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# Process-Integrated Matrix Engineering and Drying Strategy for Stabilization of Fibrinolytic Protease from Bacillus tequilensis toward Biomanufacturing Applications

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# Abstract

Stabilizing enzyme functionality during downstream processing remains a central challenge in scalable biomanufacturing. This study presents a process-integrated evaluation of microencapsulation strategies to preserve the activity of a fibrinolytic protease derived from *Bacillus tequilensis* HSFI-5, with emphasis on the interplay between encapsulant matrix, drying method, and enzymatic accessibility. Encapsulation systems based on maltodextrin, Arabic gum, chitosan, carrageenan, and alginate (5–15% w/v) were processed by freeze-drying and spray-drying and assessed for activity retention and microencapsulation yield. Results demonstrated that both formulation and processing conditions significantly influenced functional performance ($p < 0.05$). Lower polymer concentrations favored higher apparent enzymatic activity, whereas higher concentrations improved powder recovery, revealing a trade-off between catalytic accessibility and process efficiency. A moderate negative correlation between activity retention and yield ($r = −0.62$, $p = 0.018$) supports a matrix-dependent diffusion constraint. A comparative analysis of crude, diluted, and microencapsulated systems further showed that reduced apparent activity in encapsulated formulations is primarily due to diffusion-limited accessibility rather than complete enzyme inactivation. It was evidenced by increased activity following mechanical disruption. Among the evaluated systems, maltodextrin-based formulations exhibited a favorable balance between activity retention and yield, particularly under spray-drying conditions, indicating compatibility with scalable processing. Qualitative clot degradation assays confirmed preservation of fibrinolytic functionality after encapsulation, although with a delayed response consistent with controlled enzyme release. Morphological analysis revealed spherical, relatively smooth microcapsules that may facilitate improved hydration and substrate diffusion. Collectively, these findings establish a process-level framework linking matrix composition, drying strategy, and mass transfer behavior to enzymatic performance. This work highlights the importance of designing encapsulation systems that balance structural protection with functional accessibility, thereby providing a rational basis for developing stable, scalable enzyme formulations for biomanufacturing applications.


# Highlights

- Matrix composition critically affects enzyme stability and process performance
- The maltodextrin–Arabic gum system provides an optimal activity–yield balance
- Spray drying enables scalable recovery despite moderate activity loss
- Freeze drying preserves activity but limits industrial scalability
- Process-integrated formulation strategy supports enzyme biomanufacturing

# 1. Introduction

Thrombosis is a major cause of death from heart and blood vessel diseases worldwide (Mc Namara et al., 2019; Al-Ani et al., 2020; WHO, 2021). Standard thrombolytic therapies may be associated with high cost, adverse effects, and stability limitations. Despite the availability of established thrombolytic

enzymes such as urokinase and streptokinase, limitations related to cost, side effects, and stability continue to drive the search for alternative fibrinolytic systems with improved functional robustness (Capitanescu et al., 2016; Nailufar et al., 2016; Akhtar et al., 2017; Katz & Tadi, 2019).

*Bacillus* species are known to produce fibrinolytic proteases, which are essential thrombolytic agents (Ayanti et al., 2022; Nailufar et al., 2016). These enzymes are often found in *Bacillus* strains from fermented foods (Ju et al., 2019; Hu et al., 2019), for example, nattokinase from *Bacillus subtilis* subsp. Natto can inhibit thrombin activity in the body and increase fibrinolytic activity in patients (Ju et al., 2019). Similarly, proteases from *Bacillus* in Douchi can degrade fibrin both *in vitro* and *in vivo* (Hu et al., 2019).

Recent studies have explored fibrinolytic protease–producing bacteria isolated from Rusip, a fermented product derived from the digestive organs of the sea cucumber *Holothuria scabra* (Afriansyah & Ethica, 2023). Among these isolates, *Bacillus tequilensis* HSFI-5 (HSFI: *H. scabra* fermented intestine) produced a crude protease that exhibited higher *in vitro* clot lysis activity than the positive control, nattokinase, and demonstrated anticoagulant and antiplatelet effects (Hidayati et al., 2021; Mehrnoush et al., 2012). Nattokinase itself is a commercially available thrombolytic enzyme produced through a patented process and marketed in encapsulated form (Chen et al., 2018).

The fibrinolytic protease from HSFI-5 is a promising example of an enzyme system capable of degrading fibrin. Its effectiveness could be improved by purifying it, microencapsulating it, and testing it in living systems to assess safety and efficacy. These features make HSFI-5 a strong candidate for the development of fibrinolytic enzymes similar to nattokinase. *B. tequilensis* HSFI-5, found in marine environments, produces a potential fibrinolytic protease that may support fibrin degradation processes associated with blood clot formation (Hidayati et al., 2021). However, like other protein-based drugs, the crude enzyme can lose its activity quickly when exposed to heat, moisture, or oxygen during storage and transport. In this context, microencapsulation helps solve these problems by protecting sensitive biomolecules inside a polymer matrix (Almassri et al., 2024). Typical food and pharmaceutical ingredients, such as alginate, Arabic gum, chitosan, carrageenan, and maltodextrin, are often used for this purpose because they are safe and form good films (Cui et al., 2023).

Freeze-drying and spray-drying have been extensively used for the microencapsulation of various bioactive compounds (Rezvankhah et al., 2020; Kandasamy et al., 2022). However, their application to enzymes has received comparatively less attention. Preservation of enzymatic functionality after encapsulation represents a critical translational step linking upstream enzyme discovery with downstream biological validation and application. This study aimed to identify formulation and processing parameters that preserve fibrinolytic protease functionality during microencapsulation, thereby supporting the development of stable enzyme-based biotechnological systems. Encapsulation performance was subsequently evaluated based on enzymatic activity, yield, morphology, and functional clot degradation. This study aimed to contribute to the valorization of microbial bioresources through formulation strategies to enhance enzyme functionality. This study hypothesizes that the apparent loss

of enzymatic activity in encapsulated systems is not due to denaturation but to diffusion-limited accessibility within polymer matrices.

## 2. Materials and Methods

## 2.1. Bacterial Strain and Enzyme Production

Cells of *B. tequilensis* HSFI-5 were revived from glycerol stock and cultured in Brain Heart Infusion Broth (BHIB) to optimal growth. Proteolytic and fibrinolytic activity was confirmed by clear zones on skim milk and fibrin agar. The crude protease was collected from the culture supernatant after centrifugation and then ultrafiltered as previously described (Ferdiani et al., 2023). After ultrafiltration, the extract was then used directly for microencapsulation. Crude protease activity before encapsulation was measured and used as the reference (100% activity retention) for comparison with encapsulated formulations.

## 2.2. Carrier Material Specification

The encapsulating materials used in this study were chitosan (medium molecular weight, degree of deacetylation 75–85%, Sigma-Aldrich, USA), carrageenan (κ-carrageenan, food grade, Merck), maltodextrin (DE 10–12), and Arabic gum (Acacia senegal).

## 2.3. Microencapsulation Procedure

Crude proteases were encapsulated with maltodextrin, Arabic gum, chitosan, carrageenan, or alginate at 5, 10, and 15% (w/v) (See Fig. 1). The crude protease represents a semi-preparative enzyme system following upstream production and ultrafiltration (Ferdiani et al., 2023). For each treatment, the coating agent was added to the crude enzyme at the specified concentration. Encapsulation was performed using either freeze-drying or spray-drying. For freeze-drying, 1.25 g, 2.5 g, or 3.75 g of coating agent was dissolved in 25 mL of crude proteases for the 5%, 10%, and 15% treatments, respectively, until uniform. Samples were frozen and placed in freeze-drying equipment for 72 h (Mehrnoush et al., 2012). For spray-drying, the coating agent at each concentration was mixed with 50 mL of enzyme, and the mixture was processed in a spray dryer. The resulting samples were analyzed by Scanning Electron Microscopy (SEM) to assess capsule morphology. All experiments were performed in triplicate, and results are presented as mean values.

**Figure 1. Schematic representation of the microencapsulation process of fibrinolytic protease from Bacillus tequilensis HSFI-5.**

Crude protease extract was combined with encapsulant matrices at defined concentrations and processed using either freeze-drying or spray-drying. The workflow illustrates formulation preparation, drying pathways, and recovery of microencapsulated powders for subsequent characterization.

*Freeze-drying*

Each encapsulating agent was dissolved in 25 mL of crude enzyme solution at concentrations of 5, 10, and 15%. The obtained mixtures were frozen at − 20°C for 30–48 h, then freeze-dried for 72 h under reduced pressure. Chitosan was dissolved in 1% (v/v) acetic acid solution (pH ≈ 4.5) before mixing with the enzyme solution. The resulting powders were stored in airtight containers at 4°C until analysis, following the procedure described by Mehrnoush et al. (2012).

*Spray-drying*

Encapsulating agents were combined with 50 mL of crude enzyme solution and processed in a laboratory spray dryer at an inlet temperature of 110°C, an outlet temperature of 65°C, and a feed rate of 6 mL/min. Dried microcapsules were collected from the cyclone separator and stored in desiccators for analysis as previously described by Shahidi and Han (1993).

# 2.4. Morphological analysis

Microcapsule morphology was examined using scanning electron microscopy (SEM). Samples were attached to aluminum stubs with double-sided carbon tape and coated with a thin layer of gold to improve conductivity. Features such as particle shape, surface texture, and encapsulation integrity were evaluated from SEM images taken at 10,000× magnification (Jaidka et al., 2022).

# 2.5. Enzymatic activity and yield

Protease activity was measured spectrophotometrically using casein as the substrate, as previously described. After a 20-min incubation at room temperature, absorbance was measured at 660 nm using a UV-Vis spectrophotometer (Dewi et al., 2023; Ferdiani et al., 2023). Protease activity was reported as U $mL^{-1}$ based on tyrosine equivalents released (Pardosi et al., 2024). Yield was calculated by comparing the dry mass of microcapsules to the initial solid content of the feed solution, using Eq. (1):

$$Yield\ (\%) = \frac{weight\ of\ dried\ microcapsules}{Initial\ total\ solid\ weight}\ x\ 100$$

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Statistical analysis was performed using GraphPad Prism software with α = 0.05 (two-tailed). One-way and two-way ANOVA were applied where appropriate to evaluate the effects of encapsulant type, polymer concentration, and drying method on enzymatic activity and product yield. Tukey's post hoc test was used for multiple comparisons. In addition, a correlation analysis was conducted to examine the relationship between activity retention and microencapsulation yield. Qualitative clot lysis outcomes were assessed based on visual observation under controlled conditions.

**Qualitative observation of clot degradation**

To qualitatively assess fibrinolytic activity, fresh human blood (600 µL) was transferred into sterile microtubes and incubated at 37°C to allow clot formation. After clot stabilization, excess serum was

carefully removed. The clots were then incubated with microencapsulated protease formulations, crude enzyme preparation, or negative control under identical conditions at 37°C. Changes in clot structure and visible degradation were monitored and documented photographically after incubation, using previously described observational approaches (Ainutajriani et al., 2023). This assay was designed as a qualitative visual evaluation, and no gravimetric measurements or quantitative statistical analyses were performed.

# 3. Results

## 3.1. Microcapsule morphology

Macroscopic comparison revealed formulation-dependent differences in microcapsule appearance across encapsulant matrices, concentrations, and drying methods (Fig. 2). Freeze-dried formulations (Fig. 2A) generally formed irregular and porous structures, whereas spray-dried formulations (Fig. 2B) produced more compact powders with a finer appearance.

Differences in aggregation behavior were also observed among encapsulants. Maltodextrin-based systems produced relatively uniform, less aggregated powders, whereas chitosan and carrageenan formulations tended to form denser, more aggregated structures. These macroscopic differences suggest matrix-dependent structural organization that may influence mass transfer and enzymatic accessibility upon reconstitution.

Because macroscopic observation provides only qualitative information, detailed structural interpretation was supported by scanning electron microscopy (SEM) analysis (Fig. 6).

**Figure 2. Macroscopic appearance of microencapsulated fibrinolytic protease from Bacillus tequilensis HSFI-5 prepared using different encapsulant matrices, concentrations, and drying methods.**

**(A)** Freeze-dried microcapsules prepared with alginate, Arabic gum, carrageenan, chitosan, and maltodextrin at 5, 10, and 15% (w/v).
**(B)** Spray-dried microcapsules prepared with Arabic gum and maltodextrin at identical concentrations. Variations in powder appearance and aggregation were observed across encapsulant matrices and drying methods. Detailed structural interpretation was supported by SEM analysis (Fig. 6).

## 3.2. Enzymatic activity and formulation-dependent performance

The effects of the encapsulant matrix and drying method on enzymatic performance are summarized in Fig. 3. Both factors significantly influenced activity retention and microencapsulation yield ($p < 0.05$), with a significant interaction effect indicating that functional preservation depends on the combined effects of formulation and process conditions.

As shown in Fig. 3A, formulations containing lower polymer concentrations (5% w/v) generally exhibited higher activity retention relative to the crude enzyme baseline (3.308 U $mL^{-1}$). Maltodextrin-based

systems achieved the highest activity retention, reaching up to 79% under spray-drying conditions. Increasing the polymer concentration reduced apparent activity, consistent with diffusion-limited substrate accessibility in denser matrices.

Microencapsulation yield (Fig. 3B) showed an inverse trend, with higher polymer concentrations improving powder recovery. Correlation analysis revealed a moderate negative relationship between yield and activity retention (r = − 0.62, p = 0.018), indicating a trade-off between process efficiency and enzymatic accessibility.

**Figure 3. Effect of encapsulant matrix and drying method on enzymatic activity retention and microencapsulation yield.**

**(A)** Activity retention (%) relative to crude enzyme activity (3.308 U $mL^{-1}$).
(B) Microencapsulation yield (%) across different formulations. Values represent mean measurements used for comparative evaluation of functional preservation and process efficiency. Ma = maltodextrin, Ar = Arabic gum, Ch = chitosan, Ca = carrageenan, Al = alginate.
Figure 3A shows normalized activity retention values calculated relative to crude enzyme activity, allowing direct comparison with retention percentages summarized in Table 1. As shown in Fig. 3A, formulations containing 5% coating material generally exhibited higher retained enzymatic activity. Enzymatic activity values were normalized to crude protease activity (3.308 U/mL; set as 100%) to enable direct comparison among formulations. The resulting activity retention percentages are summarized in Table 1. With a baseline crude activity of 3.308 U/mL = 3.308 U/mL, activity retention was calculated using Eq. (2):

$$Retention\ (\%) = \frac{Activity_{encapsulated}}{Activity_{unencapsulate}}\ x\ 100$$

2
As shown in Table 1, the activity retention analysis revealed that maltodextrin 5% formulations retained up to 79% of the initial enzymatic activity following spray-drying. The apparent full retention observed in chitosan 5% formulations may reflect retention within the assay's resolution limits rather than the complete absence of catalytic loss. The combined interpretation of activity (Fig. 3A) and yield (Fig. 3B) highlights a performance trade-off inherent to polymer-based encapsulation systems.

Higher polymer concentrations generally reduced activity retention, supporting diffusion-limited accessibility within denser polymer matrices. Pearson's correlation analysis revealed a moderate negative correlation (r = − 0.62, p = 0.018, n = 12). The analysis demonstrated a negative relationship between microencapsulation yield and retained enzymatic activity, indicating that formulations producing higher powder recovery tended to exhibit reduced apparent catalytic accessibility due to increased matrix density.

Table 1
Activity retention of microencapsulated HSFI-5 protease

| Formulation | Activity retention (%) |
|---|---|
| Maltodextrin 5% | 79.2% (spray) / 66.9% (freeze) |
| Maltodextrin 10% | 68.9% |
| Maltodextrin 15% | 60.3% |
| Arabic gum 5% | 68.0% (spray) / 63.2 (freeze) |
| Arabic gum 10% | 68.4% |
| Arabic gum 15% | 49.8% |
| Chitosan 5% | 97% (freeze-drying) |
| Chitosan 10% | 24.6% |
| Carrageenan | low (< 32%) |
| Alginate 10% | 53.3% |

Although freeze-drying is generally considered gentler for protein stabilization, maltodextrin-based formulations can exhibit higher *apparent* post-processing activity after spray-drying. This formulation-dependent behavior indicates that process effects are strongly modulated by matrix composition. This mechanism is consistent with the higher activity retention observed for maltodextrin 5% under spray-drying compared with freeze-drying (Table 1).

**Figure 3B** shows how both the encapsulant type and the drying method affect product recovery efficiency. The yield of microencapsulation products varied significantly depending on the matrix and drying method used. In most cases, freeze-drying preserved activity better than spray-drying, except for maltodextrin formulations, where spray-drying yielded activity comparable to or higher than that of freeze-drying. The maltodextrin exception is mechanistically plausible because carbohydrate-rich systems can benefit from rapid dehydration during spray-drying. Fast water removal promotes the formation of an amorphous glass, which reduces the conformational mobility of proteins/enzymes. At the same time, the short residence time of droplets in the drying chamber may limit cumulative thermal damage. Conversely, freeze-drying introduces distinct stresses (freezing, concentration, and ice–interface effects) that can reduce measurable activity even when gross denaturation is limited. Therefore, for maltodextrin 5% in particular, the balance between rapid glass formation and rapid rehydration may yield higher immediately detectable activity after spray-drying. In contrast, other matrices (e.g., more viscous or gel-forming polymers) are more prone to diffusion limitations or heat sensitivity during spray drying.

When interpreted together with enzymatic activity results (Fig. 3A), yield analysis reveals a formulation-dependent trade-off between powder recovery and apparent enzymatic accessibility. This normalization

highlights that increased polymer thickness primarily affects apparent catalytic accessibility rather than enzyme presence. This was seen with chitosan 5% (freeze-dried), maltodextrin 5% (spray-dried), and Arabic gum 5% (spray-dried). Using a thicker coating increased product yield but reduced specific enzymatic activity, suggesting that increased polymer thickness may limit substrate diffusion and reduce apparent activity (Chalella Mazzocato & Jacquier, 2023).

Of all the wall materials tested, maltodextrin formulations demonstrated comparatively balanced performance between activity retention and product recovery, with good product yield, especially in spray-dried samples, and acceptable retained enzyme activity. Arabic gum yielded the highest recovery at higher concentrations, particularly during spray drying. Chitosan-based samples yielded lower amounts, primarily upon spray-drying. Carrageenan and alginate yielded moderate to low amounts in both drying methods, compared with maltodextrin and Arabic gum.

Increasing the encapsulant concentration from 5 to 10% typically led to higher yields, especially during spray-drying. Thicker polymer layers increase the solid content in the feed, stabilize droplets, and strengthen microcapsules during drying. However, higher coating levels can reduce specific enzyme activity by limiting diffusion. These findings emphasize the need to balance powder recovery efficiency with functional enzymatic accessibility.

The results show that microencapsulation performance depends strongly on both the wall material and drying method. Spray-drying improves product recovery, especially with carbohydrate-based encapsulants like maltodextrin and Arabic gum (Assadpour & Jafari, 2019). Freeze-drying gives lower but more consistent yields. Taking enzyme activity and clot lysis into account, maltodextrin at low to moderate concentrations provided a favorable balance between activity retention and product recovery, suggesting its potential suitability among the evaluated encapsulants.

**Figure 4. Enzymatic activity of fibrinolytic protease under different formulation and accessibility conditions.**

Crude enzyme, diluted (1:1), and microencapsulated systems (maltodextrin and Arabic gum) were evaluated based on measured activity (U $mL^{-1}$). Microcapsule fractions were analyzed as supernatant and vortex-treated samples to assess matrix-associated diffusion limitations. Error bars represent mean ± standard deviation where applicable. Different letters indicate statistically significant differences ($p < 0.05$).

# 3.3. Evidence of diffusion-limited enzyme accessibility

To further evaluate the effects of formulation on enzyme accessibility, enzymatic activity was compared across crude, diluted, and microencapsulated systems (Fig. 4). Crude enzyme exhibited the highest activity, while dilution (1:1) resulted in a substantial decrease, confirming concentration-dependent catalytic response.

Microencapsulated systems showed reduced apparent activity compared to crude enzyme, with values ranging from 0.27 to 0.57 U $mL^{-1}$. To distinguish between enzyme inactivation and accessibility constraints, microcapsule fractions were analyzed as supernatant and vortex-treated samples. Vortex-treated samples consistently exhibited higher activity than corresponding supernatant fractions.

For example, maltodextrin-based microcapsules increased from 0.29 U $mL^{-1}$ (supernatant) to 0.57 U $mL^{-1}$ (vortex), while Arabic gum systems increased from 0.27 to 0.49 U $mL^{-1}$. These results indicate that enzymatic activity is partially retained within the polymer matrix but is not fully accessible without mechanical disruption, supporting a diffusion-limited accessibility mechanism, although diffusion was not directly quantified.

## 3.4. Functional validation through qualitative clot degradation

Qualitative clot degradation assays confirmed that fibrinolytic activity was preserved following microencapsulation (Fig. 5). Crude enzyme produced rapid and extensive clot degradation, whereas microencapsulated formulations (maltodextrin 5% and 10%, Arabic gum 5%) showed slower and partial clot disruption.

This delayed response is consistent with diffusion-controlled enzyme release from the polymer matrix. The negative control showed no visible clot degradation, confirming that the observed effect was enzyme-dependent. Because this assay was performed as a qualitative evaluation, the results are interpreted as proof-of-function rather than quantitative comparison.

**Figure 5. Qualitative clot degradation by fibrinolytic protease from Bacillus tequilensis HSFI-5.**

Crude enzyme, microencapsulated formulations (maltodextrin 5% and 10%, Arabic gum 5%), and a negative control were incubated with pre-formed blood clots under identical conditions. Visual differences in clot degradation indicate that fibrinolytic functionality is preserved following microencapsulation. Observations represent a qualitative assessment without quantitative measurement.

The present clot degradation assay was intended as a qualitative confirmation of fibrinolytic functionality; future studies incorporating gravimetric or spectrophotometric quantification will be necessary to determine comparative lytic efficiency. Since this assay was conducted as a qualitative observational evaluation, no quantitative measurements or statistical comparisons were performed. Nevertheless, the visual outcomes indicate that detectable fibrinolytic activity is preserved after microencapsulation. However, these observations should be interpreted as qualitative confirmation of functional preservation rather than comparative thrombolytic efficiency.

## 3.5. Structure–function relationship from SEM analysis

SEM analysis of maltodextrin-based microcapsules revealed predominantly spherical particles with relatively smooth surfaces at 10,000× magnification (Fig. 6). The observed morphology indicates successful encapsulation and structural integrity after drying.

These structural characteristics are consistent with the higher apparent enzymatic accessibility observed in maltodextrin systems. The relatively smooth and discrete particle morphology may facilitate rapid hydration and substrate diffusion, whereas denser matrices are expected to impose greater diffusion resistance. This observation supports the proposed relationship between matrix structure and enzymatic accessibility in the encapsulated system.

**Figure 6. Scanning electron microscopy (SEM) image of microcapsules prepared using maltodextrin as the encapsulant matrix.**

Microcapsules produced with 5% (w/v) maltodextrin exhibit predominantly spherical morphology with relatively smooth surfaces at 10,000× magnification. Structural characteristics support successful encapsulation and are consistent with observed enzymatic accessibility.

Importantly, these structural features are consistent with the observed enzymatic behavior. The relatively smooth and discrete morphology likely facilitates hydration and substrate diffusion, contributing to the higher apparent activity observed in maltodextrin systems. In contrast, denser matrices are associated with reduced accessibility, underscoring the role of matrix architecture in governing enzyme function. Thus, SEM analysis not only confirms successful particle formation but also supports a morphology–function relationship in which particle structure may influence enzyme accessibility and apparent fibrinolytic performance following encapsulation.

# 4. Discussion

This study presents a process-integrated evaluation of microencapsulation strategies for a fibrinolytic protease derived from *Bacillus tequilensis* HSFI-5, with emphasis on the relationships among formulation design, drying method, and enzymatic accessibility. The results consistently indicate that encapsulation performance is governed not only by enzyme preservation but also by matrix-dependent transport limitations that influence measurable activity.

Macroscopic observations (Fig. 2) revealed clear differences in powder appearance and aggregation across encapsulant matrices and drying methods. Freeze-dried formulations exhibited irregular, porous structures, whereas spray-dried formulations produced more compact powders. Although these observations are qualitative, they suggest that drying-induced structural organization differs substantially between processes and may influence hydration behavior and mass transfer during enzymatic assays. These macroscopic trends are further supported by SEM analysis (Fig. 6), which confirmed that maltodextrin-based microcapsules exhibit a relatively smooth, spherical morphology, consistent with improved accessibility.

Quantitative analysis of enzymatic performance (Fig. 3) demonstrated that both encapsulant matrix and drying method significantly affect activity retention and process efficiency. Lower polymer concentrations (5% w/v) consistently resulted in higher apparent enzymatic activity, whereas higher concentrations improved microencapsulation yield but reduced activity. This inverse relationship, supported by correlation analysis ($r = -0.62$), highlights a fundamental trade-off between structural recovery and catalytic accessibility. Such behavior is characteristic of polymer-based encapsulation systems, where increasing matrix density enhances structural integrity but limits substrate diffusion.

At low maltodextrin concentration (5% w/v), atomization produces small droplets that rapidly form an amorphous “glassy” carbohydrate matrix as water evaporates. It thereby limits enzyme unfolding by rapidly reducing molecular mobility. In addition, maltodextrin solutions typically exhibit lower viscosity and better droplet formation, supporting efficient drying and reducing residence time in the hot zone; thus, the thermal exposure is brief and may be less damaging than prolonged freezing–drying stresses, such as ice-crystal formation, concentration, and interfacial adsorption during freezing. Upon reconstitution, spray-dried maltodextrin particles also tend to hydrate and dissolve rapidly, improving substrate diffusion and increasing the *measured* activity in the casein assay.

Further mechanistic insight was obtained through comparative analysis of crude, diluted, and microencapsulated systems (Fig. 4). The observed reduction in activity following dilution confirms the dependence of catalytic performance on enzyme concentration. More importantly, the difference between supernatant and vortex-treated microcapsule fractions demonstrates that reduced apparent activity in encapsulated systems is not solely due to enzyme inactivation. Instead, the increase in activity upon vortex treatment indicates that a portion of the enzyme remains functionally intact but is physically restricted within the polymer matrix. This provides indirect experimental evidence supporting a diffusion-limited accessibility mechanism.

The qualitative clot degradation assay (Fig. 5) further supports this interpretation. While crude enzyme induced rapid clot disruption, microencapsulated formulations exhibited slower and partial degradation. This delayed response is consistent with controlled enzyme release and diffusion constraints within the encapsulation matrix. The absence of quantitative measurement limits direct comparison of thrombolytic efficiency; however, the observed patterns confirm that fibrinolytic functionality is preserved after encapsulation.

SEM analysis (Fig. 6) provides a structural context for these functional observations. The predominantly spherical, relatively smooth morphology observed in maltodextrin-based microcapsules is consistent with improved hydration and substrate diffusion, which may explain the higher apparent enzymatic activity in these systems. In contrast, denser or more aggregated matrices are expected to impose greater diffusion resistance, leading to reduced measurable activity despite potential preservation of enzyme integrity.

From a process perspective, the comparison between freeze-drying and spray-drying highlights an important trade-off between structural preservation and scalability. Freeze-drying generally preserved

enzymatic activity more effectively, likely due to reduced thermal exposure, whereas spray-drying enabled higher product recovery and improved process efficiency. Notably, maltodextrin-based systems exhibited relatively high activity even after spray-drying, suggesting that rapid formation of an amorphous carbohydrate matrix may limit protein denaturation while facilitating rehydration and substrate diffusion.

Importantly, this study does not aim to identify a single optimal encapsulant but rather to demonstrate that encapsulation performance is application-dependent. Systems designed for immediate enzymatic accessibility benefit from lower polymer concentration and more hydrophilic matrices, whereas applications requiring enhanced structural protection may favor denser or gel-forming polymers. This formulation-dependent performance underscores the need to align matrix selection with intended functional outcomes.

Several limitations should be acknowledged. First, the present study focuses on immediate post-processing performance and does not evaluate long-term stability or release kinetics. Second, clot degradation was assessed qualitatively, and quantitative validation will be required to support translational claims. Third, although the accessibility analysis (Fig. 4) provides mechanistic insight, replication across all formulations remains limited and warrants further validation.

Overall, the findings establish that the apparent loss of enzymatic activity in microencapsulated systems is largely governed by diffusion-limited accessibility rather than by complete enzymatic inactivation. This work provides a process-oriented framework that links encapsulation matrix design, drying strategy, and mass transfer behavior to functional enzyme performance, thereby supporting the development of scalable biomanufacturing strategies for enzyme-based applications.

# 5. Conclusion

This study demonstrates that microencapsulation preserves measurable fibrinolytic protease activity from *Bacillus tequilensis* HSFI-5 while introducing formulation-dependent constraints on enzyme accessibility. The results show that both encapsulant matrix and drying method significantly influence apparent enzymatic performance, with maltodextrin-based systems providing higher immediate activity and denser polymer matrices exhibiting reduced accessibility.

Comparative analysis of crude, diluted, and microencapsulated systems, including vortex-treated fractions, indicates that reduced apparent activity in encapsulated formulations is primarily governed by diffusion-limited accessibility rather than complete enzymatic inactivation. In addition, the observed trade-off between activity retention and product yield highlights a key design consideration in encapsulation systems, where increased structural recovery may be associated with reduced catalytic accessibility.

From a biotechnological perspective, these findings emphasize that encapsulation performance should be evaluated not only in terms of enzyme preservation but also in relation to transport-related limitations

within the polymer matrix. The results provide a formulation-oriented framework for selecting encapsulation strategies based on the intended functional outcome, particularly when balancing immediate enzymatic accessibility and structural protection. Future studies should further address long-term stability, release kinetics, and quantitative functional assays to support translational applications of encapsulated fibrinolytic enzymes. This framework may be transferable to other enzyme systems requiring a balance between structural stabilization and functional accessibility. It extends beyond fibrinolytic proteases and may inform formulation strategies for other enzyme systems requiring controlled accessibility and structural stabilization

## Declarations

### Ethical Approval

This study was reviewed and approved by the Health Research Ethics Commission, Faculty of Public Health, Universitas Muhammadiyah Semarang, Indonesia (Approval No. 377/KEPK-FKM/UNIMUS/2020).

### Consent to Participate

Informed consent was obtained from all individual participants whose blood samples were used in this study.

### Consent to Publish

All authors consent to the publication of this manuscript.

## Competing Interests

The authors declare that they have no competing interests.

## Funding

Not applicable.

## Author Contribution

SNE conceptualized the study, secured funding, supervised the project, and coordinated overall research activities. DSZ and OKR contributed to the conceptualization and development of the methodology. MZBA performed microencapsulation experiments and data acquisition. IC conducted the data analysis and drafted the manuscript; DSZ and SNE curated data and prepared visualizations. RS performed analyses of enzymatic activity and yield. All authors reviewed and approved the final manuscript.

# Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Figures

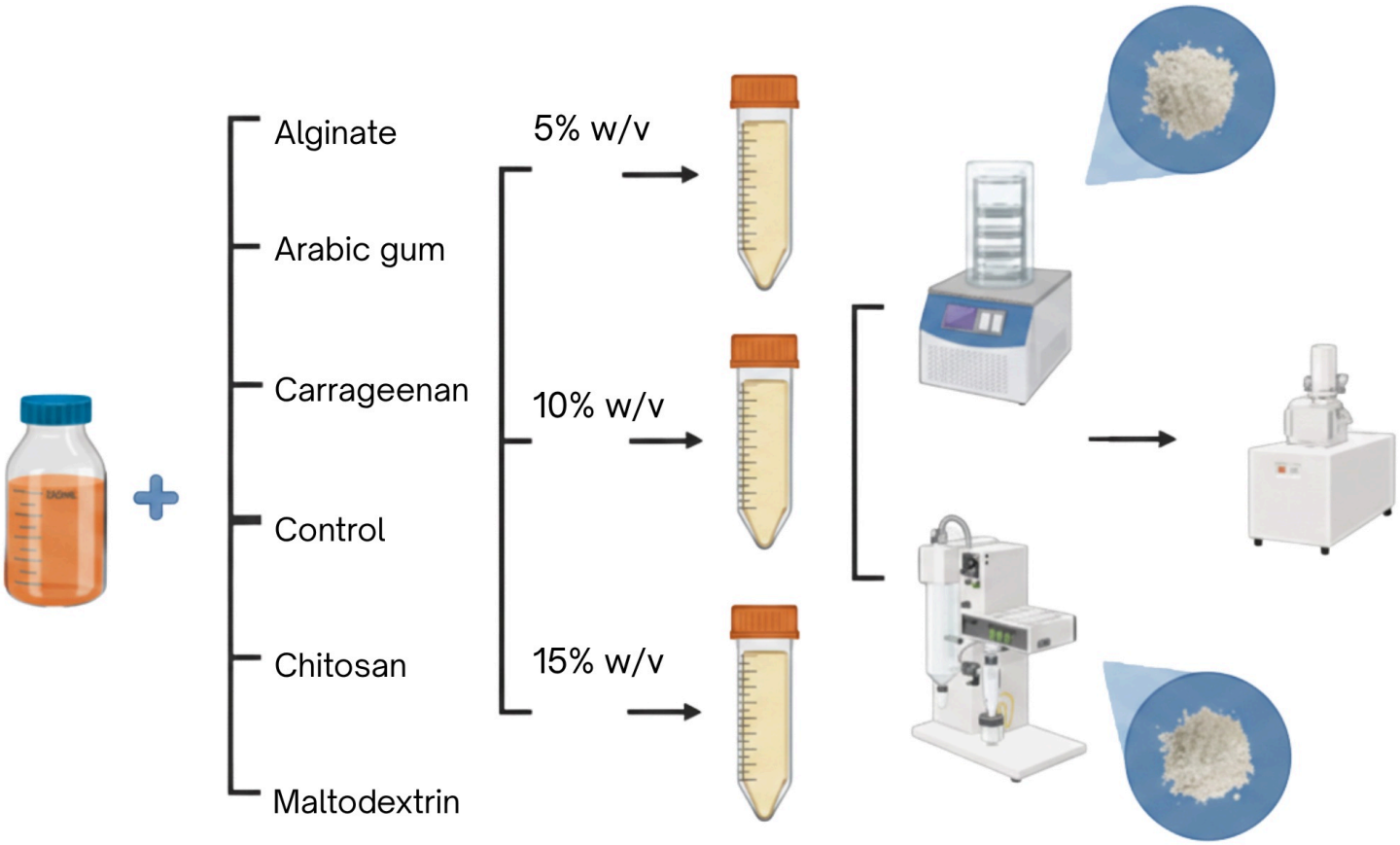
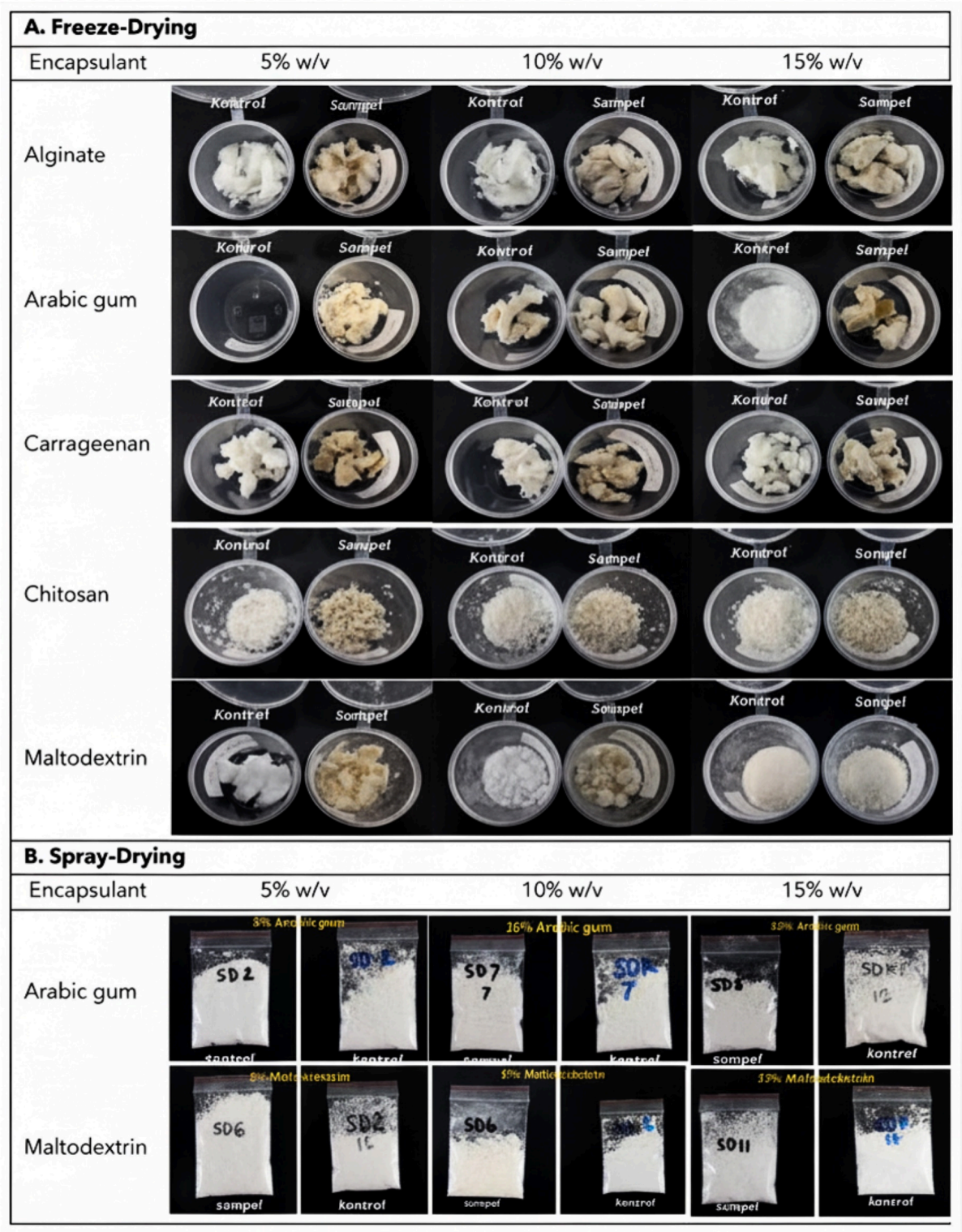



**Figure 1**

**Schematic representation of the microencapsulation process of fibrinolytic protease from *Bacillus tequilensis* HSFI-5.**

Crude protease extract was combined with encapsulant matrices at defined concentrations and

processed using either freeze-drying or spray-drying. The workflow illustrates formulation preparation, drying pathways, and recovery of microencapsulated powders for subsequent characterization.




## Figure 2

**Macroscopic appearance of microencapsulated fibrinolytic protease from *Bacillus tequilensis* HSFI-5 prepared using different encapsulant matrices, concentrations, and drying methods.**

**(A)** Freeze-dried microcapsules prepared with alginate, Arabic gum, carrageenan, chitosan, and maltodextrin at 5, 10, and 15% (w/v).

**(B)** Spray-dried microcapsules prepared with Arabic gum and maltodextrin at identical concentrations. Variations in powder appearance and aggregation were observed across encapsulant matrices and drying methods. Detailed structural interpretation was supported by SEM analysis (Figure 6).

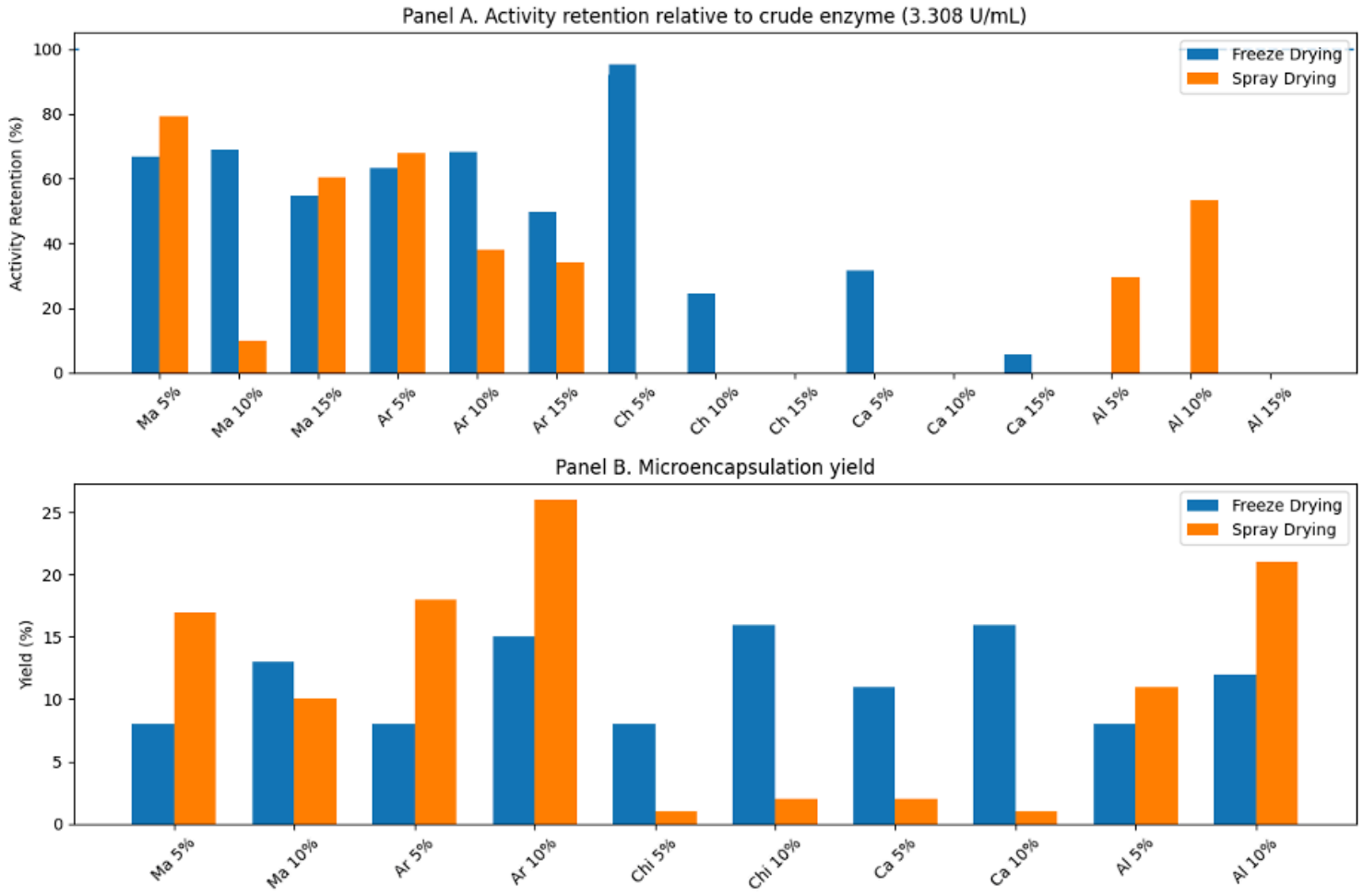



**Figure 3**

**Effect of encapsulant matrix and drying method on enzymatic activity retention and microencapsulation yield.**

**(A)** Activity retention (%) relative to crude enzyme activity (3.308 U $mL^{-1}$).

(B) Microencapsulation yield (%) across different formulations. Values represent mean measurements used for comparative evaluation of functional preservation and process efficiency. Ma = maltodextrin, Ar = Arabic gum, Ch = chitosan, Ca = carrageenan, Al = alginate.

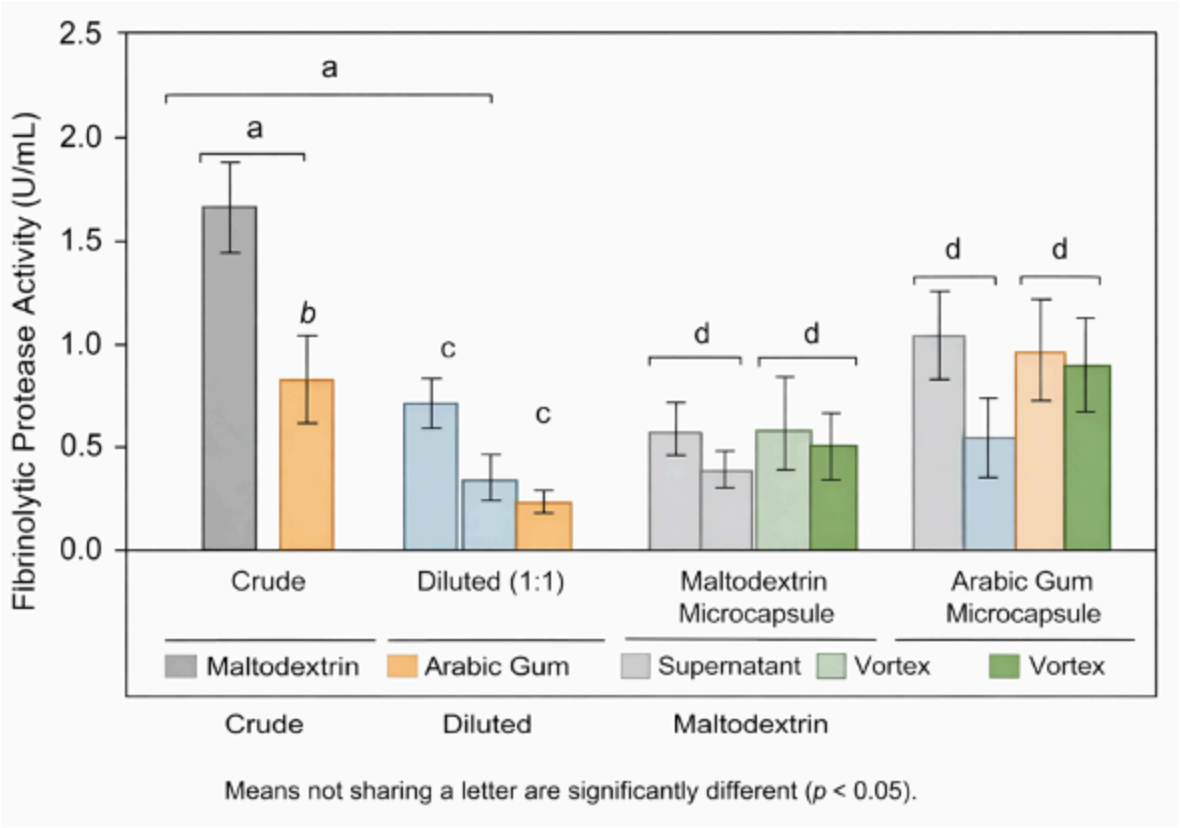



**Figure 4**

**Enzymatic activity of fibrinolytic protease under different formulation and accessibility conditions.**

Crude enzyme, diluted (1:1), and microencapsulated systems (maltodextrin and Arabic gum) were evaluated based on measured activity (U $mL^{-1}$). Microcapsule fractions were analyzed as supernatant and vortex-treated samples to assess matrix-associated diffusion limitations. Error bars represent mean ± standard deviation where applicable. Different letters indicate statistically significant differences ($p < 0.05$).

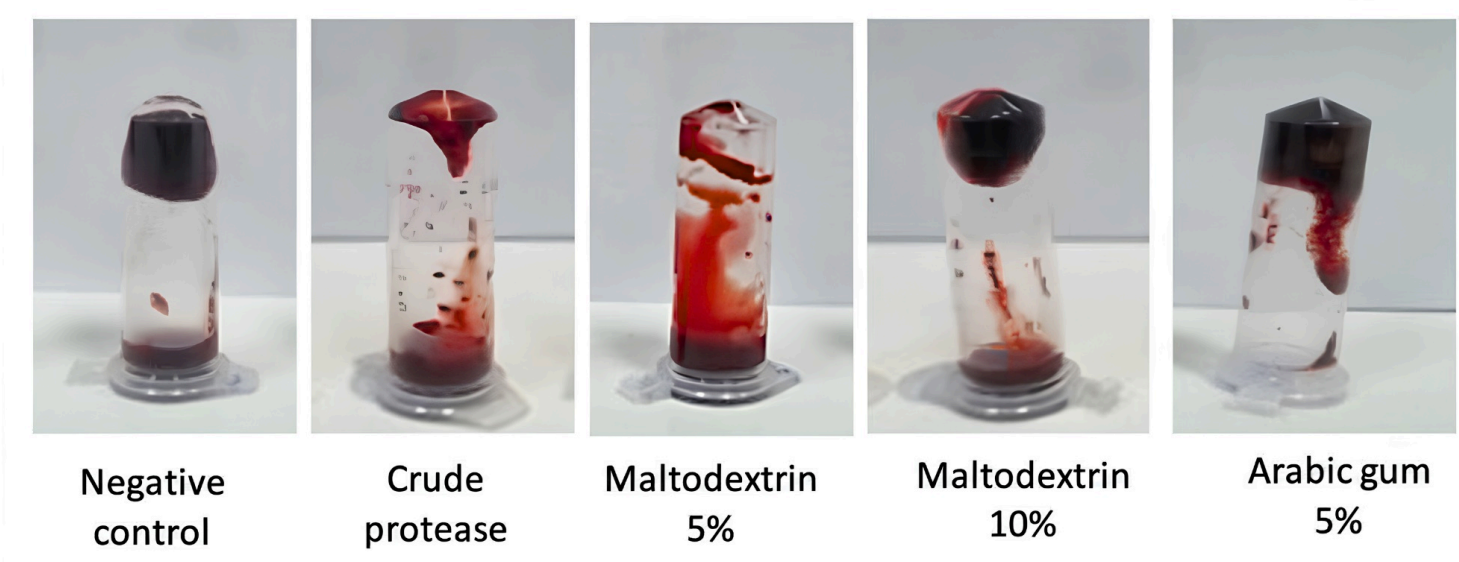


**Figure 5**

**Qualitative clot degradation by fibrinolytic protease from *Bacillus tequilensis* HSFI-5.**

Crude enzyme, microencapsulated formulations (maltodextrin 5% and 10%, Arabic gum 5%), and a negative control were incubated with pre-formed blood clots under identical conditions. Visual differences in clot degradation indicate that fibrinolytic functionality is preserved following microencapsulation. Observations represent a qualitative assessment without quantitative measurement.

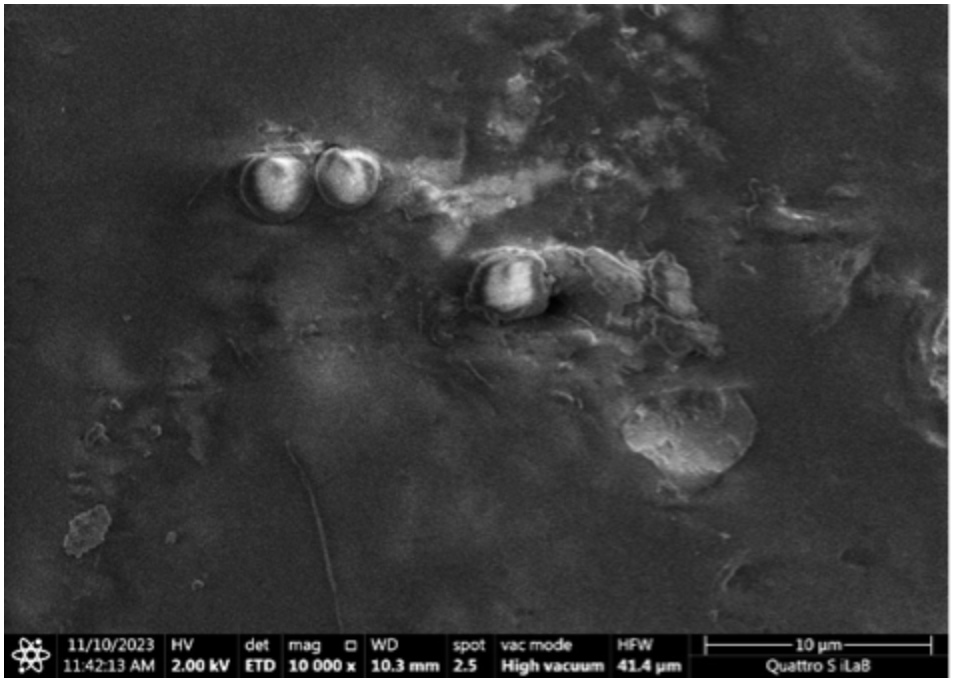



**Figure 6**

**Scanning electron microscopy (SEM) image of microcapsules prepared using maltodextrin as the encapsulant matrix.**

Microcapsules produced with 5% (w/v) maltodextrin exhibit predominantly spherical morphology with relatively smooth surfaces at 10,000× magnification. Structural characteristics support successful encapsulation and are consistent with observed enzymatic accessibility.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract.jpg