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Immobilization of enzymes for synergy in polymers to produce lactose free milk

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Stabilization of the enzyme lactase is of interest in obtaining lactose-free foods. Optimization of the lactase enzyme activity was investigated using various immobilization methods, including encapsulation, and electrospinning. The physical properties of the immobilized lactase, such as thermal stability, mechanical strength, and resistance to denaturation have been enhanced and evaluated. The activity of the immobilized lactase was compared to that of the free enzyme. Additionally, lactase was examined in combination with glucose oxidase and catalase to reduce feedback inhibition of glucose and the toxicity of H_2O_2. The core–shell electrospinning technique was employed, wherein lactase was encapsulated within an aqueous core and shielded by a non-biodegradable shell. Electrospinning enhanced the enzyme's stability, reduced diffusion limitations, and improved reusability. The enzyme activity remained stable for four weeks and retained its activity after three months of storage at 4 °C in phosphate-buffered saline (PBS). This approach represents a preliminary investigation into the potential benefits of enzyme synergy.

Keywords Lactase, Glucose oxidase, Catalase, Lactose, Immobilization, Electrospun fibers, Biopolymers

Lactose intolerance is the inability to digest lactose, a sugar found in many bovine milk products, due to insufficient lactase enzyme activity in the small intestine[1]. Globally, approximately 65 out of every 100 adults suffer from lactose intolerance[2]. The prevalence of this condition varies between countries and regions. For example, in Northern Europe, the incidence is less than 10%, while in parts of Asia and Africa, it may be as high as 95%. In India, 66.6% of individuals in Southern India are lactose intolerant, compared to 27.4% in Northern India[3]. Individuals with lactose intolerance are also more likely to experience related conditions such as gastroesophageal reflux disease (8–20%) and irritable bowel syndrome (5%). To alleviate symptoms, many individuals in India turn to traditional Ayurvedic remedies[4].

Lactose malabsorption is becoming more common among the elderly, with studies indicating that up to 50% of healthy elderly individuals in Western societies are affected by this condition[5]. This increase is primarily attributed to small intestine bacterial overgrowth, rather than mucosal factors[6]. As milk and dairy products are significant sources of dietary calcium, lactose malabsorption can lead to inadequate calcium intake and increase the risk of osteoporosis[7].

One solution to lactose intolerance is the production of lactose-free milk, which involves the breakdown of lactose into glucose and galactose, making it more digestible for those with lactose intolerance. A common and efficient method for achieving this is by adding the lactase enzyme (β-galactosidase). However, a major challenge in using lactase enzymes is their deactivation and removal during the re-pasteurization process[8]. This often results in a product that is sweeter and more viscous than regular milk, potentially lacking some naturally occurring nutrients[9]. Additionally, lactose-free milk is considerably more expensive than regular milk, especially in developing countries like India, where it can cost up to three times that of regular milk. Furthermore, it is not stable when added to milk, and technical difficulties arise when attempting to reuse the enzyme.

To address these challenges, strategies for stabilizing lactase activity, such as immobilization and encapsulation, have been extensively explored[10,11]. The optimization of lactase enzyme activity has been examined using various methods, including electrospinning, and the activity of immobilized lactase has been compared with that of the free enzyme. Various methods for enzyme immobilization have been investigated in the literature. For instance, lipase has been encapsulated in K-carrageenan, demonstrating stability across a pH range of 6–9 and at temperatures up to 50 °C in various organic solvents[12]. Research by Wong and Jin further explored the stability of immobilized enzymes on different polymer nanofibers[13,14], while Mission compared immobilized lactase on

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functionalized nanoparticles and nanofibers[15]. In the present study, we specifically focus on electrospinning as an immobilization technique for lactase. Other methods for enzyme immobilization were also tested, including the entrapment of lactase in carrageenan hydrogel beads and chitosan[16]. Stoilova et al.[16] found that lactase immobilized on microfibrous mats exhibited enhanced pH and temperature stability. Similarly, Estevinho et al.[17] utilized Ca(II)-alginate with biopolymers added via a spray-drying process, further contributing to the understanding of enzyme stabilization techniques.

In the dairy industry, current methods for enzyme immobilization include physical trapping techniques such as entrapment, microencapsulation, and cross-linking, as well as the use of nanoparticles. In this study, we compared the stability and enzymatic activity of lactase immobilized in core–shell polymer nanofibers with its activity as a free enzyme, as well as its activity in alginate, alginate-carrageenan, and alginate-gelatin beads. We specifically focused on the electrospinning of polymer nanofibers due to several advantages over other immobilization methods, making it a rapidly emerging technique in the dairy industry. Polymer nanofibers can be either monolithic or structured as a two-component core–shell structure[18], where two concentrically aligned nozzles are used, with a non-soluble polymer forming the outer shell layer. In our approach, the shell was made of non-biodegradable poly(vinylidene fluoride-co-hexafluoropropylene) (PVDF-HFP) polymer, which serves to protect the enzyme, prevent contamination of the milk by the water-soluble PVP (polyvinylpyrrolidone) core polymer, and provide mechanical stability compared to other methods. This matrix offers protection against fluctuations in environmental conditions, thereby reducing enzyme leakage and enhancing long-term stability by entrapping the enzyme for extended periods.

Additionally, we addressed the issue that hexose products inhibit lactose hydrolysis[19]. To overcome this, we added glucose oxidase to enhance lactase activity by reducing free glucose, which acts as a feedback inhibitor. Catalase was also incorporated to remove the hydrogen peroxide (H_2O_2) produced by glucose oxidase[20]. The use of multiple enzymes has been shown to improve reaction rates and final product concentrations by modifying the reactant through successive reactions. Furthermore, the activity of lactase was evaluated in the presence of potential activators such as magnesium, manganese, calcium, and potassium.

Materials and methods

Chemicals and materials

Enzymes

Lactase from *Aspergillus oryzae* (beta-galactosidase) (CAS-9031-11-2, specific activity ≥ 8.0 units/mg solid), glucose oxidase from Aspergillus niger (> 100,000 units/g solid), and catalase (CAS-9001-05-2, EC-1.11.1.6, specific activity 2000–5000 units/mg protein) from bovine liver were obtained from Sigma-Aldrich.

Characteristics of the enzymes

The lactase from A. oryzae has a pH optimum of 4.5 and cleaves lactose into its monosaccharide components, glucose, and galactose. Glucose oxidase from A. niger has a pH optimum of 5.5 and is a dimer consisting of two equal subunits with a molecular mass of 80 kDa. It converts glucose to gluconic acid and is used in the food industry to remove glucose.

Biopolymers

Sodium alginate was purchased from MP Biomedicals, France. Chitosan (medium molecular weight @75,000) was obtained from Fluka Bio-Chemika, and K-Carrageenan (22048, Sigma-Aldrich) was used.

Other reagents and polymers

Alpha-lactalbumin from bovine milk type I, D-glucose and lactose, poly (vinylidene fluoride-co-hexafluoropropylene) (PVDF-HFP) Mw-400 K, polyvinylpyrrolidone (PVP) MW-1300 K, tetrahydrofuran (THF), and dimethylformamide (DMF) were purchased from Sigma-Aldrich. D-galactose was obtained from BDH Chemicals Ltd, Poole, England. All chemicals used in the experiments were of analytical grade, with high-quality solvents and deionized water.

Assay kits

Lactose, D-glucose, and D-galactose assay kits were purchased from Megazyme (Bray Business Park, Ireland).

Methods

Measurement of catalytic activity of enzymes

To measure lactase enzyme activity, glucose, and galactose concentrations were determined using the Megazyme kits.

(I) *Determination of glucose, galactose, and lactose*: Concentrations of these sugars were determined using the Megazyme kits. D-glucose is determined by phosphorylation to glucose-6-phosphate (G-6-P) and then oxidized by nicotinamide-adenine dinucleotide phosphate (NADP +) to gluconate-6-phosphate with NADPH formation. The amount of NADPH formed is stoichiometric with the amount of D-glucose present. Lactose is first hydrolyzed to form D-galactose. Galactose is then determined by interconversion to α-galactose, which is oxidized to D-galactonic acid and NADH in the presence of β-galactose dehydrogenase. The amount of NADH formed is stoichiometric with the amount of lactose/galactose, which is measured by an increase in absorbance at 340 nm.

(II) *Determination of sugars in milk*: The sugars in milk are determined as explained above. The sample is prepared by taking 1 g of milk into a 100 mL volumetric flask, adding 60 mL of distilled water, and storing it at 50 °C for 15 min. 2 mL of 3.6% (w/v) potassium hexacyanoferrate (II) and 2 mL of 7.2% (w/v) zinc sulfate

solution are added and mixed step by step. 10 mM NaOH is added and diluted with distilled water, mixed vigorously, and filtered. A sample is taken and determined by the above processes.

Influence of different conditions on enzyme activity

Free enzyme: Lactase (0.01 g/mL) and glucose oxidase (0.0034 g/mL) were dissolved in phosphate-buffered saline (PBS) and studied in a total volume of 4 mL of 250 mM lactose solution. These enzyme concentrations were used unless noted otherwise. Several different conditions were examined affecting free enzyme activity, including temperature, rate of shaking, pH, activity, stability over time, and activity in the presence of divalent ion activators, which is further explained in the results part.

Methods for enzyme immobilization

Alginate

0.01 g/mL of the lactase and 0.0034 g/mL of the glucose oxidase solutions were added to 1 mL of 40% sodium alginate and kept under stirring for a few hours. The solution was pipetted dropwise into 0.1 M calcium chloride to form uniform beads. After ten minutes, the beads were washed thrice with PBS and used for further studies.

Chitosan

Chitosan beads were prepared following the method of Chan et al.[21] 2 g of chitosan flakes were dissolved in 60 mL of 5% (v/v) acetic acid, mixed for two hours, and kept overnight. The solution was added dropwise into 500 mL of 0.5 M NaOH and left for one hour. The spherical beads were removed by filtration, rinsed three times with distilled water to remove residual NaOH, and vacuum dried. 0.1 g of dried chitosan beads were dissolved in 1 mL of 0.3% glutaraldehyde at 25 °C and 150 rpm for three hours, then washed thrice with water to remove the glutaraldehyde. 1 mL of 0.8 mg/mL lactase enzyme was added to the chitosan beads and kept in a shaker for six hours at 37 °C and 450 rpm, then washed with PBS. The enzyme activity was measured.

Carrageenan hydrogels

Carrageenan hydrogel formation was slightly modified from Zhang et al.[22]. The enzyme solutions were added to a 1% (w/v) carrageenan solution and kept under stirring for a few hours. The solution was injected manually into 3% $CaCl_2$ solution using a syringe and left for a few hours for bead hardening. The beads were then washed three times with distilled water.

Alginate-carrageenan hydrogel beads

The formation of alginate-carrageenan hydrogel beads was slightly modified from the procedure described by Popa et al.[23]. The same basic procedure was followed, but the polymer-enzyme solution was poured dropwise into a precipitating solution composed of 25 mL of a 1:1 ratio of 3 wt.% KCl and 3 wt.% $CaCl_2$, along with 5 mL of 3.65% glutaraldehyde, and left to harden for 18 h. After bead formation, the beads were washed thoroughly three times with distilled water to remove residual glutaraldehyde, as its presence could be harmful. The glutaraldehyde removal was confirmed using analytical methods to ensure that the residue was effectively eliminated. After each experiment, the beads were stored in 0.1 M acetate buffer at pH 4.5 and kept at 4 °C to maintain enzyme stability.

Alginate-gelatin crosslinked beads

The method of crosslinking the enzyme in alginate-gelatin beads was modified from Freitas et al.[24] A 4% (w/v) sodium alginate and 2.5% (w/v) gelatin polymer-enzyme solution were poured dropwise into a precipitating solution composed of 25 mL of a 1:1 ratio of 3 wt.% KCl and 3 wt.% $CaCl_2$, along with 5 mL of 3.65% glutaraldehyde, and left for 18 h for bead hardening. The beads were then washed three times thoroughly with distilled water as explained above. After every experiment, beads were stored in 0.1 M acetate buffer, pH 4.5, at 4 °C.

Electrospinning

As described earlier by Letnik et al.[25] a core–shell electrospinning technique was used for enzyme immobilization (lactase and glucose oxidase). The system allows for the fabrication of microtubes in an electrostatic field. Two separate syringes continuously ejected solutions from a spinneret with co-axial capillaries at the rate of 0.5 and 5 mL/hour respectively. The needle was fixed at a distance of 10 cm from the tip of a 30 cm diameter flatbed rotating disk collector in an electrostatic field of 900 V/cm, the temperature was maintained at $23.5^{\circ}C \pm 3^{\circ}C$ throughout the process. The internal capillary of this spinneret facilitated the flow of the enzymes (lactase 10 mg/mL, glucose oxidase 3 mg/mL) in an aqueous PVP solution while being enveloped by the external capillary containing a non-biodegradable polymer solution. The core solution consisted of 15 wt.% PVP in deionized water. The shell solution consisted of 15% PVDF-HFP in a solvent mixture of THF/DMF in a weight ratio of 6.5:35. If nanofibers are water-soluble, they may swell or dissolve away, leading to enzyme leakage. Therefore, a water-insoluble shell was used for co-electrospinning with enzymes. The electrospun fibers were collected on the surface of a PBS bath and wound onto plastic carriers. Carriers were stored in PBS solution to prevent the aqueous core of the fibers from drying out.

Extra high-resolution scanning electron microscopy (XHR-SEM) was performed using the Magellan™ 400L. Samples (alginate-gelatin beads and electrospun fibers) were vacuum-dried and gold-coated. The drying caused the hollow fibers to collapse, forming a ribbon structure.

Cross-linked enzyme aggregates (CLEA)

CLEA were prepared following the method of Guauque-Torres et al.[26] by preparing lactase (15 mg/mL) and glucose oxidase (5 mg/mL). 120 µL of each enzyme, separately and together, and BSA (170 µL, 30% w/v) were

homogenized for 10 min, and an ammonium sulfate solution was added, initiating precipitation (500 mg/mL, at 4 °C with intermittent shaking). After two hours, a glutaraldehyde solution (200 µL, 25–26%) was added, and cross-linking was allowed to continue for 24 h. in the refrigerator at 4 °C. A ~ 2 mL pellet was washed with 4 mL of water and re-suspended after low-speed centrifugation. Measurements of activity were conducted in a final volume of 6 mL in PBS.

Results and discussion

Influence of different conditions on enzyme activity

Temperature and shaker speed

The effect of shaking rate (rpm) and temperature (°C) on lactase activity was tested. An increase of 74% was observed when the shaking rate was increased from 300 to 400 rpm and the temperature from 37 to 50 °C. The enhancement was 25–35% for electrospun enzyme under the same conditions.

pH

The pH optimum for lactase activity was 3.8, lower than the pH 5 reported by Park et al.[27] An increase in pH from 3.9 to 4.9, followed by 6.5, resulted in a decrease in enzyme activity by 35% and 45%, respectively[28,29].

Effect of activators

The influence of three divalent metals on lactase activity was compared for the free enzyme. Manganese and magnesium were added at two different concentrations, and calcium (see Table S1). Compared to the free enzyme without activators, manganese had a similar effect on the enzyme at the two concentrations tested compared to the control. After one hour of incubation, activity increased by approximately 25%, followed by a reduction with continued incubation (Table S1). For magnesium, the lower concentration (3 mM of MgSO4) shows a negative impact on the activity whereas at higher concentrations (30 mM) shows10–20% increase in the activity. For calcium ions, a 100 mM concentration had a similar effect as Manganese. Compared to the free enzyme without activators, there was almost a 10–20% increase in enzymatic activity.

Storage and stability of free enzyme

The activity of the free enzyme in solution increased by 20% when stored at 4 °C for seven days.

Activity and stability of immobilized enzymes in various polymers

Lactase entrapped in alginate beads

The activity of lactase immobilized in alginate beads showed a steady decrease over several weeks. Enzyme leakage was assumed due to the porosity of the gels[23,24,27,30]. The lactase activity was measured as glucose formed (g/mL) at 50 °C at 450 rpm for two hours. After seven days, the enzyme activity had decreased by 57% from the initial activity, and after four weeks of storage at 4 °C, it was only 25% of the original activity.

Lactase entrapped in blended biopolymers

Due to enzyme leakage from alginate beads, lactase was immobilized in alginate beads blended with other polymers. Enzyme activity was measured in terms of glucose formed (g/mL) after two hours at 37 °C and 300 rpm. The immobilization of enzymes in chitosan and carrageenan hydrogel beads had no significant effect on enzyme activity. In contrast, alginate combined with carrageenan or gelatin demonstrated higher stability and activity. After one month of storage at 4 °C, lactase activity decreased by 13% and 9%, respectively. For alginate combined with carrageenan and crosslinked with glutaraldehyde, enzyme activity decreased by almost 50% after two weeks, thus this immobilization method was not tested for longer storage periods. However, crosslinking the enzyme with alginate and gelatin resulted in enzyme stabilization (Table 1).

The immobilization methods involving enzyme embedding in various polymers, such as alginate crosslinked with carrageenan, did not show significant effects after two weeks. On the other hand, alginate crosslinked with gelatin-maintained enzyme activity for approximately one month. Despite the advantages of these methods, several limitations exist, such as enzyme instability during temperature and pH fluctuations, limited reusability, slow diffusion of substrates to the enzymes in the gel, uncontrolled enzyme distribution, and potential gel collapse if the matrix is too weak, leading to enzyme activity loss.

Time	Alginate	Alginate-carrageenan on crosslinking	Alginate-carrageenan hydrogel beads	Alginate-gelatin on crosslinking
First day	0.556 ± 0.02	0.521 ± 0.05	0.553 ± 0.02	0.540 ± 0.01
1 week	0.354 ± 0.05	0.575 ± 0.09	0.636 ± 0.04	0.615 ± 0.04
2 weeks	0.159 ± 0.02	0.362 ± 0.03	0.616 ± 0.02	0.623 ± 0.02
3 weeks	0.138 ± 0.01	–	0.618 ± 0.02	0.617 ± 0.01
2 months	–	–	0.542 ± 0.05	0.575 ± 0.02
3 months	–	–	0.536 ± 0.06	0.569 ± 0.03

Table 1. Stability of lactase entrapped in various biopolymers. Lactase is entrapped in different biopolymers and its activity is expressed in terms of the concentration of glucose formed (g/L) at different time intervals. Data are expressed as mean ± SD (n = 3).

To address these limitations, we focused on the electrospinning method, which is often considered a superior approach compared to the aforementioned methods. Electrospinning increases enzymatic activity by providing more active sites, enhances stability and reusability through matrix protection on the fiber surface, and allows for controlled enzyme loading and even distribution. In some cases, the electrospun fiber matrix can mimic biological systems, enhancing enzyme–substrate interactions. Due to these advanced features, we chose to employ the electrospinning method for the production of lactose-free milk.

Figure 1a presents SEM images showing the surface morphology of the alginate-gelatin beads with and without enzymes. The outer surface of the alginate-gelatin beads without enzymes appeared more "wrinkled" compared to the beads with immobilized enzymes.

Electrospinning

Activity and stability of the enzyme in electrospun fibers

The electrospun fibers exhibited uniformity with an average diameter of 3.5 µm (Fig. 1b(III)). These fibers function as microtubes, acting as enzymatic reactors. The enzymes are encapsulated within the fibers, while water, lactose, glucose, and other small molecules are able to pass through the tube walls. A few large pores (averaging 50 nm in size) are observed in the tube walls; however, these pores are not interconnected, preventing large molecules, such as enzymes, from escaping through the walls. No enzyme was detected on the exterior of the fibers. Pore size was observed to increase after use, with an average size of 100 nm (Fig. 1(b)(I)–(II)).

The catalytic activity of the electrospun enzyme was compared to that of the free enzyme and the enzyme entrapped in alginate beads over a 12-week period. The electrospun enzyme exhibited an increase in activity starting from day one, showing 57% of the activity of the free enzyme. By the fourth week, the activity reached 95% of that of the free enzyme and decreased to 70% by the tenth week. After three months, the electrospun enzyme retained 34% of its initial activity compared to the free enzyme and demonstrated reusability for at least eight cycles (Fig. 2).

Effect of glucose oxidase on lactase enzyme

To address the issue of inhibition of lactose hydrolysis by hexose products[31], we attempted to enhance lactase activity by adding glucose oxidase, which is expected to act as a feedback inhibitor to reduce free glucose levels. To evaluate whether glucose oxidase would enhance lactase activity, we mixed it with lactase. However, a complicating factor emerged, as the activity of glucose oxidase was found to be rapidly reduced when mixed with lactase. In the case of free enzymes, glucose oxidase (10 mg/mL) enhanced lactase (5 mg/mL) activity at 37 °C over a period of several days.

We also assessed the activity by varying lactose concentrations and observed that glucose oxidase influenced lactase activity over a short period of 15 min. A higher concentration of lactase (10 mg/mL) and a lower concentration of glucose oxidase (3.3 mg/mL) were tested as free enzymes. Within 30 min, glucose oxidase enhanced lactase activity by 50%, which decreased to 15% after one hour and to 7% after two hours, after which glucose oxidase showed no further effect (Table 2).

When the enzymes were electrospun together in polymer fibers, glucose oxidase enhanced lactase activity at 37 °C for a short period. However, with longer incubation times, no further advantage was observed, and in fact, lactase activity decreased in the presence of glucose oxidase.

Effect of catalase on lactase and glucose oxidase enzyme activity

We also investigated whether catalase would enhance the activity of glucose oxidase when mixed with lactase enzyme. Different concentrations of catalase, lactase, and glucose oxidase were tested at various temperatures. Significant results were observed with lactase (0.01 g/mL), glucose oxidase (0.0034 g/mL), and catalase (0.0068 g/mL) at 37 °C and 400 rpm. The enzymes were immobilized in alginate-gelatin beads, and their activity remained stable even after ten days of storage at 4 °C. These findings suggest that catalase can play a role in enhancing glucose oxidase activity when combined with lactase, with the immobilization method helping to preserve the enzyme activity over time.

The effect of catalase was variable, but it yielded an increase in lactase + glucose oxidase activity of up to 61%, though this enhancement was observed only for one hour. However, when immobilized in alginate-gelatin beads, the activity of lactase + glucose oxidase steadily increased after one hour and remained stable for ten days (Fig. 3). These results suggest that while catalase can temporarily enhance enzyme activity, immobilization in alginate-gelatin beads provides more sustained enzyme activity over time.

Enzyme activity in low and high-fat milk

After performing experiments with known concentrations of pure lactose, we tested the activity of the enzyme in 3% and 1% fat cow's milk. We incubated the free and entrapped enzyme in alginate beads, both alone and with glucose oxidase, as well as the electrospun enzyme, in 4 mL of 3% milk at 50 °C and 500 rpm. We found that the free enzyme resulted in only a 20% decrease in lactose, while the alginate and electrospun enzymes showed a 30% and 50% decrease in lactose, respectively, during the first hour. However, after four hours, there was little observed difference in lactose reduction between the different conditions. Glucose oxidase had no effect on lactase activity in reducing lactose in 3% milk (Table S3).

In low-fat (1%) milk, lactase was immobilized in alginate and other polymer beads and added to 10 mL of 1% milk in conical flasks. The flasks were shaken at 450 rpm at 37 °C. In low-fat milk, both the free enzyme and the enzyme immobilized in alginate showed nearly an 80% reduction in lactose within three hours, while the enzyme immobilized in alginate along with the other two polymers resulted in a 56% reduction in lactose (Table 3).

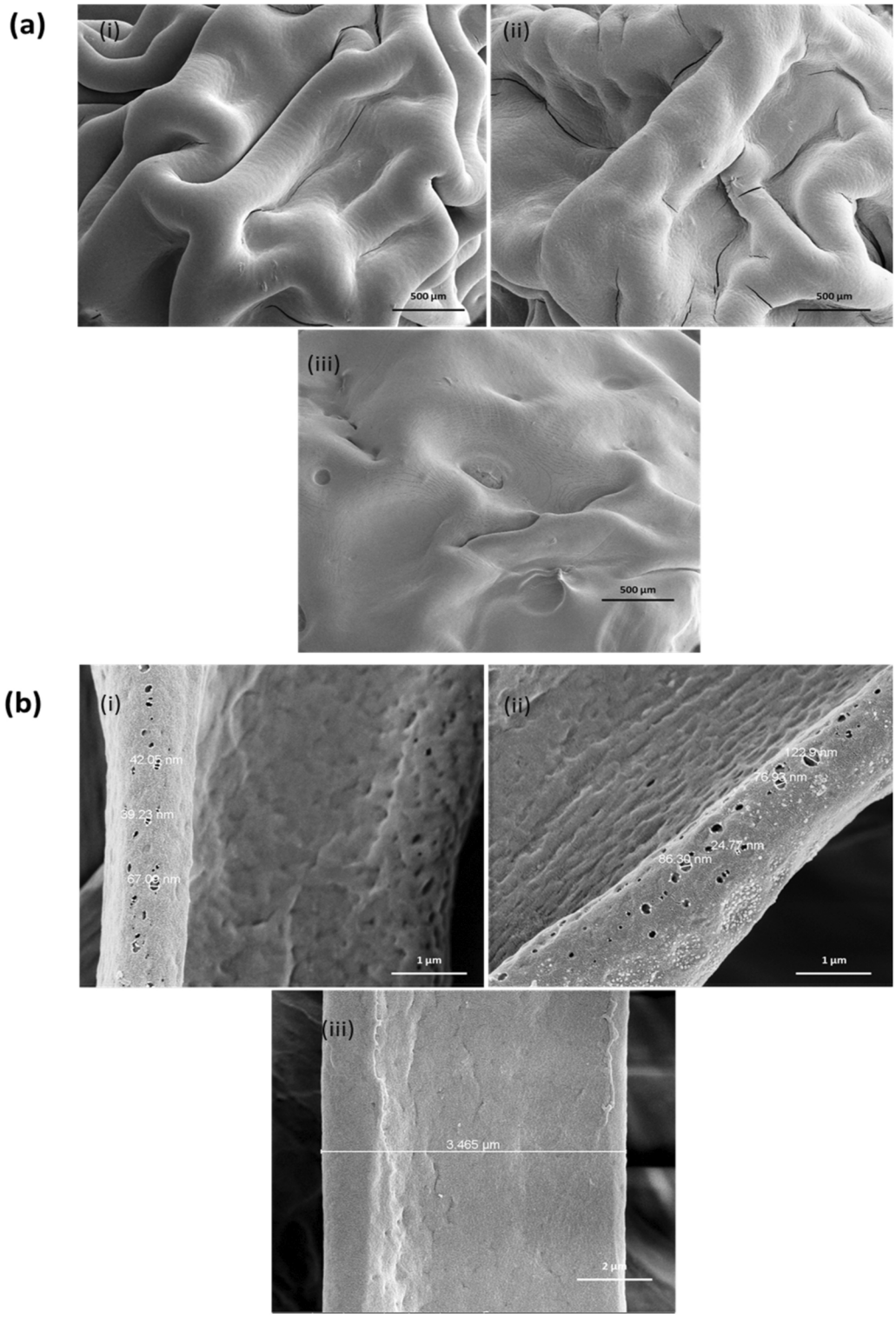

Fig. 1. (**a**) (i) XHR SEM image of alginate-gelatin beads surface. (ii) SEM image of alginate-gelatin bead with 0.01 g lactase. (iii) SEM images of alginate-gelatin beads with two enzymes (0.01 g lactase + 0.0034 g glucose oxidase). (**b**) (i) and (ii) XHR-SEM images of electrospun fibers without and with enzymes (iii) Diameter of a single electrospun fiber.

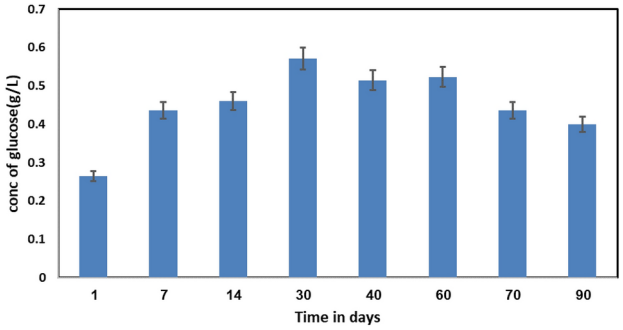

Fig. 2. Stability of lactase in electrospun fibers: Activity of lactase was expressed in terms of glucose formed in g/L on incubation for different time intervals when immobilized in electrospun fibers.

Time (h)	Glucose oxidase effect on lactase at 37 °C (amount of galactose formed (g/L))	
	Lactase alone (5 mg/mL)	Both enzymes (5 mg/mL, 10 mg/mL)
1	0.026 ± 0.002	0.036 ± 0.001
2	0.046 ± 0.001	0.075 ± 0.002
3	0.056 ± 0.001	0.111 ± 0.001
4	0.076 ± 0.003	0.152 ± 0.001

Table 2. Effect of glucose oxidase on lactase. Glucose oxidase enhances lactase activity over short periods. This effect is expressed in terms of galactose formed during incubation (g/L) at different time intervals. Data are expressed as mean ± SD (n = 3).

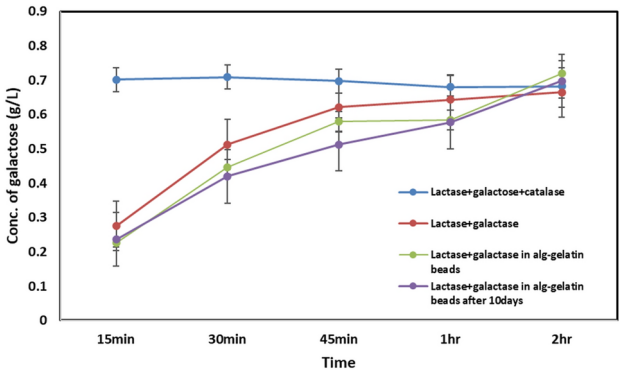

Fig. 3. Effect of catalase on lactase is measured in terms of galactose formed in g/L in different time intervals in different conditions.

Time (h)	Lactose concentration (g/L)					
	1% real milk	Free-enzyme	Alginate	Alginate-carrageenan	Alginate-gelatin	Electrospun enzyme
0	0.119					
1		0.041 ± 0.004	0.045 ± 0.002	0.086 ± 0.002	0.072 ± 0.003	0.088 ± 0.001
2		0.037 ± 0.006	0.039 ± 0.004	0.066 ± 0.003	0.069 ± 0.005	0.059 ± 0.003
3		0.022 ± 0.003	0.028 ± 0.001	0.065 ± 0.004	0.066 ± 0.002	0.050 ± 0.001

Table 3. Enzyme activity in low-fat milk: lactase activity was measured in terms of residual lactose in milk (g/L) at different time intervals and under different immobilization conditions. Data are expressed as mean ± SD (n = 3).

The enzyme reduces lactose content more effectively in low-fat milk (1%) and has no effect in high-fat milk (3%) (results are enclosed in Table S3).

Summary and conclusions

Several factors affecting the activity and stability of lactase, both in its free state and when physically entrapped within a matrix, have been thoroughly investigated. Various immobilization methods, such as encapsulation in gel matrices (e.g., alginate beads) along with other polymers and crosslinking techniques, have been compared with the electrospinning method. The electrospinning technique is considered superior in industrial applications due to its several advantages, including enhanced stability, reusability (due to the protective matrix), controlled enzyme loading, even enzyme distribution, and the provision of more active sites for substrate-enzyme interactions. Additionally, electrospinning can mimic biological systems, which further enhances enzyme–substrate interactions.

Enzymes immobilized in alginate alone exhibited poor stability, with a 50% reduction in activity within a few weeks which might be due to the porosity of the gels. To improve stability, alginate was combined with other polymers such as gelatin or carrageenan, resulting in increased enzyme activity and improved stability. High temperatures (50 °C) and rapid shaking (450 rpm) were beneficial for the activity of the free enzyme, but not for the immobilized or electrospun enzymes. Lactase activity was found to be optimal at a pH of 3.8. The presence of activators, such as $MnCl_2$ and $MgSO_4$ (30 mM), resulted in a 20–25% increase in enzyme activity.

When lactase was immobilized in alginate beads combined with other polymers such as carrageenan or gelatin and crosslinked, it exhibited activity for up to three months with a reduction in activity during storage at 4 °C. Similarly, lactase immobilized in electrospun fibers also demonstrated stability for 90 days, with enhanced activity compared to the alginate-based system. The primary difference, however, is that electrospun fibers offer greater mechanical stability and can withstand fluctuations in environmental factors. During the extraction of experimental data, all parameters were maintained consistently. We hypothesize that storing the enzyme in the substrate solution for one week may have increased the pore size on the surface of the fibers, thereby facilitating greater substrate flow to the enzyme and enhancing its activity.

In general, performing sequential chemical reactions in a single pot, particularly those involving organic compounds, is challenging due to various factors. Dunn et al.[32] proposed that synthesizing one kilogram of an organic compound, such as sildenafil citrate, could save over 95% in solvent usage if the reaction were carried out in a "one-pot" format[33], which also offers other potential savings. Inspired by this, we attempted a "one-pot" reaction using biological reagents by immobilizing three enzymes together and investigating the effect of glucose oxidase and catalase on lactase activity. While the addition of these enzymes enhanced lactase activity, the effect was observed only for short periods, suggesting that further investigation is required in this area.

In this study, the lactase enzyme, when immobilized in electrospun fibers, was shown to be stable for up to three months and reusable for at least eight cycles. After testing the activity of immobilized lactase in a solution with a known lactose concentration, we examined its performance in real milk, which represents the practical application of the immobilization methods. The enzyme's activity was tested in both low-fat and high-fat milk, with a focus on a short-term evaluation. Long-term stability studies and the parameters affecting enzyme performance in low and high-fat milk were considered beyond the scope of the current manuscript and will be addressed in a future version of this work, with a more comprehensive explanation. Future research will also focus on utilizing these immobilized enzymes in a "tea bag" format, which could enable the convenient, low-cost production of lactose-free milk. A multi-enzyme system may provide a valuable solution for the dairy industry and related fields, addressing the growing demand for lactose-free milk while preserving the nutritional content and taste of native milk.

Data availability

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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Gayathri peta— Writing – original draft, Examining, Investigation. Ron Avrahami, Eyal Zussman—Preparation of electrospun fibers. J.Stefan Rokem—Writing, review & editing. Charles Greenblatt—Supervision, writing, review, and editing.

Competing interests

The authors declare no competing interests.

Additional information

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