BIOCONVERSION OF OLIVE OIL INDUSTRY WASTES FOR LIPASE PRODUCTION AND IMMOBILIZATION ON CLAY PEBBLES AND JADE STONES: POSSIBLE APPLICATIONS IN DETERGENTS

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Abstract. Due to their ability to catalyze the synthesis of esters in non-aqueous environments and hydrolyze glyceric esters in aqueous environments, lipases are ideal catalysts for a large number of industrially relevant bioconversions. For this reason, this study aims to product and immobilize lipase of *Aspergillus tubingensis* strain MO503 on clay pebbles and jade stones in order to use it as a possible detergent additive. In this objective, lipase activity and proteins rates were followed before and after immobilization, Scanning Electron Microscopy (SEM) and Fourier-Transform Infrared Spectroscopy (FTIR) analysis were carried out. The growth kinetics of the microorganism showed its ability to bioconvert olive oil industry wastes to lipase. The immobilization of the lipase enzyme showed a clear difference on clay pebbles and jade stone surfaces after lipase immobilization. The formulation of the detergent with this immobilized enzyme showed a total and partial elimination of oil from the cotton and synthetic fabric pieces respectively which unveil a potential application of immobilized lipase as an additive in local detergents.
Keywords: *Fourier-transform infrared spectroscopy (FTIR), fungal lipase, immobilization, purification, scanning electron microscopy (SEM)*

Introduction

Bioconversion is a process that allows for the conversion of a substrate into a new product using organisms, biological systems, or their enzymes (Lopes et al., 2024). This mechanism enables effective waste management by employing methods that have a direct impact on the environment, such as anaerobic digestion, fermentation, and composting (Zou et al., 2024). The scalability and efficiency of bioconversion processes require ongoing research and development (Siddiqui et al., 2024).

With their specificity, biomolecules called enzymes, acting as biological catalysts, are able to catabolize a specific substrate in a short time and improve effectiveness of transformation processes. This makes them popular molecules in several industries such as agri-food, pharmaceutical and detergent fields (Hammami et al., 2018). Because of their easy production by microbial culture on agri-food waste and to their stability in organic solvents, microbial enzymes, can easily adapt to difficult conditions of industrial technological processes (Zhao et al., 2021). For this reason, microbial lipases are therefore widely used in environmentally friendly processes especially in the textile, detergent and agro-food industries (Singh et al., 2021; Khmaissa et al., 2023)

For the sake of reducing the use of environmentally harmful chemicals products, many industries have chosen lipases for their ability to hydrolyze triglycerides and release the

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fatty acids after their action (Khan et al., 2019). Detergent industries use these enzymes because of their ability to be active at the presence of surfactants; in acidic or alkaline environments and at high temperatures (El-Ghonemy and Ali, 2021; Khmaissa et al., 2022).

The instability, the price and non-recycling of free lipase reduced its use in several applications, which allowed the immobilized enzyme to replace it (Zhao et al., 2022). Enzyme immobilization allows fixing this biomolecule on a matrix surface without affecting the diffusion of the substrate on active site (Nemiwal et al., 2022). Five methods can be used to immobilize enzyme, namely: adsorption, embedding, polymerization, cross-linking and covalent bounds (Guisan et al., 2022). In the latter, bounds are formed between ionizable groups of enzyme and the present ionizable groups on material used like support (Aggarwal and Ikram, 2022).

The alumina, silica, alginate and clay richness of environment, helped different applications areas such as green chemistry and agriculture, which are interested in these materials, particularly clay pebbles known for their porosity, lightness, aeration and stability (Calabi-Floody et al., 2012). The interest of enzymes stability at certain temperatures, after immobilization, has allowed many researchers to determine the efficiency of the pebbles beads as a good host matrix able to keep the enzyme stable (Hass Caetano Lacerda et al., 2020; Menezes-Blackburn et al., 2011). On the other hand, Jade stone, particularly used as an ornament in jewelry in East and South Asia, can be used like support for enzyme immobilization, because it refers to two different silicate minerals: the silicate of sodium and aluminum (jadeite) or the silicate of calcium and magnesium (nephrite) (Manrique-Ortega et al., 2019). These elements are very good adsorbents and can enhance thermal stability of lipases after immobilization (Alagöz et al., 2021; Ovsyannikov, 2009).

In this respect, the current study focuses on microbial lipase immobilization on clay pebble and jade stone. It attempts to determine its effect on oil spots before and after immobilization for potential uses as a detergent additive.

Materials and methods

Material

The lipase enzyme used in the current study is produced by *Aspergillus tubingensis* strain (MO503) and characterized by our team during previous study (Benlounissi et al., 2012). Clay pebbles used were bought from artisanal hand potter (Constantine, Algeria). All products were obtained from Sigma-Aldrich (Germany). Milli-Q water (ZHUOYUE) was used to prepare all solutions and reagents used were of analytical-reagent grade.

Preparation of clay pebbles and jade-stones for Immobilization

Clay pebbles (Ø≈3 mm) and jade-stones (S≈4 mm^2) have been hydrated with pure water and dried at room temperature (25±1°C) for 30 min. The Glutaraldehyde at 4% was used as a Cross-linking agent. It was Prepared in 200 mM Carbonate buffer pH 9.2, drying for approximately 1 h at room temperature, then rinsing with carbonate buffer to remove excess Glutaraldehyde. 100 µL of the purified enzyme (5 µg/mL) were deposited on the support and left 2 h at room temperature (25±1°C). After drying, the support was rinsing with phosphate buffer pH 7.4 in order to remove excess unbound enzyme. Non-enzymatic control supports were prepared according to the same protocol (Ben Ameur Villain, 2012).

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Protein assay

Proteins of samples were quantified using bovine serum albumin (Sigma-Aldrich) as standard according to (Lowry et al., 1951). Assays were conducted in triplicate.

Lipase activity assay

Determination of lipase activity was based on olive oil and clear Arabic gum. The reaction was carried out for 2 h at 37°C then supplemented with ethanol. The titrating allows determining amount of oleic by the hydrolysis products with NaOH using thymolphthalein as indicator. Knowing that one enzyme unit is defined as the amount of enzyme catalyzing the formation of one micromole of oleic acid in 2 h, at pH 7.5, 37°C, the lipase activity was determinate against the control sample that prepared and treated using boiled enzyme (Adham and Ahmed, 2009). Assays were conducted in triplicate.

Effect of the lipase as a detergent additive

To study the action of the purified lipase on the lipids containing in oil stains used as samples and evaluate the wash performance of lipase as detergent additive, various tests were carried out with local detergent on two kinds of fabrics, cotton and synthetic (4 cm × 4 cm) (*Table 1*); each fabric pieces spotted with 50 µL of olive oil. The different pieces of fabric were subjected to the same treatment according to *Table 1,* at 37°C with stirring at 100 rpm/30 min, then rinsed with running water and dried at room temperature (25±1°C). The fabrics parts were observed and photographed (Akmoussi-Toumi et al., 2018). The darkness and brightness are determined by observing the surface of the spots after shining a beam of light LED (100 lm/W) at a 90° angle. The lipids levels were calculated by Röse-Gottlieb method (Laiterie, 1958).

Table 1. *The tests carried out to evaluate the effect of lipase as a detergent additive*

Lipase	**-**	**ILCP**	**ILJS**	**-**	**FL**	**FL**	**ILCP**	**ILJS**
Local detergent (100 µL)	-	-	-	+	-	+	+	+
Fabric pieces (cotton and synthetic)	+	+	+	+	+	+	+	+

ILCP: immobilized lipase on clay pebbles; ILJS: immobilized lipase on jade stone; FL: free lipase; +: present; -: absent

In the same conditions and given the large amount of free lipase, it was possible to test the effect of purified enzyme on the same fabrics, spotted with olive oil.

Scanning electron microscopy (SEM)

The morphology of clay pebbles and jade-stones used as supports of lipase immobilization were examined using a Scanning Electron Microscope (SEM) JSM-7610F (Massachussets, USA).

Fourier-transform infrared spectroscopy (FTIR)

The supports-samples, crude and with immobilized lipase, were rinsed with KCl, 0.1 M solution for 60 min to remove impurities and uncoated particles (Chen et al., 2020),

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then placed in the Fourier-transform infrared spectrophotometer IRAffinity-1S (Shimadzu, Kyoto, Japan) for analysis, spectra were recorded.

Results and discussion

Scanning electron microscopy (SEM)

The Scanning Electron Microscope was used to observe the morphologies of clay pebbles and jade-stones before and after, activation and lipase immobilization. *Figure 1* shows smooth and rough parts on clay pebbles before treatment (*Fig. 1a, b* and *c*) with an inhomogeneous surface. After activation with glutaraldehyde and lipase immobilization the clay surface looks smoother (*Fig. 1a'*), covered with a light crust with a several shiny spots on the surface compared to non-immobilized enzyme (*Fig. 1c*). This appearance seems to be caused by the lipase coverage of the clay beads surface.

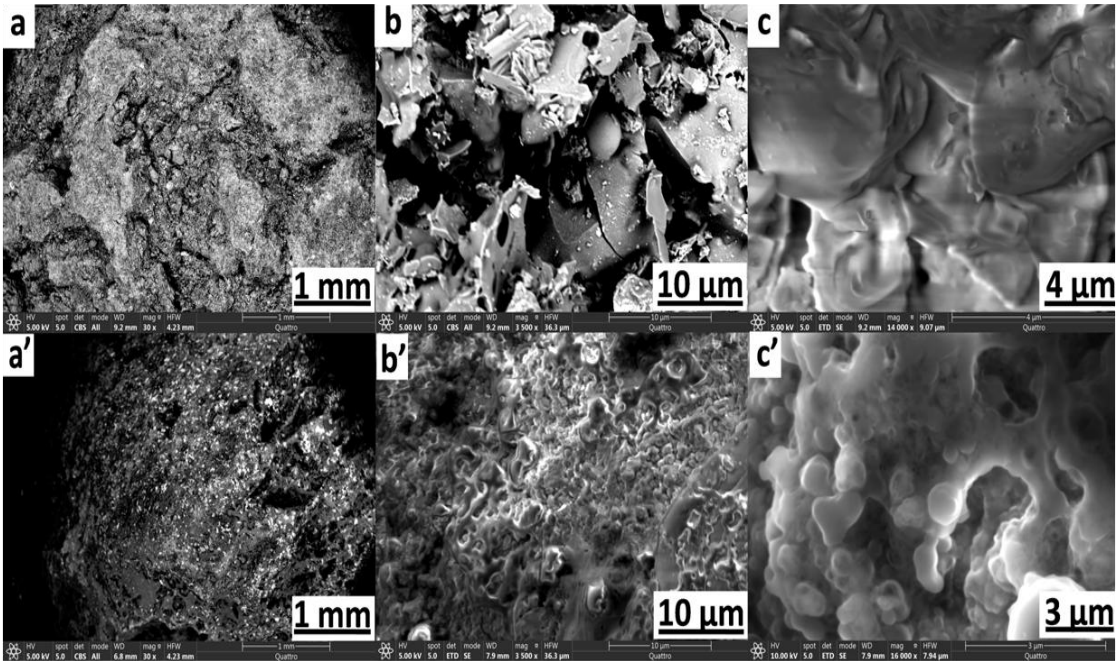

Figure 1. *Morphology of clay pebble under SEM crude clay pebble: (a) M.30x, (b) M.3500x and (c) M.14000x. Clay pebble after lipase immobilization: (a') M.30x, (b') M.3500x and (c') M.16000x*

The Scanning Electron Microscope used let appear, as showing in *Figure 1*, that glutaraldehyde activation decreases the surface area of clay pebbles which seems more porous and less smooth after immobilization (*Fig. 1c'*). However, it was reported during immobilization of laccase in clay aggregate, that the chemical activation increases the surface area of material and the pore size formed looks bigger (Anita et al., 2020). Another research demonstrated that immobilization on crude bentonite, after acid-base treatment, enlarge the interlamellar spacing and reduce the particle size (Anas et al., 2020). However, it was reported that glutaraldehyde treatment of bentonite create a surface rougher (Li et al., 2013)**.** In the case of jade stone immobilization, *Figure 2* shown more porous and less smooth surface after immobilization (*Fig. 2a'*), confirmed after observation under SEM at M.4000x (*Fig. 2b'*). Research has shown that jade-stone

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surface composition changes after interaction with the components of external environment (Cheng et al., 2004)**.**

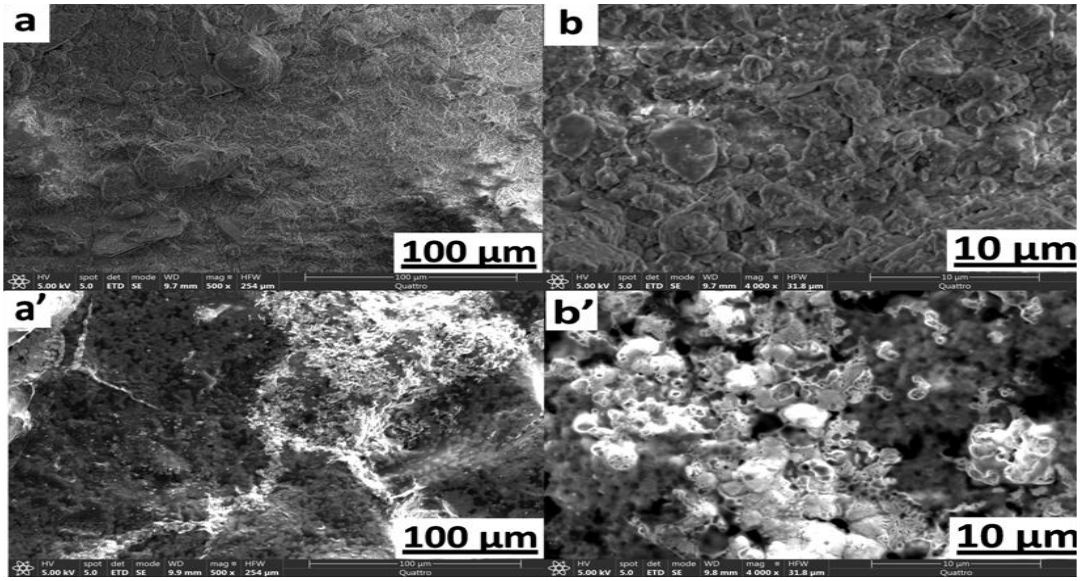

Figure 2. *Morphology of jade-stone under SEM Crude jade-stone: (a) M.500x and (b) M.4000x. Jade-stone after lipase immobilization: (a') M.500x and (b') M.4000x*

Infrared spectroscopy (FTIR)

The FTIR spectra of the clay were recorded over the spectral range 400–4000 cm^{-1}. *Figure 3* shows IR spectra of the clay pebbles before and after lipase immobilization. In the spectra before immobilization (*Fig. 3* – curve *B1*), the absorption bands show characteristic absorption peaks at 470.63; 622.93; 1056.99, 1489.04, 1622.29, 2287.94, 2301.25 and 2972.30 cm^{-1}.

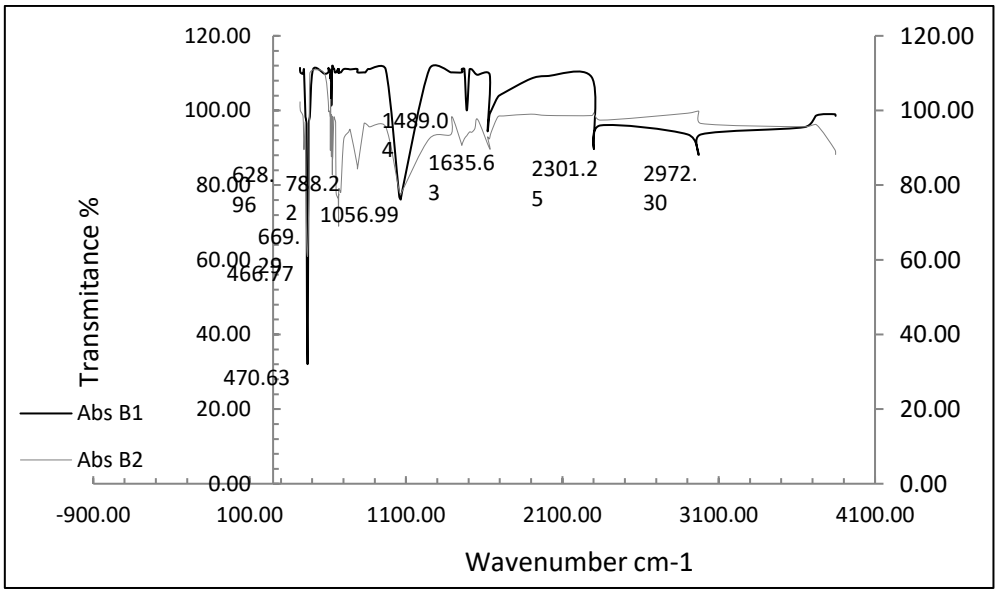

Figure 3. *FTIR spectra of clay pebbles; (B1) before lipase immobilization, (B2) after lipase immobilization*

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In the same way, the kaolinite material showed a high intensity of the peak at 1100.78 cm^{-1} (Djebbar et al., 2012). The FTIR spectra of purified montmorillonite clay showed that peaks between 836 and 875 cm^{-1} are attributed to AlMg-OH and AlFe-OH bending vibrations while the peak at 1035 cm^{-1} was attributed to Si–O stretching vibration for layered silicates (Patel et al., 2006).

After lipase immobilization, *Figure 3* (curve *B2*), showed that the number of peaks has increased. The presence of extra peaks, in clay spectra after lipase immobilization at 466.77; 628.96; 669.29; 788.22; 1056.99; 1457.67 and 1635.63 cm^{-1} can be attributed to the influence of molecule. The results obtained by another study assigned the band at 830.38 to Si–O–Al (Madejová, 2003).

The emerging peaks at 669.29 and 788.22 cm^{-1} is assigned to a new chemical bound between enzyme and support which could correspond to Si–O bond as demonstrated during a thermal and FTIR spectroscopy study of calcined brick clay (Peyne et al., 2017). Moreover, the decrease observed in the peaks at 466.77 and 1056.99 cm^{-1} suggests that the immobilization process modified clay pebble spectrum (Gou et al., 2005).

Figure 4 show IR spectra of the jade-stone over the spectral range 400–4000 cm^{-1}, in the spectra before immobilization (*Fig. 4* – curve *C1*), the absorption bands shows characteristic absorption peaks at 466.77; 616.30; 702.20; 790; 856.39; 1056.99; 1457.67; 1521.83; 1898.98; 2330.65 and 2972.30 cm^{-1}. A high intensity of the peak appearing at 466.77; 856.39 and 1056.99 cm^{-1}.

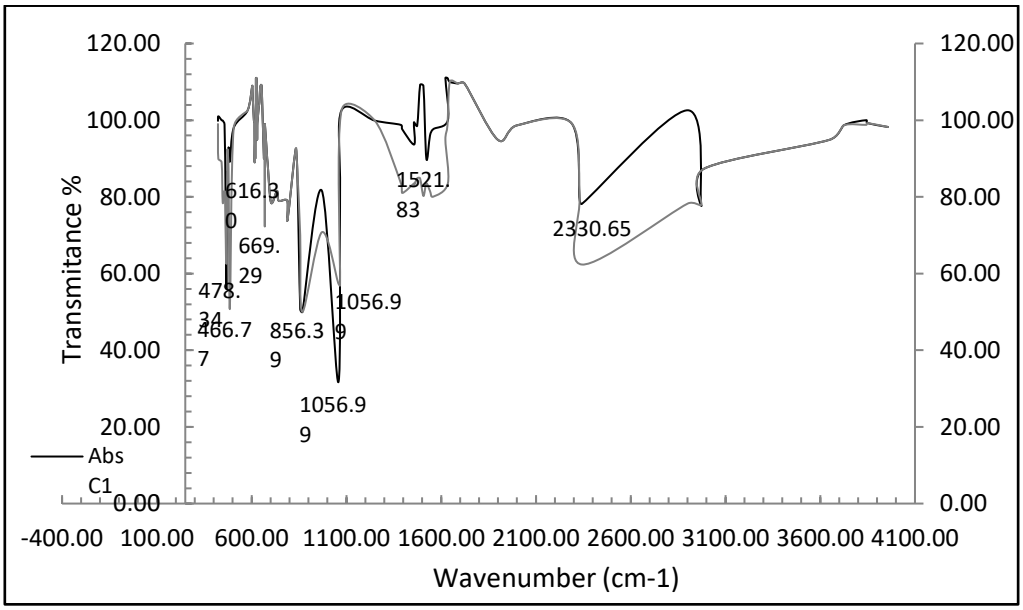

Figure 4. *FTIR spectra of jade-stone; (C1) before lipase immobilization, (C2) after lipase immobilization*

After the immobilization (*Fig. 4* – curve *C2*) a decrease in absorbance indicating binding mechanisms between lipase groups and jade stone (Qu et al., 2019). The same work showed high-intensity peaks at 550 and 1035 cm^{-1} and low intensity peaks at 823 and 924 cm^{-1} knowing that peaks within the 600 to 800 are the result of symmetrical stretching vibration of Si–O–Al.

It is worth noting that due to its aldehyde group, glutaraldehyde can exhibit a characteristic stretching band around 1730-1740 cm^{-1}, which is attributed to the stretching

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vibration of the C = O bond. On the other hand, C-H bonds can display stretching bands in the region of 2800-3000 cm^{-1} typically observed for the methyl and methylene groups. Glutaraldehyde can also generate starching bands in the region of 1200-1500 cm^{-1} due to C-C bonds (Silverstein et al., 2014).

Effect of immobilization on lipase activity and proteins

The immobilization of the lipase on the clay pebbles (CP) and Jade stone (JS) by chemical bounds, as shown in *Figure 5,* revealed a drop of 20.75% and 44.15% of lipase activity and 8% and 32.51% of proteins respectively after immobilization. Knowing that the enzymatic activity and protein levels, before and after immobilization, were 752 and 596 A.U., and 4.86 and 4.47 $g.L^{-1}$; and 752 and 420 A.U., and 4.86 and 3.28 $g.L^{-1}$ for lipase immobilized on clay pebbles and jade stone respectively. All assays were conducted in triplicate. All assays were conducted in triplicate. The error bars in *Figure 5* represent the standard deviation of these three replicates, indicating the variability in the results obtained.

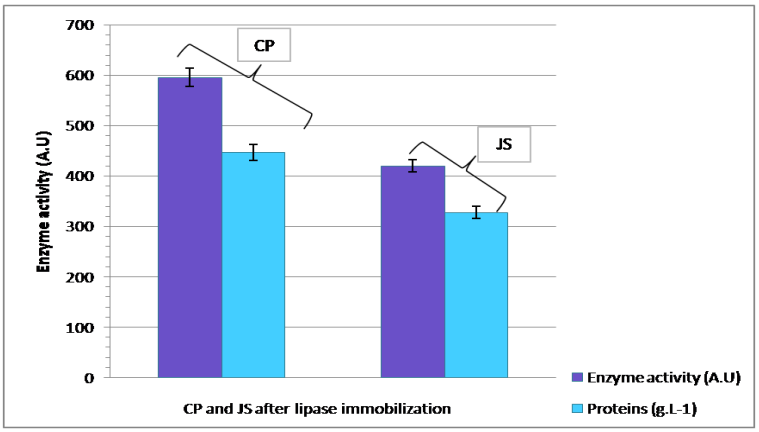

Figure 5. *Lipase activity and protein concentration after immobilization. CP: clay pebbles; JS: jade-stone*

The immobilization of the lipase on the clay pebbles and Jade stone by covalent bounds had reveals a drop of rate proteins after immobilization can be explained by a loss of enzyme during immobilization, or by appearance of bonds between the support and the enzyme which interfere with the lipase and substrate linking. Some studies have disclosed that the catalytic activity can be influenced during the manipulation which sometimes leads to enzyme denaturation and low accessibility of the substrate at the active site (Palmer, 1985)**.** This can cause an alteration of the enzymatic activity and the physical properties of the system due to the use of incompatible compounds (Vial, 2005).

Effect of lipase as an additive of local detergent on olive oil spots

The study of the effect of purified free and immobilized enzyme on olive oil spots made on pieces of cotton and synthetic fabrics, allowed determine the diameters (mm) of the stains before and after the enzyme action, as reported in *Table 2*.

The formulation of the detergent and immobilized enzyme on clay pebbles and jade stone showed a total elimination of olive oil from the cotton and synthetic pieces (*Table 2*) and a partial elimination in the case of the free enzyme. The brightness of fabrics was slightly reduced after the action of the detergent and immobilized enzyme formulation,

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suggesting that intensive washing using hot water or washing machine can effectively restore fabric clarity. It was demonstrated that the addition of lipase produced in a commercial detergent completely eliminated the butter stain, as compared to the control and detergent alone (Abol-Fotouh et al., 2021a).

Table 2. *Effect study of immobilized lipase produced by A. tubingensis (MO503), before and after immobilization, as an additive of local deactivated detergent on olive oil spots made on cotton and synthetic fabrics. Results presented in mm*

	N.C	P.C	BUF	FL	ILCP	ILJS	DET	ILCP+DET	ILJS+DET
Cotton fabric	0	30	30	27	26 mm	28	28	0	0
Darkness	/	+++	+++	+	/	+	/	/	/
Brightness	+++++			+	+		++	++++	++++
Synthetic fabric	0	30	30	21	22	22	19	0	0
Darkness	/	+++	++	+	/	/	/	/	/
Brightness	+++++	/	/	/	+	+	+	++++	++++

N.C: negative control; P.C: positive control; BUF: buffer; FL: free lipase; ILCP: Immobilized lipase on clay pebbles; ILJS: immobilized lipase on jade stone; DET: detergent; ILCP + DET: Immobilized lipase on clay pebbles + detergent; ILJS + DET: immobilized lipase on jade stone + detergent; /: total absence; +: low presence; +++++: high presence

It should be noted that a previous study had already been conducted on this topic (Khelalef et al., 2024), with the same free lipase added to the detergent eliminated approximately 95% of olive oil spots on cotton fabric.

Effect of local commercial detergent on lipase activity

The effect of local commercial detergent on lipase activity is reported on *Figure 6* that reveal a negative effect of detergent on the free and immobilized lipase activity in the presence of the fatty substance: olive oil. In this case, the enzymatic activity decreased 38; 22.45 and 23.47% for free, immobilized on clay pebbles and immobilized on jade stone enzyme respectively.

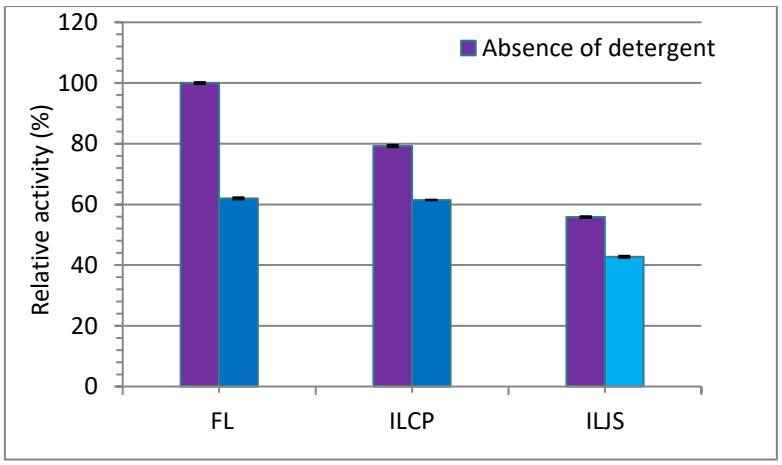

Figure 6. *Influence of local detergent on activity of the purified immobilized lipase produced by A. tubingensis strain (MO503). FL: free lipase; ILCP: immobilized lipase on clay pebbles; ILJS: immobilized lipase on jade stone*

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Finally, the results of effect of local commercial detergent on lipase activity can be explained by the direct inhibition effect of detergent on lipase activity. According to a study, certain surfactant concentrations can lead to significant substrate diffusion limitation in the microenvironment (Guncheva et al., 2007). On the other hands, works demonstrated that the immobilization lipase from *Burkholderia cepacia* on the polyacrylic support increased the lipase activity and reduction the reaction time (Dulęba et al., 2022).

Conclusion

The purified lipase from *A. tubingensis* strain has significantly enhanced the removal of olive oil spots from cotton and synthetic fabrics. The obtained results suggest that the increasing the concentration of free lipase and employing intensive washing with hot water or a washing machine can effectively restore fabric clarity. These results unveil a promising application for this biomolecule as additive in a local detergent. Moreover, for better control of the lipase, it is necessary to determine its stability during a certain number of uses and days in the detergent, while closely monitoring the enzyme in new conditions.

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Conflict of interests. The authors declare no competing interests.

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