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# Immobilization with Ca–Alg@gelatin hydrogel beads enhances the activity and stability of recombinant thermoalkalophilic lipase

Kezban Yildiz Dalginli[1]*, Onur Atakisi[2]

[1] Department of Chemistry and Chemical Processing Technologies, Kars Vocational High School Kafkas University, Kars, Turkey
[2] Department of Chemistry, Faculty Science and Letter, Kafkas University, Kars, Turkey

*** Corresponding author:**
e-mail:
kezbandalginli@gmail.com

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**Abstract**
This study aims at the immobilization and characterization of thermoalkalophilic lipases produced recombinantly from *Bacillus thermocatenulatus* BTL2 and *Bacillus pumilus* MBB03. For this purpose, immobilization of the produced enzymes in calcium-alginate@gelatin (Ca–Alg@gelatin) hydrogel beads, immobilization optimization and characterization measurements of the immobilized-enzyme hydrogels were conducted. Optimum temperature and pH values were determined for *B. thermocatenulatus* and *B. pumilus* MBB03 immobilized-enzyme hydrogels (60–70 °C, 55 °C and pH 9.5, pH 8.5). Thermal stability was determined between 65 °C and 60 °C of *B. thermocatenulatus* and *B. pumilus* MBB03 immobilized enzymes, respectively. The pH stability was determined between pH 7.0–11.0 at +4 °C and pH 8.0–10.0 at +4 °C, respectively.
In conclusion, the entrapment technique provided controlled production of small diameter hydrogel beads ($\sim$ 0.19 and $\sim$ 0.29) with negligible loss of enzyme. These beads retained high lipase activity at high pH and temperature. The activity of Ca–Alg@gelatin-immobilized lipase remained relatively stable for up to three cycles and then markedly decreased. With this enzyme immobilization, it may have a potential for use in esterification and transesterification reactions carried out in organic solvent environments. We can conclude that it is one of the most promising techniques for highly efficient and economically competent biotechnological processes in the field of biotransformation, diagnostics, pharmaceutical, food and detergent industries.

**Keywords**
alginate, gelatin, hydrogel beads, thermoalkalophilic lipase, immobilization, characterization

## 1. INTRODUCTION

In industrial applications, the biocatalyst used in enzyme-catalyzed reactions needs to be reused or used continuously for both technical and economic reasons. Enzyme immobilization allows an increase in the usability of a dissolved enzyme in industrial applications in order to enable it to be repeatedly and continuously used, to increase its stability and to increase its useful life (Bickerstaff, 1995; Edelman and Wang, 1992; Katchalski-Katzir, 1993). In addition, immobilized enzymes eliminate the disadvantages of free enzymes and allow them to be more stable (Chaplin and Bucke, 1990). In general, it is extremely important that immobilized enzymes as well as their catalytic properties have been developed economically in industrial applications (Clark, 1994). Immobilization techniques have been developed to get the most out of the purified enzymes. The most important advantage of the immobilization process is the easy use of enzyme preparations, as well as the easy separation from the reaction products catalyzed by the enzyme (Chaplin and Bucke, 1990). The easy separation of the enzyme from the product facilitates enzyme applications and supports a reliable and efficient reaction technology. Enzymes are generally soluble. Therefore, enzymes can only be used once in the absence of expensive separation. In addition, immobilization provides a cost advantage by eliminating the need to regenerate the enzyme in order to create an enzyme-catalyzed process at the first step (Tischer and Wedekind, 1999). In recent years, immobilization technology has been developing rapidly and has become an increasingly rational design issue, although it is seen that there is a need for further development (Bickerstaff, 1995).

The entrapment technique, which is one of the immobilization techniques, can be defined as the physical entrapment of lipase into a support matrix, fibers, lattice structure, a cavity or a mesh (Mammarella and Rubiola, 2005; Won et al., 2005). One of the simplest and most widely practiced procedures of lipase entrapment is the use of alginate and gelatin polymers as matrices through trapping (Guisan, 2006; López et al., 1997). Immobilization in sodium alginate (Na–Alg) gel provides great convenience in immobilization studies because it is a fast and easily applicable method. The entrapment in an insoluble calcium alginate (Ca–Alg) gel is a fast, nontoxic and inexpensive versatile method for the immobilization of enzymes and cells (Fraser and Bickerstaff, 1997). The alginate gel has a pore structure that prevents enzyme leakage

but does not block substrate access to the enzyme. Gelatin gels provide moderate immobilization conditions. The most striking reason for the use of gelatin in enzyme immobilization is its long shelf life (Emregul et al., 2006). In addition, its cheap and easy availability makes gelatin generally preferred for enzyme, cell and tissue immobilization without the need for any ion, molecule, salt pH adjustment for gel formation (Scardi, 1987).

Today, lipases are widely used in the production of pharmaceuticals, cosmetics, biodiesel, emulsifiers, flavors and fragrances, the synthesis of many organic and lipophilic antioxidants and also in the pretreatment of lipid-rich wastewater (Melani et al., 2020). Therefore, the development of new support materials has caused an increasing interest in processes related to the use of immobilized lipases. Therefore, the synthesis and characterization of new and diverse supports for lipases has become increasingly common in the literature (Ismail and Baek, 2020; Sharifi et al., 2020). Also, due to its low density and high brittleness, gelatin is rarely used alone and is often used for blending with different entrapment materials. It is well known that blending is an effective and convenient method to improve performance and to easily capture the enzyme in the beads during immobilization (Dong et al., 2006). For this purpose, in this study, an ideal entrapment material was tried to be created by using a mixture of both polymer materials (Ca–Alg@gelatin). In our study, recombinant thermophilic lipases produced from our previous study, *Bacillus thermocatenulatus* BTL2 and *Bacillus pumilus* MBB03 were immobilized in Ca–Alg@gelatin hydrogel mixture through the entrapment method. In addition, immobilization conditions such as optimum Alg concentration, Gel concentration, $CaCl_2$ percentage, enzyme amount and bead size were optimized for the obtained immobilized beads. Some biochemical characterization parameters such as optimum pH and temperature activity, pH and temperature stability, metal ions, detergent, organic solvent and inhibitory agent activity of immobilized lipases were investigated.

## 2. MATERIAL AND METHODS

### 2.1. Materials

Thermoalkalophilic lipase from *Bacillus thermocatenulatus* BTL2 and *Bacillus pumilus* MBB03 was produced from Biochemistry Department of Chemistry, Faculty Science and Letter, Kafkas University (Kars, Turkey). Gelatin, sodium alginate and calcium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). All substrates p-nitrophenyl-octaonate (pNPO, Caprylate C8), p–nitrophenyl deconate (pNPD-C10) from Sigma Chemical Co. (St. Louis, MO) were used to assess the lipase activity. Ethanol, Methanol, Acetone, HCl and Acetic acid was purchased from Merck A.G (Darmstadt, Germany). Phenyl Methyl Sulfonyl Fluoride (PMSF, Sigma-Aldrich), Sodium Deoxycholate, Sodium Taurocholate, Tween 80, Tween 20, TritonX–100, DMSO, Xylene, n–Butanol, Dimethylformamide (DMF), Acetonitrile, Glycerol, 1,4-dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO) and CAPS from AppliChem. All other chemicals were reagent grade.

### 2.2. Immobilization of thermoalkalophilic lipases

Immobilization of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 lipases in Ca–Alg@gelatin hydrogel mixture was performed through Norouzian's method (Norouzian et al., 2002). For this purpose, 0.5 g Gel was weighed and dissolved in 10 mL distilled water at 40 °C for 30 minutes, then 0.2 g Na–Alg was added to it and mixed. The lipase enzyme solution was added to the hydrogel mixture with slow mixing. With the help of an injector, hydrogel beads were obtained by dropping into it 50 mL of 250 mM $CaCl_2$ solution placed in ice. The hydrogel beads were stirred on a magnetic stirrer for 90 minutes and then filtered and washed with distilled water. The filtering and washing waters were stored for protein determination. In addition, lipase activities of the hydrogel beads and washing waters were determined through spectrophotometric method. Immobilized beads were stored in the refrigerator (+4 °C) until used in 0.05 M Tris–HCl buffer (pH 7.2) to cover. For this purpose, optimization of immobilization conditions was carried out. The protein concentration of the enzymes was determined with the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard, at 595 nm.

$$\text{Immobilization yield } (\%) = \frac{a_{\text{imm}}}{a_{\text{free}}} \cdot 100 \qquad (1)$$

where:
$a_{\text{imm}}$ – specific activity of immobilized enzyme (mmol/mg protein),
$a_{\text{free}}$ – specific activity of the free enzyme (mmol/mg protein).

$$\text{Loading yield } (\%) = \frac{C_i \cdot V_i - C_f \cdot V_f}{C_i \cdot V_i} \cdot 100 \qquad (2)$$

where:
$C_i$ – initial protein concentration,
$V_i$ – initial volume of enzyme solution,
$C_f$ – protein concentration in total filtrate,
$V_f$ – total volume of the filter (Won et al., 2005).

### 2.3. Assay of lipolytic activity

Lipase activity was also estimated using a spectrophotometric assay (Winkler and Stuckman, 1979) with p-nitrophenyl esters as a substrate. The absorbance of p-nitrophenol released was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per min under standard assay conditions.

## 2.4. Optimization of hydrogel parameters

Five main immobilization parameters were used to determine the optimum amount of immobilization of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 lipases. These parameters are; 1.5–3% (w/v) Gel concentration, 10–30% (w/v) Na–Alg concentration, 100–300 mM $CaCl_2$ concentration, 1–5 mL enzyme solution and 2–4 mm bead diameter. The bead diameter was optimized using a caliper. The bead size was changed by using syringes with different needle diameters (0.45 × 13 mm (insulin injector), 0.60 × 32 mm (blue injector), 0.70 × 32 mm (black injector) and 0.80 × 38 mm (green injector)) (Won et al., 2005).

## 2.5. Biochemical characterization of hydrogel beads

### 2.5.1. Effects of pH on immobilized lipases activity and stability

To investigate the optimal pH, immobilized-lipase hydrogel activity was assayed at various pH from 4.0 to 12.0 in the following buffers: 50 mM sodium acetate buffer (pH 4.0–5.0), 50 mM $KH_2PO_4$ buffer (pH 6.0–7.0), 50 mM Tris–HCl buffer (pH 8.0–9.0) and 50 mM glycine-NaOH buffer (pH 10.0–12.0). The pH stability in the range of 4.0–12.0 was examined by incubating the enzyme solution for 15 days at +4 °C with different buffers and then the residual activity was determined.

### 2.5.2. Effect of temperature on immobilized lipases activity and stability

The immobilized-lipase hydrogel activity was measured in the range of 10–100 °C using the standard activity assay procedure at related temperatures. Thermostability of the lipase was investigated by measuring the residual activity after incubating the enzyme solution at 40–90 °C at various times from 15 min to 30 min in 50 mM Tris–HCl Buffer (pH 9.5 for *B. thermocatenulatus* BTL2-immobilized-lipase and pH 8.5 for *B. pumilus* MBB03-immobilized-lipase).

### 2.5.3. Effect of metal ions on immobilized lipase activity

Various metal ions ($CaCl_2$, NaCl, $CuCl_2$, KCl, $BaCl_2$, $MgCl_2$, $MnCl_2$, $HgCl_2$, $NiCl_2$, $ZnSO_4$, $CuSO_4$, $CoSO_4$, $FeSO_4$, $MgSO_4$) at final concentrations of 0.1, 0.5 and 1.0 mM were added to the enzyme in 50mM Tris–HCl buffer, pH (pH 9.0 for *B. thermocatenulatus* BTL2-immobilized-lipase and pH 8.0 for *B. pumilus* MBB03-immobilized-lipase) and the solution was preincubated at room temperature for 5 min and then assayed for lipase activity. The relative activity of the enzyme was calculated by comparing it with the enzyme incubated under the similar conditions without metal ions.

### 2.5.4. Effect of some detergents on immobilized lipase activity

The effect of some detergents on immobilized-lipase hydrogel activity was determined using ionic detergents (sodium deoxycholate, sodium taucolate, and sodium dodecyl sulfate) and non-ionic detergents (Tween 80, Tween 20, Triton X-100). CHAPS as zwitterionic detergent was also used to study the effect of certain detergents at various final concentrations. Relative activity was calculated and enzyme solution without detergent was used as a comparing reference. Detergent at the final concentration of 0.1%, 0.5%, and 1.0% on the lipase was investigated and the remaining enzyme activity was examined with the standard assay method. Relative activity was calculated by comparing it with control (enzyme incubated without surfactants).

### 2.5.5. Effect of some organic solvents on lipase activity

The effect of some organic solvents on immobilized-lipase hydrogel activity was determined by using ethanol, methanol, isopropanol, n–butanol, acetone, dimethylsulfoxide (DMSO), xylene, dimethylformamide (DMF), glycerol. 10% stock solutions of these organic solvents were prepared and activity determinations were made under optimum conditions by adding final concentrations in the reaction mixtures to the range of 0.01–1%. The test medium without any organic solvents was considered as control and the activity of the control group was accepted as 100. The activity changes of the test medium containing organic solvent were compared with the control and % relative activities were calculated.

### 2.5.6. Effect of some chemical and inhibitory materials on immobilized lipase activity

The effect of the inhibitors and the chemical substance on the immobilized-lipase hydrogel activity was determined by preparing 10 mM stock solutions of $\beta$–mercaptoethanol, phenyl methyl sulfonyl fluoride (PMSF), 1,4–dithiothreitol (DTT), EDTA in order to give final concentrations in the reaction mixtures of 0.01–1 mM, and activity determinations were made under optimum conditions. The enzyme/inhibitor mixture was then taken to assay the lipase activity. Relative activity was calculated and enzyme solution without inhibitor was used as comparing reference.

### 2.5.7. Reusability of immobilized lipases

To determine the reusability of the immobilized lipases in the Ca–Alg@gelatin hydrogel, 0.003 and 0.004 g beads were weighed, respectively. With the help of the substrate solution prepared in 0.5 M Tris–HCl buffer (pH 7.2), the activity was determined by filtering the immobilized enzyme from the reaction medium without stopping the reaction with acetone-ethanol mixture. Activity assays were repeated until the lipase activity of the beads stopped.

2.5.8. Storage stability of immobilized lipases

In order to determine the storage stability of the immobilized lipases, the activity assays of the immobilized enzyme were carried out at two-day intervals for a total of 30 days.

## 2.6. Statistical analysis

Statistical analyses were performed in triplicate, and average values and standard deviation (mean ± SD) were reported. The statistical significance was evaluated by using the SPSS 16.0 software package (SPSS ver. 16.0 for Windows professional edition). A one-way analysis of variance (ANOVA) was performed and was followed by Duncan's test to estimate the significance at the 5% probability level.

# 3. RESULTS AND DISCUSSION

## 3.1. Entrapment in Ca–Alg@gelatin hydrogel

Figure 1a shows the beads obtained through the entrapment of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 lipases in Ca–Alg@gelatin hydrogel mixtures. In the study, the protein amounts of $CaCl_2$ solutions and washing waters in which the lipase solution and immobilized enzyme were dropped were determined by using these standard graphics. The percentage of immobilization was determined by calculating the amount of unattached protein and attached protein from the protein amounts found. For *B. thermocatenulatus* BTL2 immobilized-lipase, the percentage of immobilization was 73% and the immobilization efficiency was 63%, and for *B. pumilus* MBB03, the percentage of immobilization was 82% and the immobilization efficiency was 74%. The specific activity of immobilized *B. thermocatenulatus* BTL2-lipase enzyme was 6.9 U/mg protein. The specific activity of *B. pumilus* MBB03 immobilized-lipase enzyme was also 3.4 U/mg protein. From these results, it is seen that the immobilization percentage and % efficiency of *B. pumilus* MBB03 immobilized-lipase is better. It can be said that *B. pumilus* MBB03 lipase in immobilized form preserves its own active centre structure better. Cheirsilp et al. (2009) found in their study that immobilization efficiencies ranged from 97.8–99.6% and had little correlation with alginate concentration. In addition, when lipase from pig pancreas was successfully immobilized onto genipin-functionalized chitosan beads, Khan et al. (2020) found that the activity efficiency and immobilization amount of lipase under optimal conditions were 68% and 105 mg/g, respectively.

## 3.2. Optimization of immobilization parameters

The concentration of the polymer, i.e. the gel, used as the entrapment material and the concentration of the entrapment solution are important parameters in the entrapment of the enzyme. For this reason, the effects of Na/Alg and Gel concentrations, $CaCl_2$ concentration, enzyme amount and bead size on the lipase activity were investigated and their optimum values were determined.

Figure 1b shows the results from activity assays made with the beads obtained to determine the optimum gelatin concentration to be used in the immobilization of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 lipases. When the protein assay and the activity results of the immobilized lipase were evaluated, the highest enzyme activity was observed at 20% (2g) Gel concentration for *B. thermocatenulatus* BTL2 lipase and 25% (2.5 g) Gel concentration for *B. pumilus* MBB03 lipase. In the immobilization study of lipase enzyme immobilized on Ca–Alg@gelatin hydrogel beads, increasing Gel concentration increased the immobilization efficiency. At a low concentration of Gel, unstable and brittle beads formed and caused poor immobilization efficiency. On the contrary, it was observed that bead formation became more difficult at concentrations higher than *B. thermocatenulatus* BTL2 lipase 20% (w/v) and *B. pumilus* MBB03 lipase 25% (w/v). For this reason, it was decided to use 20%, 25% (w/v) Gel, which were optimum Gel concentrations. Various concentrations of Na–Alg were prepared to change the relative degree of gel formation that would create different pore size of the alginate matrix. The optimum Na–Alg concentration was 2% and 2.5% for *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 lipase, respectively (Fig. 1c). It was found that at low sodium alginate concentration (less than 2% and 2.5%), the enzyme leaked out easily due to the large pore structure of the beads. In contrast, high concentration of sodium alginate increased the bead strength, but when the sodium alginate concentration was increased above the optimal level, bead formation was difficult, and immobilization efficiency decreased.

$CaCl_2$ cationic solution is used as the gel inducing system in entrapments performed through a mixture of Na–Alg–Gel. Won et al. (2005) used $CaCl_2$ drip solution for alginate+gelatin gels as stated in their study in 2005. In this study, $CaCl_2$ dropping solution was used and the optimum calcium chloride concentration was determined as 250 mM for *B. thermocatenulatus* BTL2 lipase and *B. pumilus* MBB03 lipase (Fig. 1d). 250 mM gave the highest retained lipase as well as immobilized activity. In the study, it was observed that as the $CaCl_2$ concentration increased, the immobilization efficiency increased, and the immobilization efficiency did not change after 250 mM $CaCl_2$ concentration. With these data, it can be said that the most suitable gel structure of the immobilized hydrogel forms obtained is at a concentration of 250 mM. Enzyme leaks may occur due to the large pore structure of the gel obtained at low concentrations of $CaCl_2$, and the enzyme activity does not change at high concentrations of $CaCl_2$ because it is difficult for the substrate to penetrate into the gel due to the formation of a tight gel structure. Hydrogel beads prepared through Na/Alg, Gel, and $CaCl_2$ concentrations lower than the de-

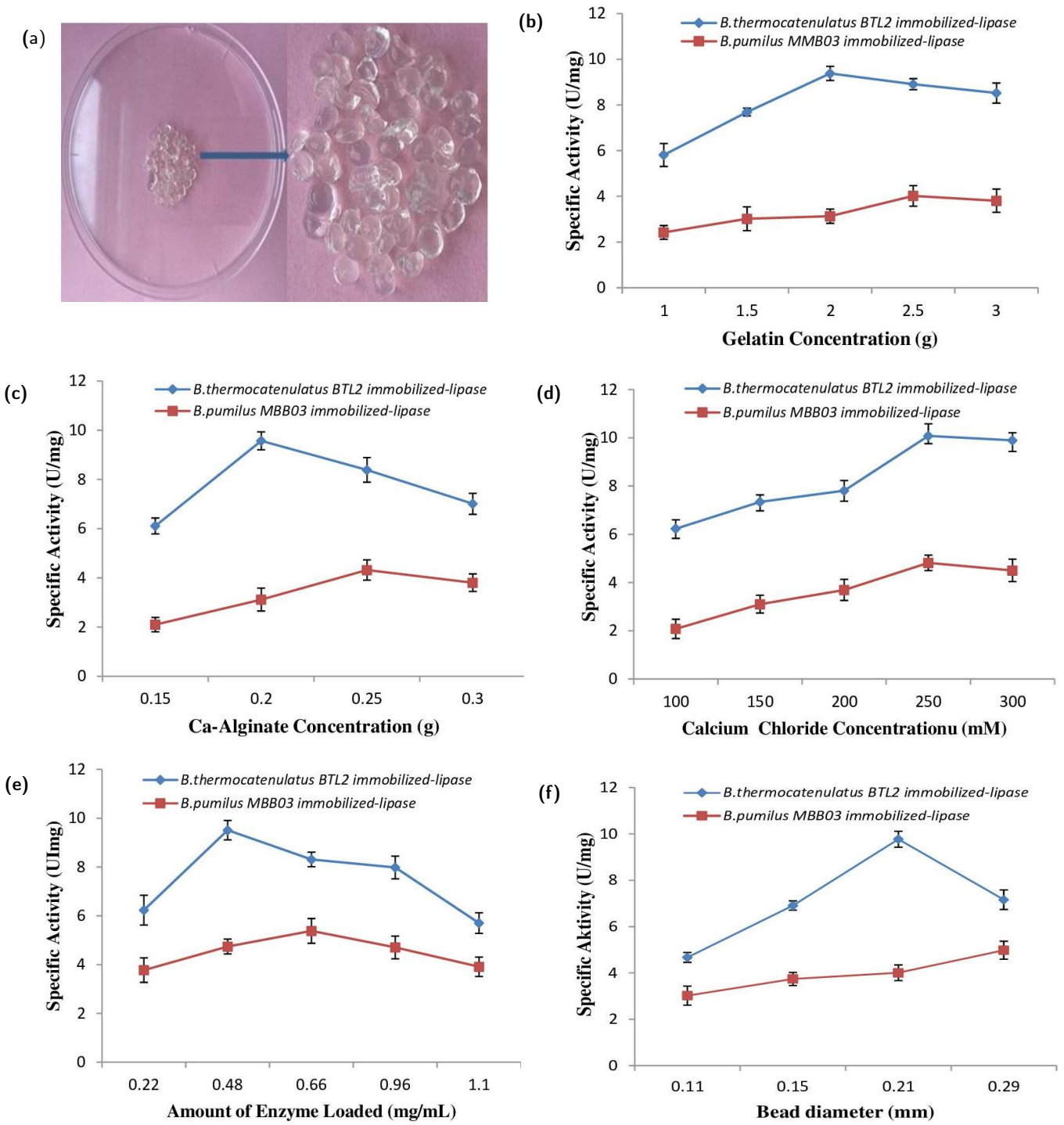


Figure 1. Hydrogel beads obtained by of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-lipases in Ca–Alg@gelatin mixtures (a). The effect of gelatin concentration (b), alginate concentration (c), $CaCl_2$ concentration (d), enzyme concentration (e) and bead size (f) on the immobilization of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 lipases in Ca–Alg@gelatin mixtures.

termined optimum concentrations were brittle and enzyme leakage into the substrate solution was high. At their optimum concentrations, bead hardness was improved and lipase activity and retained activity increased as enzyme leak was reduced. In the study of immobilization of lipase enzyme immobilized on Ca–alginate gel beads similar to this study, it was observed that the immobilization efficiency increased as the alginate concentration used at 1–2% (w/v) concentration increased, but the immobilization efficiency did not change as the $CaCl_2$ concentration used at 50–300 mM concentration increased (Won et al., 2005). Ozyilmaz and Gezer (2010), in their study, found that the best results of *Can-*

*dida rugosa* and porcine pancreatic lipase immobilized on Ca–alginate gel, were 2.5% Na–Alg and 2.5 M $CaCl_2$ for *Candida rugosa* and 2.5% Na–Alg and 2.0 M $CaCl_2$ for porcine pancreatic lipase. In the study where *Pseudomonas sp.* lipase was immobilized to alginate beads, an alginate concentration of 1.5–2.5% (w/v) and a $CaCl_2$ concentration of 50–200 mM were used. In their study, it was reported that the enzyme activity increased as the alginate concentration increased, and the enzyme activity decreased as the $CaCl_2$ concentration increased (Cheirsilp et al., 2009). Rakmai et al. (2015) used 1–3% (w/v) sodium alginate and 1–3% (w/v) gelatin concentration and 100–300 mM $CaCl_2$ concentration. Mondal et al. (2006) in their immobilization study of *Pseudomonas cepacia* lipase in 2006, determined 20% w/v alginate concentration and 60 mM $CaCl_2$ concentration. In addition to these studies, Vujčić et al. (2011) used gelatin concentration at 20% (w/v), and Trabelsi et al. (2014) used alginate concentration at 1, 2 and 3 concentrations. This information obtained thanks to literature research supports this study.

The effect of the amount of enzyme loaded on Ca–Alg@gelatin hydrogel beads on the catalytic activity was investigated by studying five different enzyme concentrations. It was observed that the activity increased proportionally with the amount of enzyme, but decreased after a certain amount. It was observed that the optimum enzyme concentration was 0.048 mg/mL of *B. thermocatenulatus* BTL2 lipase and 0.066 mg/mL of *B. pumilus* MBB03 for the optimization of the amount of enzyme loaded on the beads (Fig. 1e). Cheirsilp et al. (2009) observed that as the loaded enzyme concentration increased, lipase activity increased, but immobilization activity decreased. In addition, Vaidya et al. (2008) observed in their study that lipase activity increased as the amount of enzyme loaded similarly increased. In this study, it was determined that as the amount of enzyme loaded increased after a certain rate, lipase activity increased, but immobilization activity decreased. This may be due to the limitation of substrate diffusion into the lipase immobilized in the beads. That is, at high enzyme concentration in the gel matrix, it may block access for the substrate. Considering these conditions, it was accepted that the concentrations of 0.048 mg/mL and 0.066 mg/mL, respectively, were suitable for lipase immobilization with the determined optimum alginate, gelatin and $CaCl_2$ concentrations.

When the effect of bead size on activity was studied, the highest activity was determined in beads with a diameter of 0.19 mm (with a 0.70x32 mm diameter black-tipped syringe) for *B. thermocatenulatus* BTL2–hydrogel beads and 0.29 mm (with a 0.80 × 38 mm diameter green-tipped syringe) for *B. pumilus* MBB03–hydrogel beads. The diameter of 0.19 mm and 0.29 mm beads was determined as the optimum bead size by taking the average of 10 beads obtained using a black and green tip injector (Fig. 1f).

The increase in bead diameter resulted in a decrease in immobilization efficiency. In the enzyme immobilization system through the entrapment, intraparticle mass transfer may be related to bead size dependency, which has a significant effect on the substrate conversion rate. In other words, smaller bead diameter is considered as higher immobilization efficiency by increasing mass transfer (Dey et al., 2003; Talekar and Chavare, 2012). In the study of immobilization of lipase enzyme immobilized on Ca–alginate gel beads by Won et al. (2005) the bead size obtained through different needle diameters was measured with an optical microscope and it was observed that the specific activity decreased as the bead size increased. In the immobilization study of *Pseudomonas sp.* lipase with sodium alginate, beads with a diameter of 2.03, 2.53, and 3.02 mm were obtained and immobilized lipase activity decreased as the bead size increased (Cheirsilp et al., 2009). Other studies have reported that as the bead size increased due to mass transfer resistance, the immobilized enzyme activity decreased (Knezevic et al., 2002; Fadnavis et al., 2003). In this study, maximum activity was observed when black and green tipped injectors were used. The immobilization conditions of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 lipases in Ca–Alg@gelatin hydrogel beads were optimized as shown in Table 1.

Table 1. The optimization of immobilization parameters depending on gelatin, Ca–Alg, $CaCl_2$ concentrations and enzyme amount, bead size.

| Immobilization parameters | *B. thermocatenulatus* BTL2 immobilized lipase | *B. pumilus* MBB03 immobilized lipase |
|---|---|---|
| Gelatin concentration | % 20 | % 25 |
| Ca–Alg concentration | % 2 | % 2.5 |
| $CaCl_2$ concentration | 250 mM | 250 mM |
| Enzym amount | 0.48 mg/mL | 0.66 mg/mL |
| Bead size | 0.19 mm | 0.29 mm |

*Experiments were performed in triplicates and ± standard errors are reported.

## 3.3. Biochemical characterization of Ca–Alg@gelatin hydrogel beads

### 3.3.1. Effects of pH on immobilized-lipase activity and stability

The best activities were obtained at pH 9.5 for *B. thermocatenulatus* BTL2 immobilized-lipase p–nitrophenyl deconate and at pH 8.5 for *B. pumilus* MBB03 immobilized-lipase p–nitrophenyl octanate as a result of the determination of activity (Fig. 2a). It is known that lipases generally show activity at alkaline pH (Fojan et al., 2000). In our study, according to the graphs showing the effect of pH on the immobilized enzyme activity, it was determined that the enzyme showed very low activity at acidic pH's and its ability to

hydrolyze p–nitrophenyl ester substrates at slightly alkaline pHs was higher. It shows that the immobilization application increased the optimum pH value of both immobilized lipases but did not make a significant change on them. Looking at the literature, it has been reported that free enzymes show maximum activity at 7.5 pH (Quyen et al., 2003), 8 and 8.5 pH compared to immobilized enzyme (Kim et al., 2002; Litantra et al., 2013). This increase in the pH value of immobilized enzymes compared to free enzymes can be explained by the fact that the enzyme exhibits optimum activity at higher pH values due to the immobilization of the enzyme in the bead structure. Chiou and Wu (2004) studied the pH between 3 and 11 to determine the optimum pH in the immobilization study of the *Candida rugosa* enzyme, and they determined the optimum pH as 7 for both the free enzyme and the immobilized enzyme (Chiou and Wu, 2004). When *Candida rugosa* lipase was immobilized on chitosan, the optimum pH was 8.0 for free lipase and 9.0 for immobilized lipase (Hung et al., 2003). When the lipase enzyme was immobilized on PVC, chitosan, chitin, agarose, sepharose, and trisacryl, the optimum pH was 7.5 for free lipase and 8.5 for immobilized lipase (Shaw et al., 1990). The literature research and the data obtained in the study are in line with each other.

One of the benefits of using immobilized enzymes is that they are generally more stable under a wide variety of process conditions than free enzymes. Therefore, additional pH and thermal stability studies were carried out. It was observed that *B. thermocatenulatus* BTL2 immobilized-lipase hydrogel showed high stability in a wide pH range. At the end of the first day, it was observed that 70% of the initial activity of the *B. thermocatenulatus* BTL2 immobilized-enzyme hydrogel was preserved at pH of 4.0, 5.0, 6.0, almost all at pH of 7.0, 8.0 and 9.0, and 70% at pH of 10.0 and 11.0. At the end of the fifth day, it was observed that almost all enzymes were preserved at pH of 7.0, 8.0 and 9.0. At the end of the tenth day, it was observed that 60% of the initial activity of

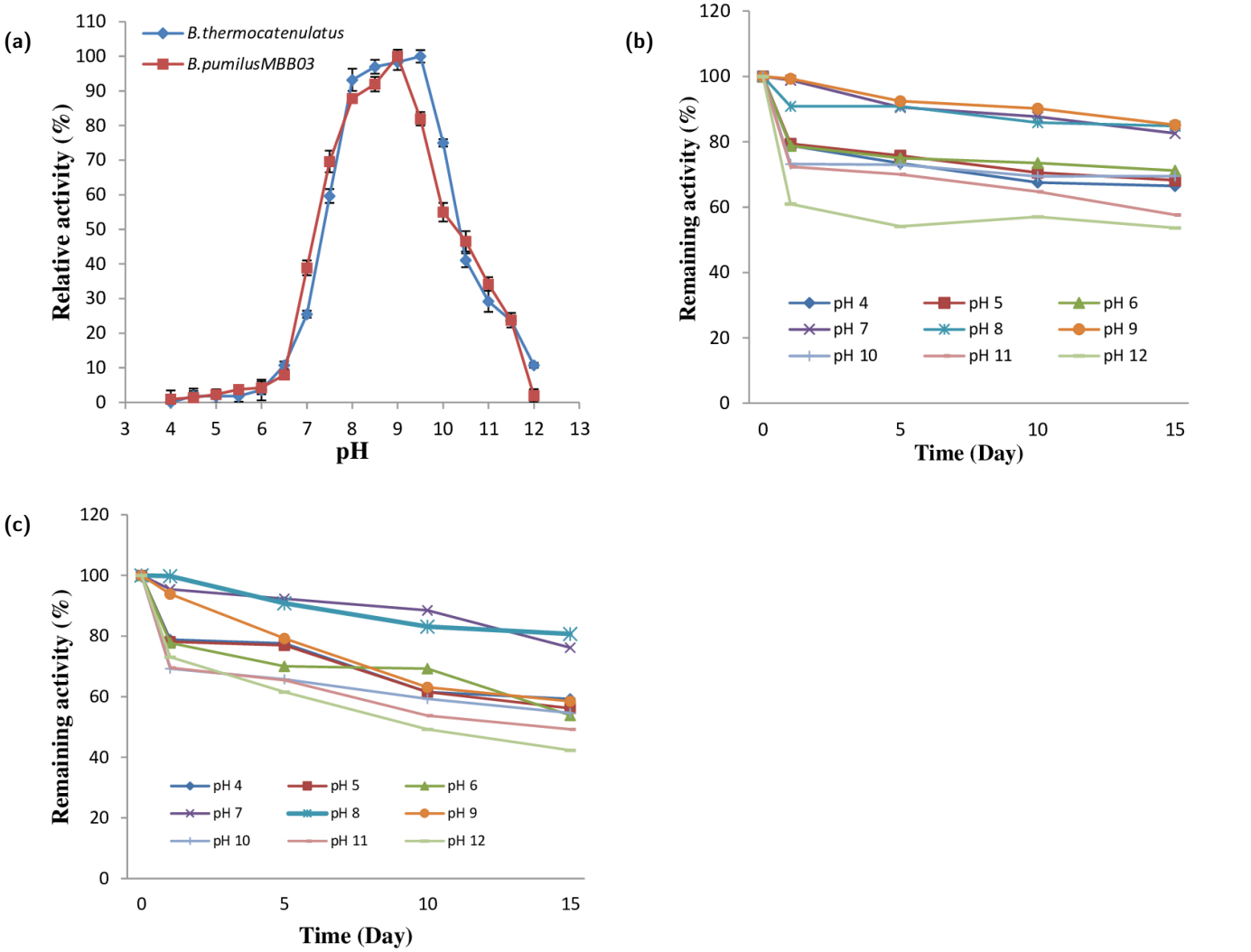


Figure 2. Effects of pH on *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-lipase activities (a). Effects of pH on *B. thermocatenulatus* BTL2 immobilized-lipase (b) and *B. pumilus* MBB03 immobilized-lipase stability (c). pH 9.5 for *B. thermocatenulatus* BTL2 and pH 8.5 for *B. pumilus* MBB03 immobilized-lipase was taken as 100%.

the enzyme was preserved at 4.0 pH, 70% at 5.0 and 6.0 pH, 80% at 7.0 and 8.0 pH, 90% at 9.0 pH, and 60% at pH of 10.0 and 11.0. At the end of the fifteenth day, it was observed that 60% of the initial activity of the enzyme was preserved at 4.0 and 5.0 pH, 70% at 6.0 pH, and 80% at 7.0, 8.0, 9.0, 10.0 and 11.0 pH. Vaidya et al. (2008) in the study of the immobilization of the *Candida rugosa* enzyme determined that 86.19% and 42.64% of the immobilized enzyme were preserved at pH of 9.0 and pH 10.0. Also in this study, at the end of the first day, it was observed that 70% of the initial activity of the *B. pumilus* MBB03 immobilized-enzyme was preserved at pH of 4.0, 5.0, 6.0 and almost all at pH of 7.0, 8.0 and 9.0. At the end of the fifth day, it was observed that almost 70% of the enzymes were preserved at pH of 4.0, 5.0 and 6.0, 90% at pH of 7.0 and 8.0, and 79% at pH of 9.0. At the end of the tenth day, it was observed that 80% of the initial activity of the enzyme was preserved at pH of 7.0 and 8.0. At the end of the fifteenth day, it was observed that 50% of the initial activity of the enzyme was preserved at pH of 4.0, 5.0, 6.0, 9.0 and 10.0, 76% at pH of 7.0, and 80% at pH of 8.0 (Fig. 2c). In our study, it was observed that the bead gel structure of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 hydrogel beads deteriorated in the phosphate buffer pH range of 6.0–7.5. This situation caused the enzyme immobilized inside the bead to leak out. It is believed that this deterioration in the bead structure was caused by the phosphate ions. Because the biggest disadvantage of calcium alginate beads is that they are sensitive to cations such as magnesium and sodium chelating agents such as phosphate and lactate, and because of the displacement of these chelator substances with Ca ions in Ca–alginate, it causes the destabilization of the Ca–alginate and the disintegration of the gel (Cheetham et al., 1979; Knezevic et al., 2002). However, it was found that Ca–Alg@gelatin hydrogel beads were stable in Tris/HCl buffer and increased lipase activity. In addition, it can be said that both enzymes obtained were stable in a wide pH range and preserved their stability especially in the optimum pH and alkaline pH ranges, which makes them suitable for use in the detergent industry and wastewater treatment studies.

#### 3.3.2. Effects of temperature on immobilized-lipase activity and stability

The temperature at which bacterial lipases generally exhibit optimum activity is in the range of 30–60 °C. In this study, the optimum temperature of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-enzymes were determined as 65–70 °C and 55 °C, respectively (Fig. 3a).

The fact that most of the *B. thermocatenulatus* BTL2 lipase activity is preserved over a wide temperature range indicates that this enzyme can be easily used at any temperature from 40 to 100 °C and has great advantages in terms of various industrial applications. In the literature studies, it was reported that the optimum temperature for lipase immobilized with Ca–alginate was 50 °C, *Candida rugosa* free lipase immobilized on chitosan showed high activity above 30 °C, while immobilized lipase showed high activity above 40 °C. It was observed that the immobilized lipase remained active 23% at 60 °C (Hung et al., 2003; Omar et al., 1988). When the lipase enzyme was immobilized on PVC, chitosan, chitin, agarose, sepharose, and trisacryl, the optimum temperature for the immobilized lipase was 45 °C (Shaw et al., 1990). In this study, it is believed that immobilized enzymes show activity at high temperatures, and the enzyme is not affected by increasing temperatures in the Ca–Alg@gelatin hydrogel structure obtained and the enzyme structure is preserved. Considering the optimum pH and temperature properties of the obtained immobilized enzyme forms, these enzymes can be used in organic synthesis studies and as detergent additives because their temperatures are above 40 °C and they have alkaline pH.

Thermal stability is important characteristic information in immobilized enzyme applications. In general, the activity of the immobilized enzyme is more resistant to temperature and denaturating agents than the free enzyme. It was determined that the *B. thermocatenulatus* BTL2 immobilized-lipase preserved approximately 89% and 80% of its activity at 40 °C, after 15 and 30 minutes, respectively. It was observed that the enzyme preserved approximately 83% and 71% of its activity at 50 °C and after 15 and 30 minutes of incubation, respectively. It was determined that the enzyme preserved approximately 79% and 68% of its activity at 60 °C and after 15 and 30 minutes of incubation, respectively. It was determined that the enzyme preserved approximately 57% of its activity at 70 °C and after 15 and 30 minutes. It was observed that the activity of the enzyme was below 50% after 15 and 30 minutes at 80 °C and 90 °C (Figure 3b). It was determined that *B. pumilus* MBB03 immobilized enzyme preserved approximately 84% of its activity at 40 °C and after 15 minutes, and 69% of its activity after 30 minutes. It was observed that the enzyme preserved approximately 74% and 61% of its activity at 50 and 60 °C and after 15 minutes, and lost 46% and 65% of its activity after 30 minutes, respectively. It was observed that the activity of the enzyme decreased after 15 minutes and 30 minutes at 70 °C, 80 °C and 90 °C (Fig. 3c). Vaidya et al. (2008) reported that at 50 °C, the free enzyme could preserve only 62.11% of its activity, while the immobilized enzyme could protect 94.76%, at 70 °C the free enzyme could preserve 9.33% of its activity, while the immobilized enzyme could preserve 9% of its activity at the same temperature. They determined that they were able to protect their 39.10. It was observed that *Candida rugosa* free lipase immobilized on chitosan was 12% active, while immobilized lipase was 23% active at 60 °C (Hung et al., 2003). Chiou and Wu (2004) in the study where they immobilized the *Candida rugosa* enzyme on chitosan with the activation of hydroxyl groups, observed the enzyme for thermal stability at temperatures ranging from 25–60 °C for 1 hour and found

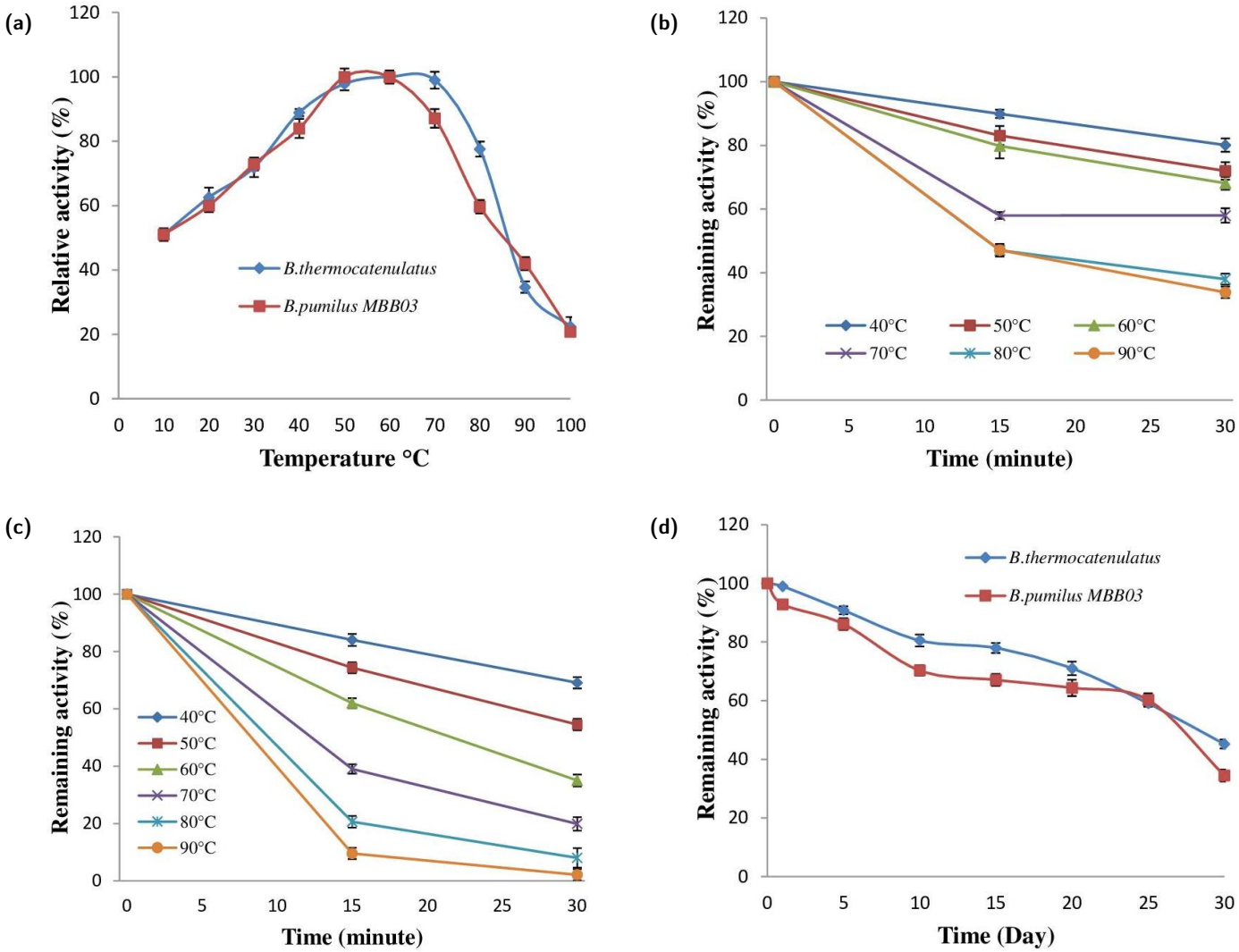


Figure 3. Effects of temperature on activity of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-lipases (a). Effects of temperature on *B. thermocatenulatus* immobilized-lipase (b) and *B. pumilus* MBB03 immobilized-lipases stability (c). Effects of room temperature on *B. thermocatenulatus* lipase and *B. pumilus* MBB03 lipase stability (d). The activity of the non-heated enzyme was taken as 100%.

that after 30 °C the free enzyme began to lose its activity. They determined that at 60 °C, 60% of the activity of the immobilized enzyme and only 20% of the free enzyme could be preserved. Differently, they found that the immobilized lipase with TiO2 supports preserved more than 95% of the enzyme activity for 5 hours at 50 °C (Zhou et al., 2021). It is thought that the thermal stability is more stable at the optimum or closest temperature value in activities related to the free enzyme, and outside the optimum temperature ranges, the free enzyme may lose its activity in general due to the denaturing of its three-dimensional structure. Enzyme immobilization provides thermal stability to immobilized enzymes. In line with the data in this study, it can be said that the immobilized-lipases of *B. pumilus* MBB03 and *B. thermocatenulatus* BTL2 have similar thermal stability compared to the lipases obtained from other microorganisms. In addition, when the immobilized enzymes were examined, it was observed that they generally preserved their activities better than the free enzyme at high temperatures such as 50–70 °C. This allows immobilized enzymes to be used for esterification and transesterification reactions in organic solvent environments.

In general, if an enzyme is in a solution, it is not stable during storage and its activity gradually decreases (Arıca and Bayramoğlu, 2004). If the *B. thermocetenulatus* BTL2 immobilized-enzyme was at room temperature, it was determined that almost all of the immobilized-enzyme was preserved at the end of the first day. At the end of the 5th day, it was observed that 90% of the immobilized-enzyme was preserved. At the end of the 10th and 15th days, it was determined that 80% and 77% of the immobilized-enzyme were preserved, respectively. At the end of the 20th and 25th days, it was observed that approximately 70% and 59%

of the immobilized-enzyme was preserved, and 45% at the end of the 30th day. It was observed that if the *B. pumilus* MBB03 immobilized-enzyme had beeb at room temperature, 92% of the immobilized-enzyme would have been preserved at the end of the 1st day and 86% at the end of the 5th day. It was observed that 70% and 67% were preserved at 10 and 15 days, respectively, 64% and 60% at the end of 20 and 25 days, and 34% at the end of 30 days (Fig. 3d). In the study, it was observed that both *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-enzymes had enzyme escapes from the gel bead structure after the 5th day. It was concluded that the decrease in enzyme activities at the end of the 5th day was due to enzyme leakage. In line with these data, room temperature thermal stability properties of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-enzymes show that the enzyme can be used in industrial applications. It was stated that immobilized *C. antarctica* A lipase retained its 60% activity during a 28 day storage period at 4 °C in a refrigerator and 25 °C in an incubator (Ondul et al., 2012).

### 3.3.3. Effects of metallic ions on immobilized-lipase activity

It was observed that $BaCl_2$, $CoSO_4$, $NiCl_2$ and $MgSO_4$, $CuSO_4$ metal ions affected the immobilized enzyme activity of *B. thermocatenulatus* BTL2 in different amounts. $BaCl_2$ and $CoSO_4$ metal ions decreased the activity by 19%, 13% at 0.1 mM concentration, respectively, $NiCl_2$ metal ion decreased the activity by 19% at 0.1 mM concentration and 15% at 1 mM concentration, and $MgSO_4$ metal ion decreased the activity by 25% at 1mM concentration compared to the control sample. It was determined that $CuCl_2$ and $ZnSO_4$ metal ions increased the activity by 63% and 32%, respectively at 1 mM concentration and $HgCl_2$ metal ion increased the activity by 18% at 0.5 mM concentration and 61% at 1mM concentration compared to the control sample. It was determined that $FeSO_4$, $MnCl_2$, $MgCl_2$ metal ions generally increased the immobilized enzyme activity compared to the control. When it comes to the *B. pumilus* MBB03 immobilized-enzyme, it was observed that $ZnSO_4$, $HgCl_2$, $FeSO_4$, $CuSO_4$, $CoSO_4$, $NiCl_2$, $MgCl_2$, $MnCl_2$, $MgSO_4$ metal ions generally decreased the enzyme activity in different amounts. Differently, it was determined that $HgCl_2$, $NiCl_2$ metal ions significantly reduced the immobilized-enzyme activity by 90% and 66% at 1 mM concentrations, respectively, compared to the control sample. However, it was observed that $CoSO_4$ and $FeSO_4$ metal ions effectively increased the immobilized-enzyme activity of *B. pumilus* MBB03 compared to the control (Table 2). In our study, it was determined that the sulfated and chloride compounds of some metal ions had different effects on the immobilized enzyme activity. It can be argued that the same metal ions affect the enzyme activity of different compounds in varying amounts, due to the different solubility of these compounds. Kambourova et al.

Table 2. Effects of various metallic ions on *B. thermocatenulatus* BTL2 and *B. pumilus* immobized-lipase stability.

| | Relative activity (100%) | | | | | |
|---|---|---|---|---|---|---|
| | *B. thermocatenulatus* BTL2 immobilized-lipase | | | *B. pumilus* MBB03 immobilized-lipase | | |
| Metallic ions | 0.1 mM | 0.5 mM | 1 mM | 0.1 mM | 0.5 mM | 1 mM |
| Control | 100 | 100 | 100 | 100±2.5 | 100±1.8 | 100±1.7 |
| $CaCl_2$ | 99±0.9 | 109±0.7 | 102±1.0 | 61±3.1 | 51±2.6 | 65±2.4 |
| NaCl | 105±0.7 | 104±2.4 | 100±0.8 | 102±4.1 | 73±2.7 | 67±1.02 |
| $CuCl_2$ | 97±1.4 | 94±2.7 | 163±2.4 | 89±1.6 | 69±1.4 | 81±0.9 |
| KCl | 100±1.2 | 108±3.1 | 106±2 | 75±3.2 | 71±3.5 | 62±4.3 |
| $BaCl_2$ | 81±1 | 104±4.4 | 100±2.7 | 69±0.9 | 59±0.5 | 75±1.2 |
| $ZnSO_4$ | 101±0.9 | 109±2.6 | 132±3,2 | 75±0.05 | 100±1.2 | 95±4.6 |
| $HgCl_2$ | 102±1.5 | 118±2.8 | 161±2.3 | 114±6.03 | 67±3.9 | 30±2.5 |
| $FeSO_4$ | 125±2.3 | 147±4.1 | 165±3.4 | 83±4.8 | 161±4.8 | 348±2.04 |
| $CuSO_4$ | 104±0.16 | 94±2.5 | 80±1.3 | 89±4.3 | 55±2.1 | 110±0.96 |
| $CoSO_4$ | 87±4.2 | 105±1.8 | 103±4.5 | 153±1.8 | 202±4.04 | 289±0.26 |
| $NiCl_2$ | 81±3.6 | 113±2.7 | 85±3.8 | 106±1.1 | 116±3.7 | 44±1.02 |
| $MgCl_2$ | 106±1.7 | 126±5.1 | 107±4.1 | 91±1.4 | 85±4.3 | 79±2.7 |
| $MnCl_2$ | 130±5.3 | 178±1.6 | 191±3.4 | 104±0.7 | 81±2.05 | 95±0.44 |
| $MgSO_4$ | 108±1.2 | 111±2.3 | 75±1.8 | 102±2.9 | 102±1.3 | 114±1.13 |

Values represent the means of three independent replicates, and ± standard deviations are reported.

(2003) reported that for the mechanism of action of metal ions regarding the inhibition by metals, either the catalytic site is directly inhibited, or the metal ions form a complex with the released fatty acids and change their solubility and their behavior at the interface, leading to this inhibition.

#### 3.3.4. Effects of organic solvents on immobilized-lipase activity

On the *B. thermocatenulatus* BTL2 immobilized-enzyme, it was determined that organic solvents effectively increased the enzyme activity in different amounts at all concentrations except glycerol at 1.0 mM concentration. It was determined that glycerol inhibited the enzyme activity by 24% at 1.0 mM concentration, compared to the control sample. It was determined that the organic solvents affected the enzyme activity of *B. pumilus* MBB03 immobilized in different amounts, and generally all organic solvents significantly increased the enzyme activity compared to the control, except isopropanol, n-butanol, acetone, DMSO, xylene and glycerol. It was observed that ethanol increased the enzyme activity by 87%, 55% and 22% at 0.1, 0.5 mM and 1.0 mM concentrations, respectively, compared to the control. It was determined that methanol increased enzyme activity by 35% at 0.1 mM concentration and by 15% at 0.5 mM concentration. It was determined that isopropanol increased enzyme activity by 57% at 0.1 mM concentration and by 45% at 0.5 mM concentration. It was observed that DMF enzyme activity increased by 57% at 0.1 mM concentration and by 55% at 0.5 mM concentration compared to the control. However, it was determined that isopropanol, n-butanol, aseton, DMSO, glycerol and xylene reduced enzyme activity by 23%, 38%, 25%,15% and 5% at 0.1 mM concentration, respectively (Table 3). The sensitivity of lipases to organic solvents varies (Rahman et al., 2005).

Table 3. Effects of some organic solvents and chemicals on *B. thermocatenulatus* BTL2 and *B. pumilus* immobilized-lipase stability.

| Some organic solvents/chemicals | Concentration (%) | Relative activity (100%) | |
|---|---|---|---|
| | | *B. thermocatenulatus* BTL2 immobilized-lipase | *B. pumilus* MBB03 immobilized-lipase |
| Control | – | 100 | 100 |
| Ethanol | 0.1 | 144±1.7 | 187±2.7 |
| | 0.5 | 138±2.5 | 155±1 |
| | 1.0 | 116±4.2 | 122±3.4 |
| Methanol | 0.1 | 143±3.1 | 135±3.4 |
| | 0.5 | 140±2 | 115±2.1 |
| | 1.0 | 136±1.8 | 102±2.7 |
| Isopropanol | 0.1 | 152±1.7 | 157±3 |
| | 0.5 | 130±1.6 | 145±2.4 |
| | 1.0 | 123±1.9 | 77±2.6 |
| n–Butanol | 0.1 | 149±4.7 | 135±1.4 |
| | 0.5 | 113±2.8 | 80±1.9 |
| | 1.0 | 113±4.3 | 62±3.5 |
| Acetone | 0.1 | 124±2.1 | 102±2.7 |
| | 0.5 | 124±2.2 | 85±1.6 |
| | 1.0 | 118±1.9 | 75±2.7 |
| DMSO | 0.1 | 156±1.2 | 142±3.4 |
| | 0.5 | 156±2.5 | 97±0.6 |
| | 1.0 | 143±3 | 85±1 |
| Xylene | 0.1 | 163±0.9 | 177±0.7 |
| | 0.5 | 161±1.8 | 122±4 |
| | 1.0 | 152±1.7 | 95±2.9 |
| DMF | 0.1 | 155±4.4 | 157±2.2 |
| | 0.5 | 130±3.5 | 155±2.5 |
| | 1.0 | 129±2.3 | 115±4.1 |
| Glycerol | 0.1 | 138±1.1 | 127±0.7 |
| | 0.5 | 133±3.6 | 90±3.3 |
| | 1.0 | 76±2.9 | 85±1.1 |

Values represent the means of three independent replicates, and ± standard deviations are reported.

3.3.5. Effects of detergents on immobilized-lipase activity

It was determined that some detergents and chemicals generally increased the immobilized-enzyme activity of *B. thermocatenulatus* BTL2 compared to the control sample. It was determined that some detergents and surfactants generally increased the immobilized-enzyme activity of *B. pumilus* MBB03. However, Tween 20 detergent reduced the enzyme activity by 55%, 75% and 78% at 0.1% (w/v), 0.5% (w/v) and 1.0% (w/v) concentrations, respectively, and sodium taurocholate detergent reduced the enzyme activity by 13% at 1.0% (w/v), compared to the control sample (Table 4). In line with this information, it can be said that these detergents do not affect the active site of the enzyme to a large extent, as the enzyme is immobilized in the gel structure of the immobilized-enzyme forms of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03. However, some sources state that lipases are inactive in the presence of detergents (dos Santos et al., 2014; Fernandez-Lorente et al., 2007). It is thought that these detergents denature the enzyme by interacting with the forces that protect the three-dimensional structure of the enzyme and therefore cannot preserve the stability of the enzyme. On the other hand, the residual activity is higher at higher concentrations of these detergents. It is thought that this situation may be related to the interfacial activation of lipase. High concentrations of these detergents may increase the interfacial area of the lipase, allowing it to meet with its substrate more easily. It is thought that this detergent changes its conformation at low concentration to decrease the stability of the enzyme, whereas at high concentration this conformational change is thought to increase the stability.

3.3.6. Effects of enzyme inhibitors on immobilized-lipase activity

It was determined that inhibitors and chemicals did not affect the immobilized-enzyme activity of *B. thermocatenulatus* BTL2. As for *B. pumilus* MBB03 immobilized-enzyme, it was determined that inhibitors and chemical substances generally reduced the enzyme activity in different amounts. As for *B. pumilus* MBB03 immobilized-enzyme, it was revealed that inhibitory substances, except for PMSF and $\beta$–mercaptoethanol, decreased the enzyme activity compared to the control sample. It was determined that PMSF increased the immobilized-enzyme activity by 10% at 0.1 mM concentration and reduced it by 33% at 1.0 mM concentration. Similarly, it was determined that $\beta$–mercaptoethanol increased the enzyme activity by 25% at 0.1 mM concentration and

Table 4. Effects of various surfactants and chemicals on *B. thermocatenulatus* BTL2 and *B. pumilus* lipase stability.

| Surfactants/chemicals | Concentration (%) | Relative activity (100%) | |
|---|---|---|---|
| | | *B. thermocatenulatus* BTL2 immobilized-lipase | *B. pumilus* MBB03 immobilized-lipase |
| Control | – | 100 | 100 |
| Triton X–100 | 0.1 | 164±4.6 | 210±3.3 |
| | 0.5 | 136±3.1 | 210±1.7 |
| | 1.0 | 130±2.7 | 150±2.4 |
| Tween 80 | 0.1 | 143±1.5 | 255±1.9 |
| | 0.5 | 148±2.3 | 115±3.5 |
| | 1.0 | 143±2.5 | 112±0.8 |
| Tween 20 | 0.1 | 147±5 | 45±1.2 |
| | 0.5 | 144±3.9 | 25±0.9 |
| | 1.0 | 121±2.2 | 22±2.2 |
| CHAPS | 0.1 | 135±0.5 | 227±3.6 |
| | 0.5 | 133±1.6 | 187±1.4 |
| | 1.0 | 130±2.3 | 160±4.1 |
| Sodyum deoksikolat | 0.1 | 128±1.7 | 127±2.5 |
| | 0.5 | 123±3.8 | 120±3.1 |
| | 1.0 | 123±2.4 | 115±2.7 |
| Sodyum taurokolat | 0.1 | 125±4.7 | 110±1.4 |
| | 0.5 | 118±3.3 | 90±0.9 |
| | 1.0 | 111±2.6 | 87±1.3 |
| SDS | 0.1 | 183±4.1 | 160±2.9 |
| | 0.5 | 136±2.3 | 147±3.5 |
| | 1.0 | 135±1.02 | 135±2.4 |

Values represent the means of three independent replicates, and ± standard deviations are reported.

Table 5. Effects of various inhibitors and chemicals on *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-lipase stability.

| Surfactants/chemicals | Concentration (%) | Relative activity (100%) | |
|---|---|---|---|
| | | *B. thermocatenulatus* BTL2 immobilized-lipase | *B. pumilus* MBB03 immobilized-lipase |
| Control | – | 100 | 100 |
| PMFS | 0.1 | 123±4.1 | 110±2.3 |
| | 0.5 | 116±2.8 | 92±1.8 |
| | 1.0 | 106±1.02 | 67±1.1 |
| $\beta$–merkaptoetanol | 0.1 | 240±3 | 125±4.5 |
| | 0.5 | 200±4.4 | 90±2.9 |
| | 1.0 | 140±1.7 | 80±2.5 |
| DTT | 0.1 | 310±1.5 | 92±3.3 |
| | 0.5 | 209±2.2 | 92±2.4 |
| | 1.0 | 140±4.3 | 80±1.5 |
| EDTA | 0.1 | 120±2.7 | 93±2.2 |
| | 0.5 | 113±1.6 | 77±0.89 |
| | 1.0 | 110±0.9 | 67±1.9 |

Values represent the means of three independent replicates, and ± standard deviations are reported.

decreased it by 20% at 1.0 mM concentration. It was determined that DTT decreased enzyme activity at different concentrations, and EDTA reduced enzyme activity by 33% at 1.0 mM concentration (Table 5). In line with these data, it was observed that the inhibitory substances affected the immobilized enzyme activity in different amounts. Lipases with the Ser–His–Asp/Glu catalytic triad are in the serine hydrolase class. Phenylmethylsulfonyl fluoride (PMSF), a serine inhibitor, binds to the serine amino acid in the active site of lipases and irreversibly inhibits the activity (Dharmsthiti and Luchai, 1998). Therefore, due to PMSF, similar to other lipases, *B. pumilus* MBB03 immobilized-enzyme activity decreased by 33% at 1.0 mM concentration. When *B. pumilus* MBB03 immobilized-lipase enzyme was treated with DTT at 0.1 mM concentration, which had the ability to inhibit disulfide bonds in proteins, it was determined that the immobilized enzyme lost 20% (Table 5). It can be concluded that the immobilized-enzyme was less affected by DTT due to its gel structure.

### 3.3.7. Repeated use capability

When biocatalysts are used in free enzyme form, their reuse is not possible. For this reason, immobilization forms are designed for industrially important enzymes and production studies are planned with these forms. Compared to the free enzyme, the immobilized enzyme can be used many times and for a long time. This property of immobilized enzyme is very important in industrial applications. Because, depending on continuous use, the production cost decreases. A change in enzyme activity was observed with continued use (Bayramoglu et al., 2002). In the reusability study of the lipase immobilized on Ca–Alg@gelatin hydrogel beads, it was observed that *B. thermocatenulatus* BTL2 the immobilized-lipase activity was completely preserved in the 1st cycle, 96% in the 2nd cycle, and approximately 80% in the 5th cycle, while the activity decreased in the following cycles. At the end of the 15th cycle, it was determined that approximately 13% of activity was preserved. *B. pumilus* MBB03 lipase immobilized on Ca–Alg@gelatin hydrogel beads prevented its activity completely in the 1st cycle, 92% in the 2nd cycle, about 80% in the 3rd cycle, about 70% until the 6th cycle, and the activity decreased in the following cycles. At the end of the 15th cycle, it was determined that approximately 20% of the activity could be preserved. This study showed that *B. thermocatenulatus* BTL2 immobilized-lipase can be used for up to five cycles with little loss of activity, and *B. pumilus* MBB03 immobilized-lipase can be used up to three cycles with little loss of activity (80% of their initial activity was preserved) (Fig. 4a). However, after these cycles, the loss of activity became apparent.

The reasons for these results may be due to possible enzyme leakage from hydrogel beads or accumulation of substrate or reaction products within the beads. Another possible explanation may be damage to the hydrogel beads during repeated use, observed as a reduction in bead diameter (Knezevic et al., 2002). Similar to our study, Won et al. (2005) observed that the alginate beads preserved 72% of their activity in the 3rd use in the study of lipase enzyme immobilization, which they immobilized on Ca–alginate gel beads. When *Candida rugosa* lipase is immobilized on a hydrophobic polymer support by adsorption, it has been reported that 90% of the

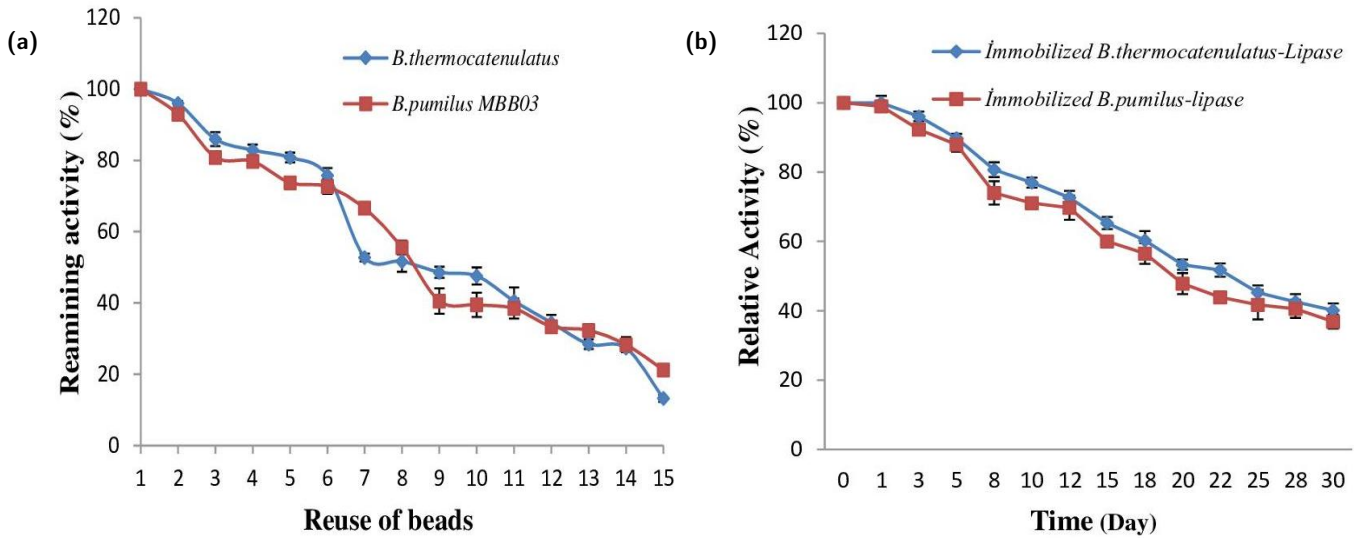


Figure 4. Reusability of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-lipases (a). Storage stability of *B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-lipases (b).

enzyme activity is preserved after 15 uses (Ruchenstein and Wang, 1993). When *Candida rugosa* lipase is immobilized on chitosan, it has been reported to show 74% activity after 10 uses (Hung et al., 2003). Chiou and Wu (2004), in the study in which they performed the immobilization of the Candida rugosa enzyme on chitosan through the activation of hydroxyl groups, observed that all of the immobilized enzyme activity at the end of the 6th use and 54% of the activity at the end of 30 days was preserved.

### 3.3.8. Storage stability of immobilized-lipase

*B. thermocatenulatus* BTL2 and *B. pumilus* MBB03 immobilized-lipases preserved almost all of their activities at the end of the 1st day, 80% and 73% of the activities, respectively, on the 8th day. However, there was a decrease in the activities on the following days, and the activities decreased approximately by 40 and 36%, respectively, at the end of the 30th day (Fig. 4b). The stability of lipase immobilized with alginate+gelatin was much lower than that obtained for lipase immobilized on heterogeneous titanium dioxide ($TiO_2$) (Zhou et al., 2021). The reasons for these results may be enzyme leakage due to damage to the hydrogel beads, which is observed as a decrease in bead diameter over time.

## 4. CONCLUSIONS

This study demonstrated that the activity profiles of lipase could be improved by immobilization on Ca–Alg@gelatin hydrogel beads. After optimization of immobilization parameters, the entrapment technique gave a higher immobilization efficiency and a higher thermal stability at basic pH's and high temperatures. The fact that most of the *B. thermocatenulatus* BTL2 lipase activity is preserved over a wide temperature range indicates that this enzyme can be easily used at any temperature from 40 to 100 °C and has great advantages in terms of various industrial applications. Considering the optimum pH and temperature properties of the obtained immobilized enzyme forms, these enzymes can be used in organic synthesis studies and as detergent additives because their temperatures are above 40 °C, they have alkaline pH and room temperature thermal stability properties. The storage stability and reuse advantages of the entrapped enzyme increased its use potential. Therefore, this work presented entrapment technique as an immobilization technique which may contribute to increasing its repeated use capability and storage stability, which might be of significant industrial interest.

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## REFERENCES

Arıca M.Y., Bayramoğlu G., 2004. Reversible immobilization of tyrosinase onto polyethyleneimine-grafted and Cu(II) chelated poly(HEMA–co–GMA) reactive membranes. *J. Mol. Catal. B: Enzym.*, 27, 255–265. DOI: 10.1016/J.MOLCATB.2003.12.006.

Bayramoglu G., Kaçar Y., Denizli A., Arıca M.Y., 2002. Covalent immobilization of lipase onto hydrophobic group incorporated poly(2–hydroxyethyl methacrylate) based hydrophilic membrane matrix. *J. Food Eng.*, 52, 367–374. DOI: 10.1016/S0260-8774(01)00128-5.

Bickerstaff G.F., 1995. Impact of genetic technology on enzyme technology. *Genet. Eng. Biotechnol.*, 15, 13–30.

Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-252. DOI: 10.1006/abio.1976.9999.

Chaplin M.F., Bucke C., 1990. *Enzyme technology*. Cambridge University Press, Cambridge.

Cheetham P.S.J., Blunt K.W., Bocke C., 1979. Physical studies on cell immobilization using calcium alginate gels. *Biotechnol. Bioeng.*, 21, 2155–2168. DOI: 10.1002/bit.260211202.

Cheirsilp B., Jeamjounkhaw P., H-Kittikun A., 2009. Optimizing an alginate immobilized lipase for monoacylglycerol production by the glycerolysis reaction. *J. Mol. Catal. B: Enzym.*, 59, 206–211. DOI: 10.1016/j.molcatb.2009.03.001.

Chiou S.H., Wu W.T., 2004. Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. *Biomaterials*, 25, 197–204. DOI: 10.1016/S0142-9612(03)00482-4.

Clark D.S., 1994. Can immobilisation be exploited to modify enzyme activity? *Trends Biotechnol.*, 12, 439–443. DOI: 10.1016/0167-7799(94)90018-3.

Dey G., Singh B., Banerjee R., 2003. Immobilization of $\alpha$–amylase produced Bacillus circulans GRS 313. *Braz. Arch. Biol. Technol.*, 46, 167–176. DOI: 10.1590/S1516-89132003000200005.

Dharmsthiti S., Luchai S., 1998. Production, purification and characterization of thermophilic lipase from *Bacillus* sp. THL027. *FEMS Microbiol. Lett.*, 179, 241–246. DOI: 10.1111/j.1574-6968.1999.tb08734.x.

Dong Z., Wang Q., Du Y., 2006. Alginate/gelatin blend films and their properties for drug controlled release. *J. Membr. Sci.*, 280, 37–44. DOI: 10.1016/j.memsci.2006.01.002.

dos Santos J.C.S., Garcia-Galan C., Rodrigues R.C., de Sant' Ana H.B., Gonçalves L.R.B., Fernandez-Lafuente R., 2014. Stabilizing hyperactivated lecitase structures through physical treatment with ionic polymers. *Process Biochem.*, 49, 1511–1515. DOI: 10.1016/j.procbio.2014.05.009.

Edelman P.G., Wang J., 1992. Biosensors and chemical sensors: Optimizing performance through polymeric materials. American Chemical Society, Washington, DC, 22–42.

Emregul E., Sungur S., Akbulut U., 2006. Polyacrylamide–gelatin carrier system used for invertase immobilization. *Food Chem.* 97, 591–597. DOI: 10.1016/j.foodchem.2005.05.017.

Fadnavis N.W., Sheelu G., Kumar B.M., Bhalerao M.U., Deshpande A.A., 2003. Gelation blends with alginate: gels for lipase immobilization and purification. *Biotechnol. Prog.*, 19, 557–564. DOI: 10.1021/bp010172f.

Fernandez-Lorente G., Palomo J.M., Cabrera Z., Fernandez-Lafuente R., Guisán J.M., 2007. Improved catalytic properties of immobilized lipases by the presence of very low concentrations of detergents in the reaction medium. *Biotechnol. Bioeng.*, 97, 242–250. DOI: 10.1002/bit.21230.

Fojan P., Jonson P.H., Petersen M.T.N., Petersen S.B., 2000. What distinguishes an esterase from a lipase: A novel structural approach. *Biochimie*, 82, 1033–1041. DOI: 10.1016/S0300-9084(00)01188-3.

Fraser J.E., Bickerstaff G.F., 1997. Entrapment in calcium alginate, In: Bickerstaff G.F. (Ed.), *Immobilization of enzymes and cells. Methods in biotechnology*, 61–66. Humana Press, New Jersey, 61-65. DOI: 10.1385/0-89603-386-4:61.

Guisan J.M., 2006. Immobilization of enzymes as the 21st century begins, In: Guisan J.M. (Ed.) *Immobilization of enzymes and cells. Methods in Biotechnology*™, vol 22. Humana Press. DOI: 10.1007/978-1-59745-053-9_1.

Hung T.C., Giridhar R., Chiou S.H., Wu W.T., 2003. Binary immobilization of *Candida rugosa* lipase on chitosan. *J. Mol. Catal. B: Enzym.*, 26, 69–78. DOI: 10.1016/S1381-1177(03)00167-X.

Ismail A.R., Baek K.H., 2020. Lipase immobilization with support materials, preparation techniques, and applications: Present and future aspects. *Int. J. Biol. Macromol.*, 163, 1624–1639. DOI: 10.1016/j.ijbiomac.2020.09.021.

Kambourova M., Kirilova N., Mandeva R., Derekova A., 2003. Purification and properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC 7. *J. Mol. Catal. B: Enzym.*, 22, 307–313. DOI: 10.1016/S1381-1177(03)00045-6.

Katchalski-Katzir E., 1993. Immobilized enzymes – learning from past successes and failures. *Trends. Biotechnol.*, 11, 471-478. DOI: 10.1016/0167-7799(93)90080-S.

Khan N., Maseet M., Basir S.F., 2020. Synthesis and characterization of biodiesel from waste cooking oil by lipase immobilized on genipin cross-linked chitosan beads: A green approach. *Int. J. Green Energy*, 17, 84–93. DOI: 10.1080/15435075.2019.1700122.

Kim H.K., Choi H.J., Kim M.H., Sohn C.B., Oh T.K., 2002. Expression and characterization of $Ca^{2+}$ – independent lipase from *Bacillus pumilus* B26. *Biochim. Biophys. Acta, Mol. Cell. Biol. Lipids*, 1583, 205–212. DOI: 10.1016/s1388-1981(02)00214-7.

Knezevic Z., Bobic S., Milutinovi A., Obradovic B., Mojovic L., Bugarski B., 2002. Alginate-immobilized lipase by electrostatic extrusion for the purpose of palm oil hydrolysis in lecithin/isooctane system. *Process Biochem.*, 38, 313–318. DOI: 10.1016/S0032-9592(02)00085-7.

Litantra R., Lobionda S., Yim J.H., Kim H.K., 2013. Expression and biochemical characterization of cold-adapted lipases from antarctic *Bacillus pumilus* strains. *J. Microbiol. Biotechnol.*, 23, 1221–1228. DOI: 10.4014/jmb.1305.05006.

López A., Lázaro N., Marqués A.M., 1997. The interphase technique: a simple method of cell immobilization in gel-beads. *J. Microbiol. Methods*, 30, 231–234. DOI: 10.1016/S0167-7012(97)00071-7.

Mammarella E.J., Rubiola C.A., 2005. Study of the deactivation of $\beta$-galoctosidase entrapped in alginate-carrageenan gels. *J. Mol. Catal. B: Enzym.*, 34, 7–13. DOI: 10.1016/j.molcatb.2005.04.007.

Melani N.B., Tambourgi E.B., Silveira E., 2020. Lipases: from production to applications. *Sep. Purif. Rev.*, 49, 143–158. DOI: 10.1080/15422119.2018.1564328.

Mondal K., Mehta P., Mehta B.R., Varandani D., Gupta M.N., 2006. A bioconjugate of *Pseudomonas cepacia* lipase with alginate with enhanced catalytic efficiency. *Biochim. Biophys. Acta, Proteins Proteomics*, 1764, 1080–1086. DOI: 10.1016/j.bbapap.2006.04.008.

Norouzian D., Javadpour S., Moazami N., Akbarzadeh A., 2002. Immobilization of whole cell penicilin G acylase in open pore gelatin matrix. *Enzyme Microb. Technol.*, 30, 26–29. DOI: 10.1016/S0141-0229(01)00445-8.

Omar I.C., Saeki H., Nishio N., Nagai S., 1988. Hydrolysis of triglycerides by immobilized thermostable lipase from *Humicola Lanuginosa*. *Agric. Biol. Chem.*, 52, 99–105. DOI: 10.1080/00021369.1988.10868641.

Ondul E., Dizge N., Albayrak N., 2012. Immobilization of *Candida antarctica* A and *Thermomyces lanuginosus* lipases on cotton terry cloth fibrils using polyethyleneimine. *Colloids. Surf., B*, 95, 109–114. DOI: 10.1016/j.colsurfb.2012.02.020.

Ozyilmaz G., Gezer E., 2010. Production of aroma esters by immobilized *Candida rugosa* and porcine pancreatic lipase into calcium alginate gel. *J. Mol. Catal. B: Enzym.*, 64, 140–145. DOI: 10.1016/j.molcatb.2009.04.013.

Quyen D.T., Schmidt-Dannert C., Schmid R.D., 2003. High-level expression of a lipase from *Bacillus thermocatenulatus* BTL2 in Pichia pastoris and some properties of the recombinant lipase. *Protein Expression Purif.*, 28, 102–110. DOI: 10.1016/s1046-5928(02)00679-4.

Rahman R.N.Z.R.A., Baharum S.N., Basri M., Salleh A.B., 2005. High-yield purification of an organic solvent-tolerant lipase from *Pseudomonas* sp. strain S5. *Anal. Biochem.*, 341, 267–274. DOI: 10.1016/j.ab.2005.03.006.

Rakmai J., Cheirsilp B., Prasertsan P., 2015. Enhanced thermal stability of cyclodextrin glycosyltransferase in alginate-gelatin mixed gel beads and the application for $\beta$–cyclodextrin production. *Biocatal. Agric. Biotechnol.*, 4, 717–726. DOI: 10.1016/j.bcab.2015.10.002.

Ruchenstein E., Wang X., 1993. Lipase immobilized on hydrophobic porous polymer supports prepared by concentrated emulsion polymerization and their activity in the hydrolysis of triacyglycerides. *Biotechnol. Bioeng.* 42, 821–828. DOI: 10.1002/bit.260420706.

Scardi V., 1987. [25] Immobilization of enzymes and microbial cells in gelatin. *Methods in Enzymology*, 135, 293–299. DOI: 10.1016/0076-6879(87)35086-4.

Sharifi M., Sohrabi M.J., Hosseinali S.H., Hasan A., Kani P.H., Talaei A.J., Karim A.Y., Nanakali N.M.Q., Salihi A., Aziz F.M., Yan B., Khan R.H., Saboury A.A., Falahati M., 2020. Enzyme immobilization onto the nanomaterials: application in enzyme stability and prodrug-activated cancer therapy. *Int. J. Biol. Macromol.*, 143, 665–676. DOI: 10.1016/j.ijbiomac.2019.12.064.

Shaw J.F., Chang R.C., Wang F.F., Wang Y.J., 1990. Lipolytic activities of a lipase immobilized on six selected supporting materials. *Biotechnol. Bioeng.* 35, 132–137. DOI: 10.1002/bit.260350204.

Talekar S., Chavare S., 2012. Optimization of immobilization of $\alpha$-amylase in alginate gel and its comparative biochemical studies with free $\alpha$-amylase. *Recent Res. Sci. Technol.*, 4(2), 1–5.

Tischer W., Wedekind F., 1999. Immobilized enzymes: methods and applications, In: *Biocatalysis – From Discovery to Application. Topics in Current Chemistry*. Vol. 200. Springer, Berlin, Heidelberg, 95–126. DOI: 10.1007/3-540-68116-7_4.

Trabelsi I., Ayadi D., Bejar W., Bejar S., Chouayekh H., Salah R.B., 2014. Effects of Lactobacillus plantarum immobilization in alginate coated with chitosan and gelatin on antibacterial activity. *Int. J. Biol. Macromol.*, 64, 84–89. DOI: 10.1016/j.ijbiomac.2013.11.031.

Vaidya B.K., Ingavle G.C., Ponrathnam S., Kulkarni B.D., Nene S.N., 2008. Immobilization of *Candida rugosa* lipase on poly (allyl glycidyl ether–co–ethylene glycol dimethacrylate) macroporous polymer particles. *Bioresour. Technol.*, 99, 3623–3629. DOI: 10.1016/j.biortech.2007.07.035.

Vujčić Z., Miloradović Z., Milovanović A., Božić N., 2011. Cell wall invertase immobilisation within gelatin gel. *Food Chem.*, 126, 236–240. DOI: 10.1016/j.foodchem.2010.11.010.

Winkler U.K., Stuckman M., 1979. Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by Serratia marcescens. *J. Bacteriol.*, 138, 663–670. DOI: 10.1128/jb.138.3.663-670.1979.

Won K., Kim S., Kim K.J., Park H.W., Moon S.J., 2005. Optimization of lipase entrapment in Ca–alginate gel beads. *Process Biochem.*, 40, 2149–2154. DOI: 10.1016/j.procbio.2004.08.014.

Zhou W., Zhou X., Zhuang W., Lin R., Zhao Y., Ge L., Li M., Wu J., Yang P., Zhang H., Zhu C., Ying H., 2021. Toward controlled geometric structure and surface property heterogeneities of $TiO_2$ for lipase immobilization. *Process Biochem.*, 110, 118–128. DOI: 10.1016/j.procbio.2021.08.004.