Biochemical Study of Bacillus stearothermophilus Immobilized Lipase for Oily Wastewater Treatment

Authors:

Abir Ben Bacha, Mona Alonazi, Humidah Alanazi, Mona G. Alharbi, Raida Jallouli, Aida Karray

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Keywords: immobilized lipase, CaCO3, wastewater treatment

Abstract:

Traditional wastewater treatments involve expensive mechanical and physiochemical methods, so researchers have been developing cost-effective, sustainable technologies that use enzymes to produce higher quality effluents and recover more energy and nutrients from wastewater. A thermostable, alkaline, and solvent-tolerant lipase was partially purified from thermophilic Bacillus stearothermophilus. The lipase displayed maximum activity at 50 °C and pH 11.0 and catalyzed both short- and long-chain triacylglycerols at similar rates. B. stearothermophilus lipase also exhibited high stability when incubated at 40 °C for 1 h with anionic and non-ionic surfactants. Studies show that thermostable enzymes can be improved through immobilization and modification of other reaction conditions. Therefore, B. stearothermophilus lipase was immobilized through adsorption on CaCO3, Celite 545, and silica gel with the CaCO3 support producing the best adsorption rate (89.33%). The optimal initial lipase activity was approximately 4500 U.g?1 after 60 min. Interestingly, 93% of the initial lipase activity was retained after six cycles, and almost 50% of the initial activity remained after 12 cycles. Furthermore, immobilization improved storage stability with 98.85% of the initial lipase activity retained after 60 days of storage at 4 °C. The biochemical characteristics of immobilized lipase shifted toward a slightly alkaline region, reaching maximum activity at pH 12. The optimal temperature of immobilized lipase was 60 °C. Immobilization also improved enzymatic stability by widening the pH range from 5?9 (for free lipase) to 4?11, and thermostability by reaching 65 °C. The application of immobilized lipase in wastewater treatment was observed through oil layer biodegradation. Notably, treating wastewater for 10 days with immobilized lipase almost removed the chemical oxygen demand (COD) from 1950.1 down to 4.04 mg.L?1. Similarly, lipid content was almost removed from 15,500 ± 546 mg.L?1 down to 12 mg.L?1. All results highlight the potential value of CaCO3-immobilized lipase as an effective biocatalyst for hydrolyzing wastewater.

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Biochemical Study of *Bacillus stearothermophilus* Immobilized Lipase for Oily Wastewater Treatment

Abir Ben Bacha [1,*], Mona Alonazi [1], Humidah Alanazi [1], Mona G. Alharbi [1], Raida Jallouli [2] and Aida Karray [3]

[1] Biochemistry Department, Science College, King Saud University, P.O. Box 22452, Riyadh 11495, Saudi Arabia
[2] Institut de Pharmacologie de Sherbrooke, Université de Sherbrooke, Sherbrooke, QC J1H 5N4, Canada
[3] Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS Route de Soukra, Université de Sfax-Tunisia, Sfax 3038, Tunisia
* Correspondence: aalghanouchi@ksu.edu.sa or abirghanouchibenbacha@yahoo.fr; Tel.: +96611-50129; Fax: +96611-4769137

Abstract: Traditional wastewater treatments involve expensive mechanical and physiochemical methods, so researchers have been developing cost-effective, sustainable technologies that use enzymes to produce higher quality effluents and recover more energy and nutrients from wastewater. A thermostable, alkaline, and solvent-tolerant lipase was partially purified from thermophilic *Bacillus stearothermophilus*. The lipase displayed maximum activity at 50 °C and pH 11.0 and catalyzed both short- and long-chain triacylglycerols at similar rates. *B. stearothermophilus* lipase also exhibited high stability when incubated at 40 °C for 1 h with anionic and non-ionic surfactants. Studies show that thermostable enzymes can be improved through immobilization and modification of other reaction conditions. Therefore, *B. stearothermophilus* lipase was immobilized through adsorption on $CaCO_3$, Celite 545, and silica gel with the $CaCO_3$ support producing the best adsorption rate (89.33%). The optimal initial lipase activity was approximately 4500 $U.g^{-1}$ after 60 min. Interestingly, 93% of the initial lipase activity was retained after six cycles, and almost 50% of the initial activity remained after 12 cycles. Furthermore, immobilization improved storage stability with 98.85% of the initial lipase activity retained after 60 days of storage at 4 °C. The biochemical characteristics of immobilized lipase shifted toward a slightly alkaline region, reaching maximum activity at pH 12. The optimal temperature of immobilized lipase was 60 °C. Immobilization also improved enzymatic stability by widening the pH range from 5–9 (for free lipase) to 4–11, and thermostability by reaching 65 °C. The application of immobilized lipase in wastewater treatment was observed through oil layer biodegradation. Notably, treating wastewater for 10 days with immobilized lipase almost removed the chemical oxygen demand (COD) from 1950.1 down to 4.04 $mg.L^{-1}$. Similarly, lipid content was almost removed from 15,500 ± 546 $mg.L^{-1}$ down to 12 $mg.L^{-1}$. All results highlight the potential value of $CaCO_3$-immobilized lipase as an effective biocatalyst for hydrolyzing wastewater.

Keywords: immobilized lipase; $CaCO_3$; wastewater treatment

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1. Introduction

Lipases, belonging to the family of carboxylic ester hydrolases in plants, animals, and microbes, hydrolyze water-soluble lipid substrates at the lipid–water interface into diglycerides, monoglycerides, fatty acids, and glycerol [1]. The actions of lipases, particularly microbial lipases, offer new possibilities for industrial processes. For example, enzymatic catalysis decreases the release of harmful products into the environment while also reducing the cost of the process [2]. Enzymes offer many advantages due to their specificity, biodegradability, and limited by-product formation. However, enzymes must be compatible and stable, as enzyme stability often limits practical applications in medical and biotechnological processes. One possible approach for stabilizing enzymes is enzyme

immobilization on a suitable matrix. From an industrial point of view, studies suggest that immobilized biocatalysts exhibit increased stability, enhanced enzyme activity, a wider pH range, and a higher affinity for substrates [3,4].

The impact of immobilization on enzyme activity depends on the enzyme source, support type, and immobilization method. Among the various immobilization techniques, adsorption remains the simplest and most cost-effective method. Different supports, such as silica, porous glass beads, alumina, diatomaceous earth, celite, and activated carbon, have already been exploited for enzyme immobilization. Adsorption reactions involve different types of bonds, such as ion exchange, Van Der Waals interactions, and hydrogen bonding [5], which are influenced by enzyme concentration, contact time, pH, and concentration of the support. Calcium carbonate ($CaCO_3$), a chemically nonreactive and nontoxic support, is a commonly used adsorbent that promotes dispersion of crude *Candida rugosa* and *Rhizopus oryzae* lipases and retention of enzymatic activity [6,7].

Industrial manufacturing processes generate several types of waste. For example, the cooling and heating industries, municipal wastewater treatment, food processing, and the production of metals, petroleum, and textiles generate liquid waste that is very rich in salts and fat. Traditional mechanical and physicochemical waste treatments are expensive and require energy; however, enzymatic hydrolysis provides a possible solution for reducing waste lipid levels down to the standards required by Tunisian standard NT 10602 (i.e., lipid levels in waste discharged into sanitation should not exceed 0.03 g/L). Since enzymes are highly selective, efficient, and specific catalysts [8], bioremediation, or enzymatic treatment, has become the most widely used method to restore polluted environments [9–12]. Although there are several reports on *Bacillus* lipases, research involving immobilization of new preferment lipases for biological treatments is still emerging.

It is now widely accepted that sustainable development involves balancing social and economic concerns with environmental concerns. This approach aims to reduce production costs while preserving the environment and promoting human health. Current effluent treatment practices reduce the polluting load of waste but involve expensive physicochemical techniques. Therefore, researchers have been developing new, sustainable wastewater treatment technologies that produce high-quality effluents, lower energy expenditure, and recover energy and nutrients.

In this study, hydrolytic enzyme activity on several oils and the biodegradation capacity of oily Wadi Hanifah water were investigated. The immobilized lipase from *Bacillus stearothermophilus (B. stearothermophilus)* isolated from olive oil mill sols was compared with the free-form lipase (previously purified in our laboratory); both were tested for oily wastewater treatment.

2. Materials and Methods

2.1. Production and Immobilization of B. stearothermophilus Lipase

B. stearothermophilus lipase was isolated from olive oil mill soil samples, as previously described [13]. After 48 h of incubation, the crude enzyme solution was precipitated at 65% saturation of ammonium sulfate. The precipitate was resuspended in 5 mL of a solution containing 25 mM Tris–HCl and 2 mM benzamidine, and treated at 70 °C for 15 min. Finally, the lipase solution was collected by centrifugation (30 min, 12,000 rpm) and stored at 4 °C. Celite 545, $CaCO_3$, and silica gel were tested as supports for enzyme immobilization, as previously described [14].

Adsorbed enzymes are generally resistant to proteolysis and aggregation due to hydrophobic bonds with support surfaces. Enzyme immobilization by adsorption is a simple and inexpensive method compatible with supports derived from polysaccharides, glass, and synthetic polymers. Adsorption is also the most widely used method for industrial processes [5]. Immobilization of *B. stearothermophilus* lipase was tested on $CaCO_3$, Celite 545, and silica gel supports. 4500 U of lipase solution was mixed with 1 g of the support and then incubated for 2 h at 4 °C with intermittent stirring. Lipase adsorption kinetics were explored for each support at 10-min intervals from 0 to 120 min. Sampling was

followed by centrifugation for 5 min at 8000 rpm, with the supernatant retained for enzyme activity. The enzyme solution was filtered, washed three times with double-distilled water, and finally dried in a vacuum desiccator for 8 h at room temperature. Adsorbed lipase activity divided by the total soluble lipase activity originally added to 1 g of the support was expressed as a percentage, as used to define the yield of immobilized lipase activity. Triplicate samples were investigated.

2.2. Effects of pH and Temperature on Lipase Activity and Stability

First, lipolytic activity was measured at 60 °C at pH values ranging from 8 to 13. The pH stability of immobilized lipase was investigated by incubating the lipase preparation for 48 h at room temperature and using appropriate buffers to test the pH from 2–13. Residual lipase activity was measured after centrifugation using the standard assay method. The measurements were performed in triplicate. Second, the activities of the immobilized and free enzymes were measured from 25 to 75 °C at pH 12. Finally, thermostability was explored at temperatures ranging from 30 to 90 °C at pH 12 after a 1 h incubation. Residual activity was measured after centrifugation of each preparation.

2.3. Influence of Various Metal Ions and Compounds

To explore the influence of metal ions and other compounds, 5 mM β-mercaptoethanol, 2 mM phenylmethane sulfonyl fluoride (PMSF), 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium dodecyl sulfate (SDS), and 2 mM metal ions, including Mn^{2+}, Ba^{2}+, Cu^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+}, were added to the enzyme solution before lipase activity was measured using the standard assay method (as described above).

2.4. Effect of Substrate Type on Free and $CaCO_3$-Immobilized Lipase Activity

Hydrolytic activities of free and immobilized lipases were measured with a pH-stat under standard conditions using an olive oil emulsion as a substrate [15]. To determine the hydrolysis efficiency of $CaCO_3$-immobilized and free lipases, the hydrolysis of four commercial oils (i.e., sunflower, palm, coconut, and corn oils) was compared under the same conditions.

2.5. Characterization of Wadi Hanifah Water

Water from Wadi Hanifah, located in Al-Riyadh City, Kingdom of Saudi Arabia, was characterized by measuring total organic carbon (TOC), chemical oxygen demand (COD), and lipid content before and after treatment with free and immobilized *B. stearothermophilus* lipase. Water samples (1 L) were enzymatically treated by adding 4500 U of free or immobilized lipase. The resulting sample was stirred at 200 rpm at room temperature. Samples were collected at 24 h intervals over 10 days for COD and TOC measurements, according to American Public Health Association protocols [16]. A negative control was also included using distilled water rather than Wadi Hanifah water. Each measurement was performed in triplicate.

2.6. Statistical Analysis

The IBM statistical package for social sciences, version 19.0 (IBM Corp., Armonk, NY, USA), was used to perform the statistical analyses. All experiments were conducted at least three times, with the results expressed as mean ± standard deviation (SD). Student's *t*-tests were used to determine the significance of observed differences in lipase activities, as well as biological treatment of Wadi Hanifah water by free and immobilized *B. stearothermophilus* lipase. Results were considered statistically significant when p values were less than or equal to 0.05.

3. Results

3.1. Immobilization of B. stearothermophilus Lipase

3.1.1. Adsorption on Different Supports

The estimated lipase adsorption on different supports is summarized in Table 1. The $CaCO_3$ support adsorbed significantly more lipase (89.33% ± 2.51) than the silica gel and Celite 545 carriers (32% and 38%, respectively) (Table 1).

Table 1. Adsorption of *B. stearothermophilus* lipase on different supports.

	Yield of Immobilized Lipase Activity (%)
$CaCO_3$	89.33 ± 2.51
Silica gel	32.33 ± 2.08
Celite 545	38.33 ± 3.05

The enzyme solution (4500 U) was adsorbed to 1 g of each support for 30 min at 4 °C. The enzymatic activities of free and immobilized lipases were measured at pH 12 and 60 °C using an olive oil emulsion as a substrate. Data are expressed as the mean ± standard deviation of triplicate measurements.

3.1.2. Optimal Immobilization Conditions

To determine the optimal conditions for immobilizing *B. stearothermophilus* lipase in aqueous solutions, different lipase amounts ranging from 1000 to 7000 $U.g^{-1}$ support were investigated (Figure 1). Immobilization yields increased to a maximum value of 4500 $U.g^{-1}$ as more lipase was loaded onto the $CaCO_3$ support. It is hypothesized that enzyme amounts beyond 4500 $U.g^{-1}$ were not retained by the matrix and removed during the washing step. The optimized enzyme concentration (i.e., 4500 $U.g^{-1}$) was applied throughout the remaining experiments.

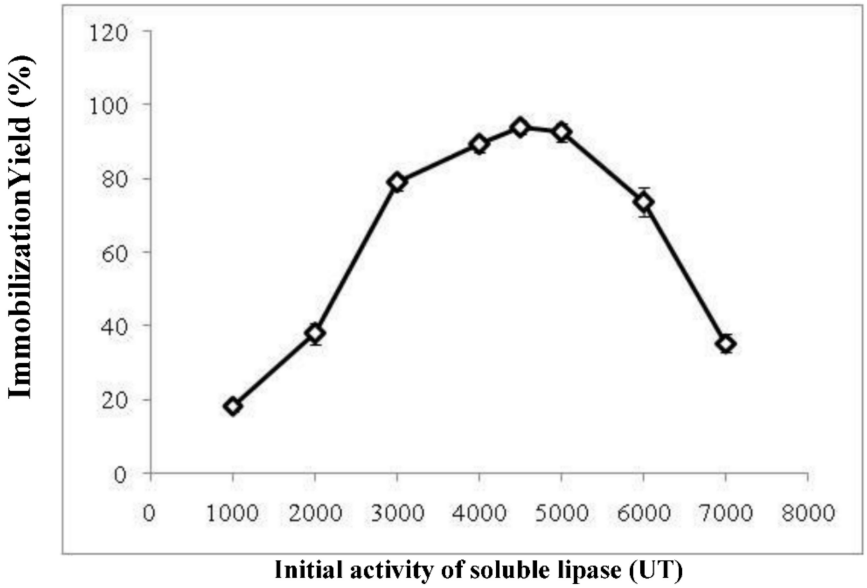

Figure 1. Yields of immobilized *B. stearothermophilus* lipase determined using different initial lipase amounts. Enzyme activity was measured using an olive oil emulsion at pH 12 and 60 °C. Data are expressed as the mean ± standard deviation of triplicate measurements.

3.1.3. Adsorption Kinetics of Immobilized *B. stearothermophilus* Lipase and Its Retention Capacity on $CaCO_3$

The kinetics of protein and lipase adsorption onto $CaCO_3$ showed that the amounts of protein and lipase loaded onto the support were below saturation (Figure 2A). For both

protein and lipase adsorption, maximum binding was observed after nearly 60 min. The hydrophobic nature of the solid substrate suggests that the adsorption of proteins is governed by hydrophobic interactions. Similar results were observed with the immobilization of *R. oryzae* and *Staphylococcus aureus* ALA1 lipases to $CaCO_3$ [6,12].

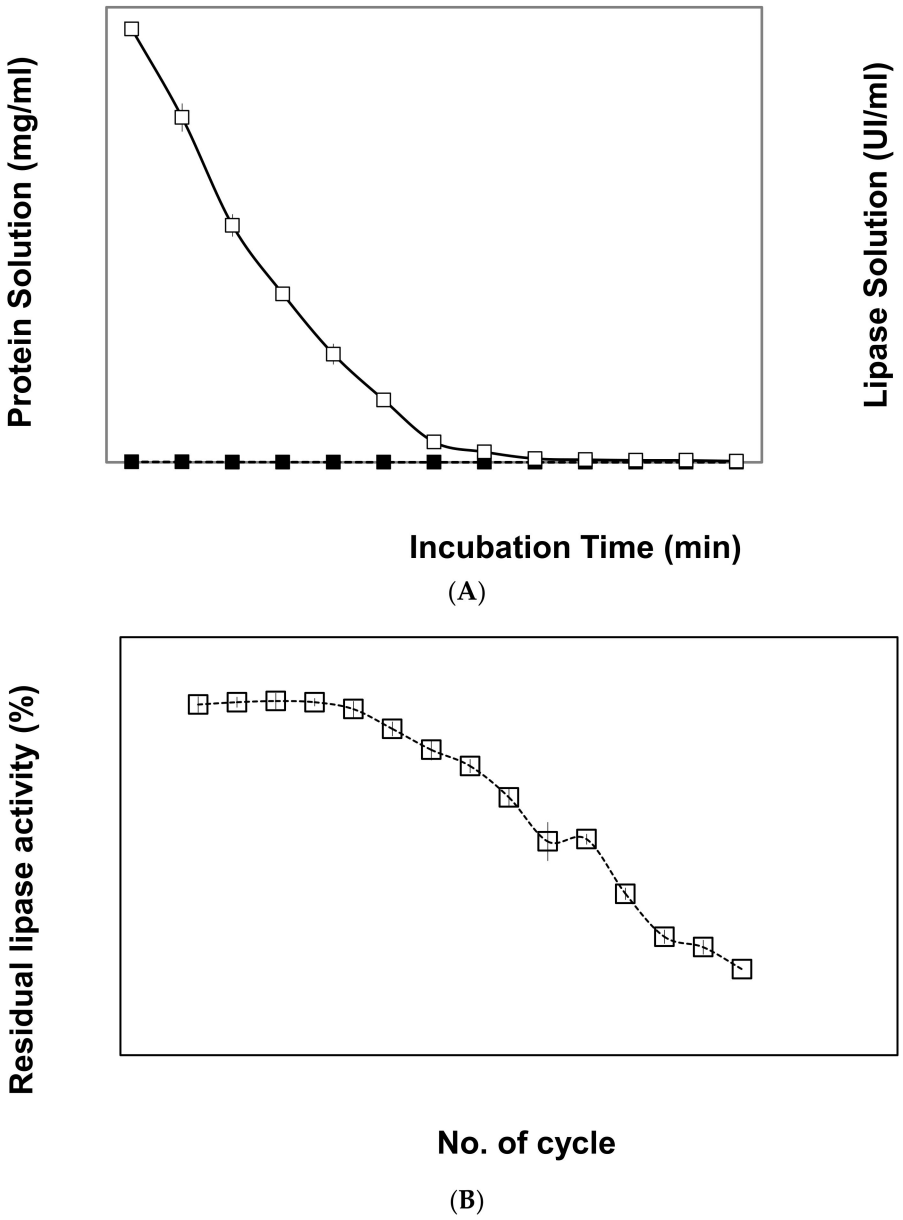

Figure 2. (**A**): Adsorption kinetics of *B. stearothermophilus* lipase (open boxes) and protein (closed boxes) on $CaCO_3$. Enzyme activity was measured using a pH-stat at pH 12 and 60 °C with an olive oil emulsion as the substrate. Data are expressed as the mean ± standard deviation of triplicate measurements. (**B**): Residual enzyme activity following repeated use of immobilized *B. stearothermophilus* lipase. Data are expressed as the mean ± standard deviation of triplicate measurements.

$CaCO_3$ provides excellent mechanical rigidity and can be reused for numerous reaction cycles [13]. Reuse of *B. stearothermophillus* lipase over 15 reaction cycles was assessed through continuous assay of residual immobilized lipase activity (Figure 2B). After the first six cycles, residual lipase activity was equivalent to 93% of its initial activity; after 12 and 15 cycles, residual lipase activity decreased to approximately 50% and 20% of its original activity. Desorption, severing of chemical bonds, or erosion of the support material possibly contributed to the observed decreases in residual activity.

3.2. Characterization of Immobilized and Free B. stearothermophilus Lipase

3.2.1. Storage Stability

Storage stability is considered the most important property for industrial applications, and it has been well established that immobilized lipases have better storage stability than free lipases [16–18]. The current study showed that after 60 days of storage at 4 °C, the relative activity of immobilized *B. stearothermophilus* lipase was over 98% of the initial activity prior to storage, while free lipase exhibited approximately 75% of its initial activity after storage (Figure 3). Interestingly, even after storage at 25 °C for 20 days, immobilized *B. stearothermophilus* lipase was fully active compared to free lipase, where only 50% of initial activity was retained.

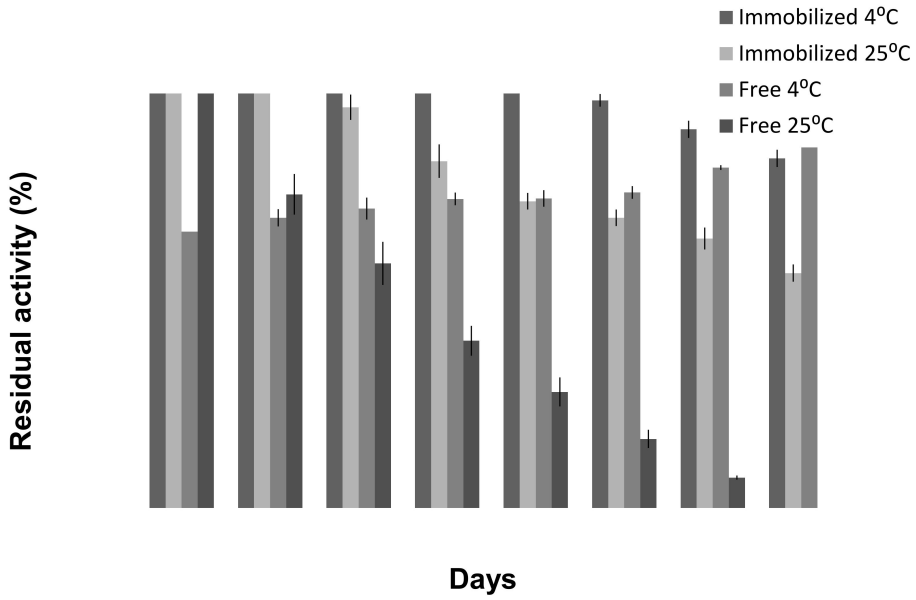

Figure 3. Activity retention rates (%) of free and immobilized *B. stearothermophilus* lipases at 4 °C and 25 °C. Data are expressed as the mean ± standard deviation of triplicate measurements.

3.2.2. pH and Thermal Stability

Several studies have shown that immobilization improves lipase stability at high pH and temperatures [19,20]. In the present study, the immobilized enzyme reached maximum activity at pH 12 (Figure 4A). This was probably due to distorted electrostatic charges after immobilization [21]. Overall, pH stability significantly increased from pH 5–9 for free lipase and from pH 4–11 for immobilized lipase (Figure 4B). Thermal stability varied significantly between free and immobilized lipase, with the optimal reaction temperature being 55 °C for free lipase and 65 °C for immobilized lipase (Figure 4C). Finally, lipase immobilization improved thermal stability from 4–50 °C (i.e., free lipase) to 4–65 °C. The enhanced thermal performance of immobilized enzymes is considered one of the main objectives of successful enzyme applications in many industrial processes.

3.2.3. Effects of Various Metal Ions and Compounds

The effect of inhibitors, activators, and metal ions on $CaCO_3$-immobilized and free lipase activity was determined. Free lipase activity was almost completely inhibited by β-mercaptoethanol followed by PMSF and EDTA, resulting in residual activities of 14%, 28%, and 42%, respectively (Table 2). Furthermore, Cd^{2+}, Co^{2+}, and Fe^{2+} ions inhibited free lipase activity similarly, reducing activity by more than 20%. Conversely, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, and Ba^{2+} did not inhibit the activity of immobilized *B. stearothermophilus* lipase (Table 2). The findings suggest that immobilization helps prevent the inhibition of *B. stearothermophilus* lipase activity by metals and other compounds. For example, immobilization of *B. stearothermophilus* lipase reduced β-mercaptoethanol-induced enzyme

inhibition from 85% to 40%. Similarly, lipase immobilization reduced EDTA- and SDS-induced enzyme inhibition from 60% to 7% and from 15% to 0%, respectively. Notably, the addition of 2 mM Zn^{2+} Ba^{2+}, Cd^{2+}, Mn^{2+} and Cu^{2+} increased immobilized lipase activity by 56%, 48%, 35%, 34%, and 31%, respectively, compared to the control.

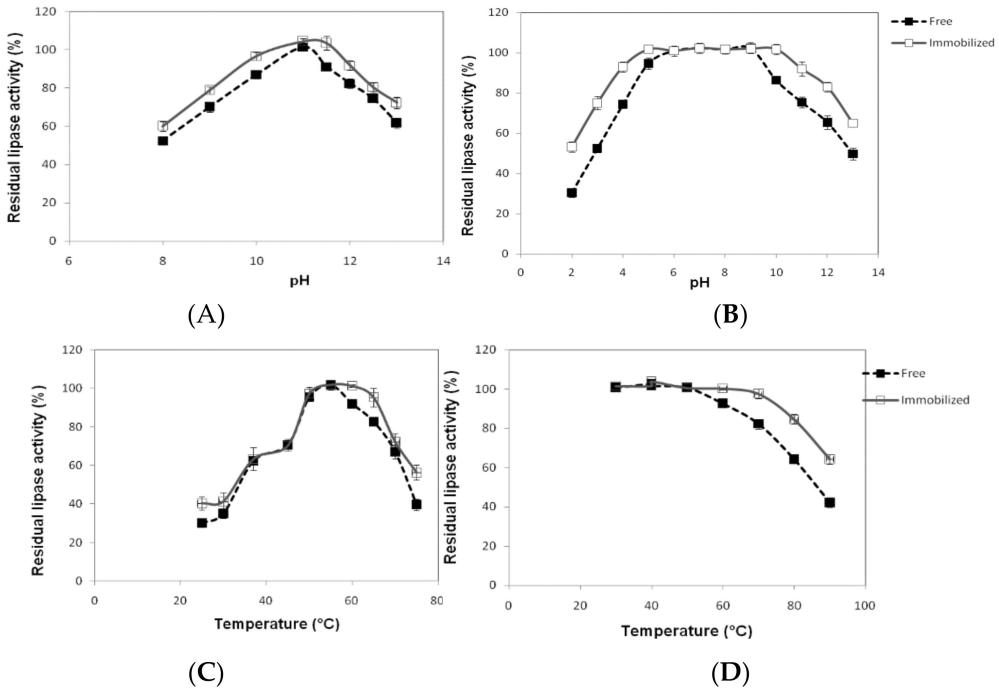

Figure 4. Effect of pH and temperature on *B. stearothermophilus* activity and stability. Effect of pH on both free and immobilized *B. stearothermophilus* lipase activity (**A**) and stability (**B**). Stability was analyzed after preincubating the enzymes for 48 h in different buffer solutions at various pH levels ranging from 2 to 13. Temperature effect on enzyme activity (**C**) and stability (**D**) of free and immobilized *B. stearothermophilus* lipases. To determine temperature stability, enzymes were preincubated at different temperatures for 1 h with activity measured under standard conditions. Data are expressed as the mean ± standard deviation of triplicate measurements.

Table 2. Effect of metal ions and other compounds on free and immobilized *B. stearothermophilus* lipase activity.

		Residual Activity (%)	
Surfactants	**Concentration (%)**	**Free Lipase**	**Immobilized Lipase**
βME	5	14.66 ± 2.8	58 ± 3.51
EDTA	5	42.33 ± 4.2	93 ± 3.05
PMSF	2	28 ± 2.5	49 ± 40.46
SDS	5	85 ± 2	104 ± 3.60
Mg^{2+}	2	126.33 ± 3.21	181 ± 3.51
Zn^{2+}	2	108.33 ± 3.33	156 ± 3.05
Mn^{2+}	2	101 ± 3	134 ± 1.73
Cu^{2+}	2	104.66 ± 3.05	131 ± 3.05
Ba^{2+}	2	104.33 ± 4.33	148 ± 3.21
Cd^{2+}	2	78.33 ± 2.06	135 ± 4.58
Fe^{2+}	2	79.66 ± 4.34	116 ± 3.60
Co^{2+}	2	75.33 ± 3.07	104 ± 4.65

3.2.4. Effect of Substrate Type on Free and $CaCO_3$-Immobilized Lipase Activity

The effect of substrate type on free and immobilized *B. stearothermophilus* lipase activity is presented in Figure 5. Free and immobilized lipase hydrolyzed all of the selected

commercial oils, revealing the potential of these lipases in the biodegradation of oil-rich wastewater. When olive oil was tested, no significant differences were observed between the activities of immobilized or free lipases. Interestingly, immobilized lipase activity was greater than free lipase activity when palm, corn seed, sunflower, and coconut oil were used as substrates (Figure 5). When compared with olive oil (100%), $CaCO_3$-immobilized lipase exhibited a preference for coconut oil (128%), followed by corn seed oil (111%), and sunflower and palm oils (92%).

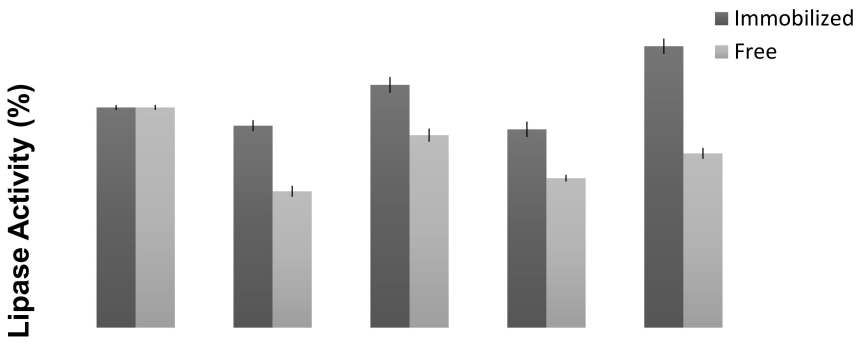

Figure 5. Effect of substrate type on free and $CaCO_3$-immobilized lipase activity. Hydrolysis was conducted at pH 12 and 60 °C. Data are expressed as the mean ± standard deviation of triplicate measurements.

3.3. Bioremediation of Wadi Hanifah Water by Free and Immobilized B. stearothermophilus Lipase

Characterization of residual water is essential for estimating levels of contamination and pollution. The main physicochemical parameters of Wadi Hanifah water, including TOC, COD, and lipid content, were analyzed before and after treatment with free and immobilized lipase (Table 3). Before enzymatic treatment, the TOC and COD contents were 205 ± 14 mg.L^{-1} and 1950 ± 67 mg.L^{-1}, respectively. Treatment with immobilized or free lipase produced varying results. During enzymatic treatment of the wastewater with immobilized *B. stearothermophilus* lipase, TOC gradually increased, reaching 1223 ± 9.8 mg.L^{-1} after 10 days, whereas treatment with free lipase produced TOC of 586 ± 9.8 mg.L^{-1} after 10 days. A significant decrease was observed in COD from 1950 to 4.3 mg.L^{-1} after treatment with the immobilized lipase for 10 days, whereas treatment with free lipase only reduced COD to 725 ± 21 mg.L^{-1}. Notably, water treatment with immobilized lipase almost completely removed all lipids, decreasing lipid content from 15,500 ± 546 mg.L^{-1} down to 12 mg.L^{-1} after 10 days of treatment. However, lipid content only decreased to 9715 mg.L^{-1} following treatment with free lipase.

Table 3. Treatment of Wadi Hanifah water by free and immobilized *B. stearothermophilus* lipase.

	Lipids Content (mg/mL)		**Total Organic Chloride (mg/L)**		**COD (mg/L)**	
Incubation Time (Days)	**Free**	**Immobilized**	**Free**	**Immobilized**	**Free**	**Immobilized**
0	15,500 ± 556.77	15,500 ± 55	205 ± 14	205 ± 12	1950 ± 62	1950 ± 50
1	13,600 ± 624.49	9533 ± 43	255.33 ± 4.72	365 ± 8.13	1721.66 ± 23	1178.33 ± 22
2	12,520 ± 457.05	5447 ± 46	320.66 ± 6.65	487.33 ± 9.7	1601 ± 26	923.33 ± 23
3	11,977 ± 415.1	2983 ± 25	361,66 ± 3.51	595 ± 57	1485 ± 34	627.66 ± 21
4	11,417 ± 289.21	1100 ± 16	394.66 ± 5.50	705 ± 78	1248.33 ± 47	234.33 ± 14
5	10,940 ± 272.54	437 ± 29	431.33 ± 6.50	791.66 ± 9.34	1115 ± 36	92 ± 5.3
6	10,580 ± 295.12	199 ± 17	480.33 ± 5.56	871.66 ± 20	1055 ± 22	61.33 ± 2.4
7	10,187 ± 105.05	93 ± 4.3	511.33 ± 5.56	960 ± 13	932 ± 21	20.66 ± 1.2
8	10,040 ± 149.33	52 ± 3.2	527 ± 41	769.66 ± 57	852 ± 20	8.83 ± 0.2
9	9850 ± 88.88	21 ± 1.5	554 ± 5.22	1186.66 ± 25	790 ± 31	5.5 ± 0.4
10	9715 ± 56.34	12 ± 1.3	586 ± 9.80	1223.33 ± 25	725 ± 21	4.33 ± 0.4

4. Discussion

To date, commonly reported thermophilic lipases have been purified and characterized from *Bacillus* sp. [13,22]. However, lipases from thermos-alkaliphilic *Aeribacillus pallidus* have recently been purified and characterized for oily wastewater treatment [23]. To meet industrial demand, biological catalysts must be improved. Enzyme immobilization may improve the stability, ease of product separation, and reuse of enzymes. Lipases can be immobilized using different methods, such as covalent attachment, adsorption, encapsulation, entrapment, and cross-linking on numerous supports [21].

In the current study, *B. stearothermophilus* lipase was immobilized on the hydrophobic $CaCO_3$ support using the adsorption method to produce a high yield of immobilized lipase (90%), compared to Celite 545 (38%) and silica gel (32%). It is possible that the hydrophobic $CaCO_3$ support causes interfacial activation of lipase during immobilization. Similar results were obtained when *R. oryzae* lipase and commercial *C. rugosa* type VII lipase were immobilized on $CaCO_3$ [6,24]. Next, the immobilization yield of *B. stearothermophilus* lipase, using different initial activities of soluble lipase, was determined to reach a maximum value of 4500 $U.g^{-1}$. The reduced immobilization yield with low initial lipase activity may be explained by a loss of lipase spatial conformation due to the maximum enzyme support contact. However, loading more than 4500 U g^{-1} of lipase negatively impacts immobilized yield, possibly due to inhibition of the active site. These results agree with immobilization experiments using $CaCO_3$ and aldehyde-Lewatit supports [25]. The adsorption kinetics of *B. stearothermophilus* lipase on $CaCO_3$ showed that maximum binding of the enzyme to $CaCO_3$ (95%) was achieved after incubation for 60 min, compared to previous studies, where the maximum binding effect of $CaCO_3$ was observed at 40 min with *S. aureus* lipase [12], *R. oryzae* [6], and *C. rugosa* lipases [26].

The advantages of lipase immobilization for industry applications have been reported, including lower energy costs, shorter processing times, and reduced risk of contamination [27,28]. The reusability of lipases also increases after immobilization. In this study, immobilized lipase was reused for 15 cycles, exhibiting a residual activity of approximately 50%. In a previous study, immobilized lipase B from *C. rugosa* [29] was reused for six cycles. Studies also show that the reusability of immobilized lipase varies, ranging from two cycles to 50 for heterologous *R. oryzae* lipase and *cepacia* lipase, respectively [30–32]. Immobilized lipases also have better storage stability compared to free lipase, and are fully active over extended periods, contributing to the stability of biocatalysts [12,26,33]. For example, this study showed that immobilized lipase could maintain over 98.85% of its initial activity after 60 days of storage at 4 °C, compared to free lipase, which retained 75% of its initial activity. For industrial use, immobilized enzymes have attracted great interest in increasing operational performance, storage periods, and thermal stability [12,30,34]. Notably, immobilized lipases exhibit higher activity than free lipases in alkaline environments [35].

$CaCO_3$ adsorption has several advantages. For example, this method is easy to implement and can regenerate enzyme-support complexes. $CaCO_3$ adsorption is also fast, fixing enzymes rapidly to the $CaCO_3$ support. $CaCO_3$ adsorption also has some disadvantages, including the orientation of the enzyme and poor accessibility to the active site. However, variations in pH and temperature can cause some enzymes to detach from the support. In the present study, maximum activity of immobilized lipase was observed at pH 12 and 65 °C, while free lipase exhibited optimal activity at pH 10.5 and 55 °C. Immobilization significantly increased lipase thermal stability from 4–65 °C, compared to 4–50 °C for free lipase. Stability at extreme pH values also improved after $CaCO_3$ immobilization widening the pH range 4–11, compared to pH 5–9 for free lipase. Significant increases in optimal temperature (37–50°) have also been observed after immobilization in a previous study [34]. Enzyme immobilization has also been shown to affect electrostatic charges, shifting the optimal pH to a slightly alkaline region [21]. Similar results were observed with *S. aureus* lipase, where immobilization on $CaCO_3$ produced significant increases in thermal stability from 10% to 70% at 80 °C [17]. In the present study, immobilization also improved enzyme stability when incubated with SDS and EDTA, and, to a lesser extent, β-mercaptoethanol

and PMSF. Moreover, the addition of 2 mM of metal ions increased the immobilized lipase activity compared to the control.

The hydrolytic activities of free and immobilized lipase were similar when olive oil was used as the substrate. However, when all the other commercial substrates were tested, immobilization improved hydrolytic activity, producing 128% for coconut oil, 111% for corn seed oil, and 92% for sunflower and palm oils. These findings were likely related to the accessibility of ester bonds to the *B. stearothermophilus* lipase, rather than to chain length specificity. In studies involving *C. rugosa* and *R. oryzae* lipases, catalytic activity improved after $CaCO_3$ immobilization when palm or olive oil was used as a substrate [36–38]. Based on the findings here and outcomes from previous studies, improving the properties of enzymes, such as thermal stability, optimal pH, temperature activity, and storage period, enhances enzyme reuse and facilitates continuous enzymatic processing. Therefore, oil layer biodegradation was explored using free and immobilized *B. stearothermophilus* lipases to treat Wadi Hanifah wastewater. Chemical oxygen demand was almost completely removed by immobilized lipase treatment, decreasing COD from 1950.1 to 4.04 $mg.L^{-1}$ after 10 days. Similarly, the initial lipid content (15,500 $\pm$ 546 $mg.L^{-1}$) decreased to 12 $mg.L^{-1}$ after treatment with immobilized lipase. Similar studies have reported successful enzymatic treatment of wastewaters using free and immobilized lipases [39–41]. However, different treatment periods, immobilization techniques, and support types were used to reduce COD and lipid content in these studies [39]. Additional studies were conducted with mixed cultures to improve the bioremediation process [40,41]. All of the results support the potential of $CaCO_3$-immobilized lipases as effective biocatalysts compared to free enzymes.

Traditional wastewater treatment methods present several disadvantages and negative effects, prompting researchers to develop new biological treatments, including enzymatic hydrolysis. Due to their high selectivity, efficiency, and specific catalysis, enzymes offer great potential in engineering. Importantly, enzymes are environmentally friendly and cheaper than traditional wastewater treatment methods. Indeed, bioremediation, which uses the enzymatic action of microorganisms for detoxifying organic contaminants (preferentially in situ), has great advantages, such as low cost, simplicity, and less environmental impacts. In this regard, biodegradation is one of the major degradation processes of hydrocarbons and synthetic dyes in nature. For heavy metals, biosorption and sequestration bioprocesses are more appropriate.

5. Conclusions

In this work, $CaCO_3$-immobilized alkaline lipase from *B. stearothermophilus* was characterized for industrial applications, revealing promising alkaline pH and temperature stability, high catalytic effectiveness, and tolerance for metal ions. The practical use of immobilized *B. stearothermophilus* lipase was confirmed through the oil layer biodegradation of wastewater. Overall, the high specific activity, selectivity, efficiency, and catalysis highlight the potential usefulness of *B. stearothermophilus* lipase in many fields of biotechnological applications, particularly bioremediation.

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