2. This finding aligns with the enzyme activity results, which suggests the susceptibility of immobilization matrices to the buffer systems employed, leading to the possibility of enzyme leakage (Shen et al., 2011). Another contributing factor to the low loading efficiency could be the absence of suitable functional groups for interaction with the enzyme, thereby failing to prevent leakage. Further, since the concentration of cross-linkers play a crucial role in loading efficiencies of associated matrices, in the cases of alginate-based matrices, the concentration of calcium ions can be a reason for the low compactness of associated matrices. Therefore, for these matrices to be effectively utilized, they may require optimization, functionalization, or reinforcement (Gur et al., 2018; & Ulu & Ates, 2017). Therefore, the loading efficiency of immobilization matrices enhanced with an additional layer of CMC or calcium phosphate demonstrated a higher capacity in our study, as illustrated in Figure 2.

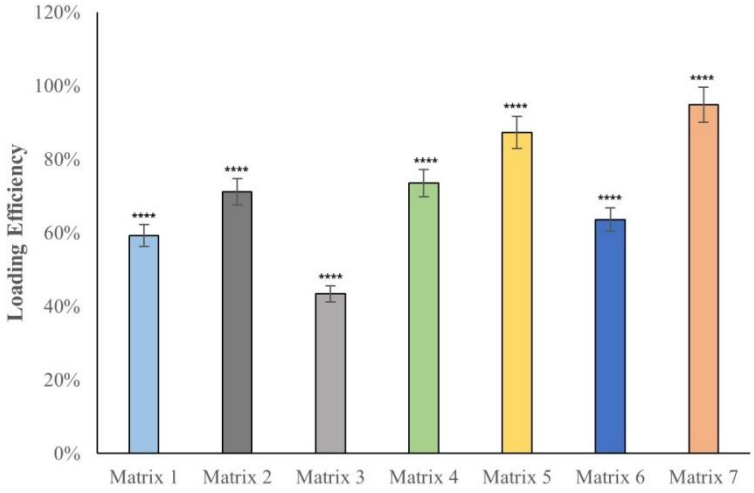

Figure2. Influence of loading efficiency of immobilization matrices on immobilization of L-asparaginase from *Ganoderma australe* GPC191 [Matrix 1: gelatin; Matrix 2: agarose blocks; Matrix 3: agar blocks; Matrix 4: sodium alginate beads; Matrix 5: sodium alginate-CMC beads; Matrix 6: alginate-gelatin fibers; Matrix 7: alginate-gelatin-calcium phosphate capsules; ** = *p*-value < 0.0001 wherein each matrix was compared to each other]**

Recycling Stability of Immobilized L-asparaginase

The cost-effectiveness of a support matrix system is not solely determined by its composition and source but also hinges on its ability to sustain multiple usage cycles without a significant decline in the activity of the immobilized enzyme. This factor holds paramount importance in the industrial application of commercial enzymes.

In our current investigation, a support matrix comprising sodium alginate-gelatin-calcium phosphate and alginate-CMC exhibited exceptional stability, as illustrated in Figure 3. This enhanced stability could be attributed to various factors, including the high mechanical strength of the capsules, limited swelling capacity, multiple layers of support, and the presence of small pore sizes (Shen et al., 2011). Additionally, the intricate nature of this matrix system may render it more resilient to the impacts of non-gelling ions, loss of cross-linking ions, and pH fluctuations.

However, the recyclability of other immobilization systems employed in the present study (as depicted in Figure 3) might have been compromised due to denaturation, enzyme loss through leaching, and instability of the immobilization matrix upon repeated usage (Zucca et al., 2016; & Chaudhari et al., 2015).

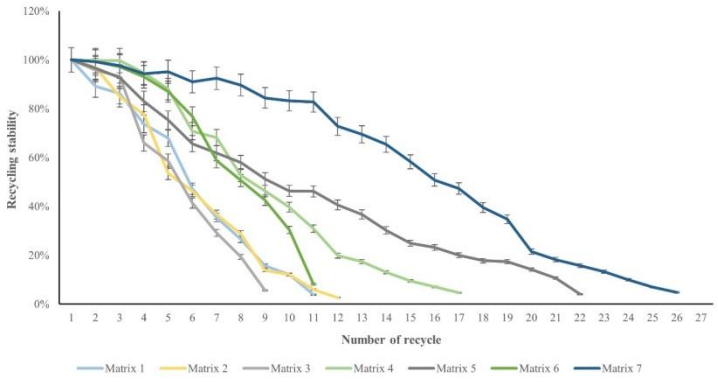

Figure3. Impact of recycling stability of immobilization matrices on L-asparaginase activity. Matrix 1: gelatin; Matrix 2: agarose blocks; Matrix 3: agar blocks; Matrix 4: sodium alginate beads; Matrix 5: sodium alginate-CMC beads; Matrix 6: alginate-gelatin fibers; Matrix 7: alginate-gelatin-calcium phosphate capsules

Storage Stability of Immobilization Matrices

Enzyme stability in storage conditions is dependent on several factors, such as the nature and number of bonds formed between the enzyme and its immobilization matrix, the degree of confinement, and the conditions in which immobilization has been performed. However, for determining its industrial usage, enzyme stability over the long duration of storage plays a critical role in programming its application (Chaudhari et al., 2015).

In the present investigation, alginate-gelatin-calcium phosphate capsules demonstrated the highest storage efficiency, with a drop below 50% only after four months, followed closely by sodium alginate-CMC beads, which dropped beyond 50% only after three months (as shown in Figure 4). This finding underscores the robustness of the immobilization matrix, as it withstands the typical adverse effects of assay conditions that often result in enzyme leaching or matrix destabilization. Moreover, it highlights the matrix's capacity to shield L-asparaginase from structural and functional denaturation by offering protection against external environmental factors.

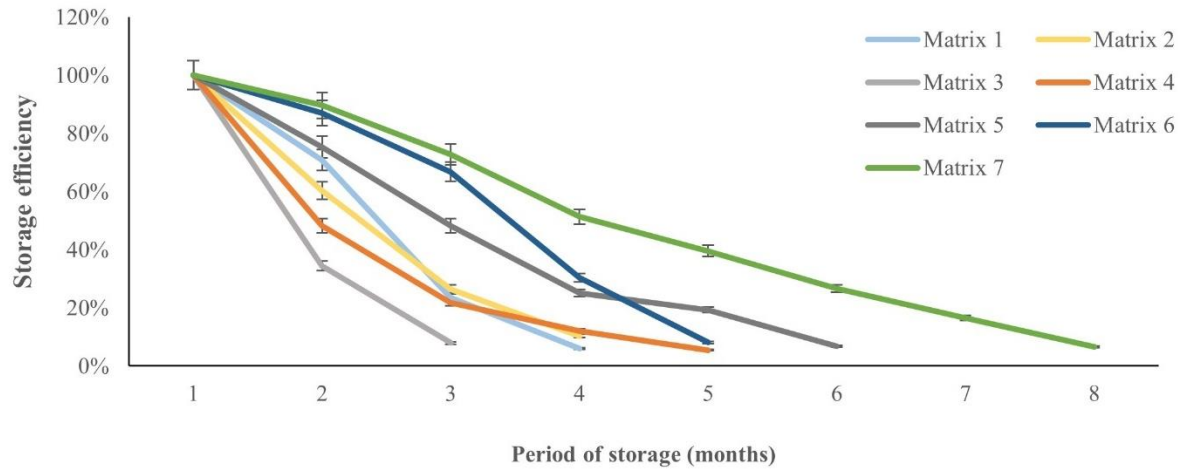

Figure4. Impact of period of storage on the efficiency of immobilization matrices used for L-asparaginase immobilization. Matrix 1: gelatin; Matrix 2: agarose blocks; Matrix 3: agar blocks; Matrix 4: sodium alginate beads; Matrix 5: sodium alginate-CMC beads; Matrix 6: alginate-gelatin fibers; Matrix 7: alginate-gelatin-calcium phosphate capsules

With respect to literature, Li et al. (2020) evaluated the immobilization system for L-asparaginase constituting agarose spheres and reported loading efficiency, storage stability, and reusability of 68.43 %, 93.21 % (after six cycles), and 72.25 % (storage of 28 days), respectively. This was comparable to the present study, where the agarose-based matrix demonstrated loading efficiency, storage stability, and reusability of 71.15 %, recyclability up to five cycles (with 54 % efficiency), and 60 % (storage after 2 months).

On comparing the use of calcium-alginate as an immobilization matrix, the enzyme activity was higher in the current investigation i.e., 45.34 U/mL as compared to Ashok et al. who reported 17.68 U/mL for L-asparaginase from *Rhizopus microsporus* IBBL-2.

However, due to the limitation of extensive fungal literature on immobilization processes for L-asparaginase, the present study contributes greatly to the enrichment of scientific knowledge in terms of the evaluation of strategies for L-asparaginase immobilization.

CONCLUSION

The present study is the first report on immobilization studies of L-asparaginase from basidiomycetes. In this investigation, when various matrices for immobilizing L-asparaginase were compared, it was evident that alginate-gelatin-calcium phosphate

capsules outperformed others in terms of efficiency, making it a promising choice for industrial use. Nonetheless, it is imperative to conduct additional research into the effects of altering the concentrations of matrix components and how this impacts enzyme properties and their interactions with the external environment. This further examination is essential to validate the suitability of the system for further use.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Code availability: GraphPad Prism software (version 9.2.0); Design Expert software (13.0 version)

Conflict of interest: The authors declare no conflict of interest in the publication.

Funding: The authors did not receive support from any organization for the submitted work.

Authors' contributions: SS and MC contributed to the concept development and design for the research. MC executed, analyzed, and interpreted the study under the guidance of SS. MC developed the manuscript draft, which was reviewed and edited by SS. All authors have seen and approved the final manuscript and its contents and are aware of the responsibilities connected to the authorship.

REFERENCES

Alam, S., Nagpal, T., Singhal, R., & Khare, S. K. (2021). Immobilization of L-asparaginase on magnetic nanoparticles: Kinetics and functional characterization and applications. *Bioresour. Technol. 339*, 125599. https://doi.org/10.1016/j.biortech.2021.125599

Amena, S., Lingappa, K., Vishalakshi, N., Prabhakar, M., & Dayanand, A. (2010). Immobilization and Characterization of L-Asparaginase from *Streptomyces gulbargensis*. *J. Pure Appl. Microbiol. 4*, 623-628.

Ashok, A., & Devarai, S. K. (2019). l-Asparaginase production in rotating bed reactor from *Rhizopus microsporus* IBBL-2 using immobilized Ca-alginate beads. *3 Biotech*. *9*, 1-10. https://doi.org/10.1007/s13205-019-1883-5

Bannikova, A., Rasumova, L., Evteev, A., Evdokimov, I., & Kasapis, S. (2017). Protein-loaded sodium alginate and carboxymethyl cellulose beads for controlled release under simulated gastrointestinal conditions. *Int. J. Food Sci. Technol. 52*, 2171-2179. https://doi.org/10.1111/ijfs.13496

Chakraborty, M., & Shivakumar, S. (2021). Bioprospecting of the agaricomycete *Ganoderma australe* GPC191 as novel source for L-asparaginase production. *Sci. Rep. 11*, 6192. https://doi.org/10.1038/s41598-021-84949-5

Chaudhari, A. S., Kar, R. J., & Singhal, S. R. (2015). Immobilization of proteins in alginate: functional properties and applications. *Curr. Org. Chem. 19*, 1732-1754.

de Alteriis, E., Parascandola, P., Pecorella, M. A., & Scardi, V. (1987). Entrapment of microbial cells within a gelatin matrix: a comparison of three procedures. *Biotechnol. Tech. 1*, 109-114. https://doi.org/10.1007/BF00159331

Gür, S. D., İdil, N., & Aksöz, N. (2018). Optimization of enzyme co-immobilization with sodium alginate and glutaraldehyde-activated chitosan beads. *Appl. Biochem. Biotechnol. 184*, 538-552. https://doi.org/10.1007/s12010-017-2566-5

Imada, A., Igarasi, S., Nakahama, K., & Isono, M. (1973). Asparaginase and glutaminase activities of microorganisms. *J. Gen. Microbiol. 76*, 85-99.

Kumar, K., Kataria, M., & Verma, N. (2013). Plant asparaginase-based asparagine biosensor for leukemia. *Artif. Cells Nanomed. Biotechnol*. *41*, 184-188. https://doi.org/10.3109/10731199.2012.716062

Li, R., Zhang, Z., Pei, X., & Xia, X. (2020). Covalent immobilization of L-asparaginase and optimization of its enzyme reactor for reducing acrylamide formation in a heated food model system. *Front. Bioeng. Biotechnol*. *8*, 584758. https://doi.org/10.3389/fbioe.2020.584758

Scardi, V. (1987). *Immobilization of enzymes and microbial cells in gelatin*. InMethods in enzymology. *135*, pp. 293-299. Academic Press. https://doi.org/10.1016/0076-6879(87)35086-4

Shen, Q., Yang, R., Hua, X., Ye, F., Zhang, W., & Zhao, W. (2011). Gelatin-templated biomimetic calcification for β-galactosidase immobilization. *Process Biochem*. *46*, 1565-1571. https://doi.org/10.1016/j.procbio.2011.04.010

Ulu, A., & Ates, B. (2017). Immobilization of L-asparaginase on carrier materials: a comprehensive review. *Bioconjug. Chem. 28*, 1598-1610. https://doi.org/10.1021/acs.bioconjchem.7b00217

Xie, J., Zhang, Y., & Simpson, B. (2022). Food enzymes immobilization: novel carriers, techniques and applications. *Curr. Opin. Food Sci*. *43*, 27-35. https://doi.org/10.1016/j.cofs.2021.09.004

Zucca, P., Fernandez-Lafuente, R., & Sanjust, E. (2016). Agarose and its derivatives are supports for enzyme immobilization. *Molecules*. *21*, 1577. https://doi.org/10.3390/molecules21111577