# Potential Application of Bacterial Lipase from *Bacillus subtilis* in the Production of Biodisel

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**This work aims to evaluate the potential of bacterial lipase of *Bacillus subtilis* in biodiesel production. Assays were performed to obtain, purify, and immobilize the enzyme and compare chemical and enzymatic transesterification tests. The Tryptic Soy Broth (TSB) culture medium without supplementation showed the highest enzymatic activity and purification yields when using 60% of $(NH_4)_2SO_4$. Citral was the most efficient agent for lipase immobilization. Even though the free enzymes showed a higher activity rate, the immobilized enzymes are associated with a better-quality product. In conclusion, the lipase enzyme showed positive potential in biodiesel production.**
**Keywords: *Bacillus subtilis*. Lipase. Biodiesel. Immobilization.**

## Introduction

The demand for sustainable fuel sources is a growing requirement for economic growth. Alternative sources of fuels are being researched to replace high-impact fossil fuels with greener options.

Biodiesel is a renewable fuel made from transesterification of biologically produced lipids. It is often carried out by a chemical process where triglycerides react with an alcohol, usually ethanol or methanol, generating two products: esters (biodiesel itself) and glycerin [1]. However, various catalysts can also be employed in the transesterification process, including alkaline, acid, and biological catalysts [2].

Lipases are triacylglycerol-acyl hydrolases that are used to replace the chemical transesterification process. Lipases have broad specificity to the substrate and their functional groups or enantioselectivity [3]. The enzymatic-driven process generates fewer byproducts or chemical wastes. However, the performance of enzymatic transesterification is limited by the need for knowledge of different enzyme potentials and methodological approaches.

Immobilization is an easy process that can be applied to improve enzyme transesterification performance. It can increase the number of reacting agents (enzymes) and possibly use them several times. It is possible because immobilization sustains the conformational structure of the enzyme [4]. This work aims to evaluate the potential of lipase bacteria from *Bacillus subtilis*, free and immobilized, in the conversion of oils into biodiesel.

## Materials and Methods

The Figure 1 presents the experimental steps of this study.

### Obtaining and activating the *Bacillus subtilis* strain

The strain of *Bacillus subtilis* came from the isolation carried out in the doctoral thesis of Tatiana Oliveira do Vale, obtained at the Microbiology and Bioprocesses Laboratory Moacyr Durham de Moura-Costa - Federal University of Bahia (UFBA).

A loop of the petri dish was removed with the microorganism and sown in Erlenmeyer of 250 mL containing 50 mL of TSB medium. The culture medium was kept at 37°C under agitation of 130 rpm for 24 hours. Then, 12-16h overnight

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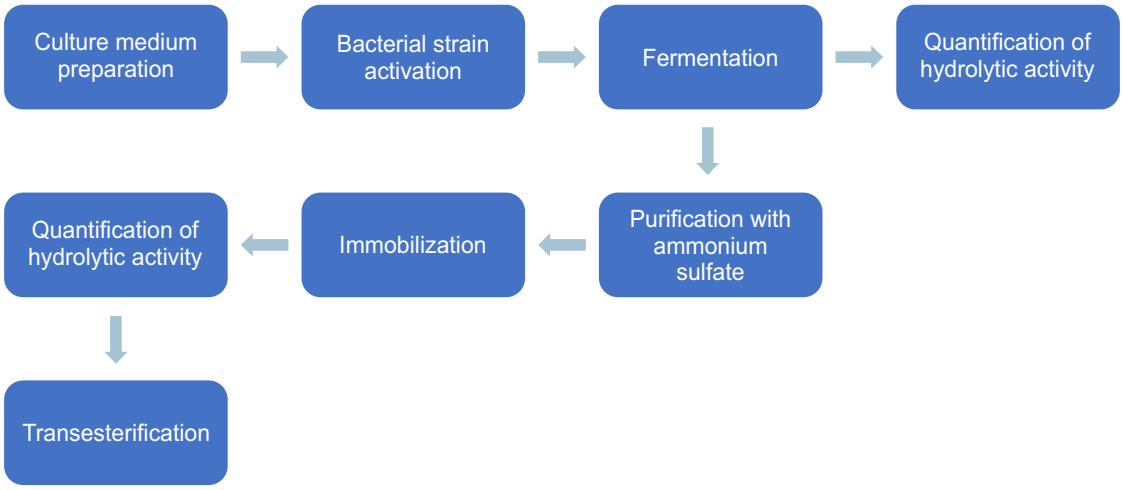
**Figure 1.** Flowchart of experimental steps performed in the study.




was performed to standardize the number of microorganisms.

Culture Medium Preparation and Fermentation

Three culture media were used for the growth of the bacteria: TSB, TSB 10x diluted and with 1% olive oil, and SMM (Synthetic Mineral Medium) with 1mL of trace element solution and supplemented with 1% olive oil; the media were sterilized in autoclave 121°C for 20 minutes.

For fermentation, 10% of inoculum was added in each fermentative media, growth was carried out for 72h, under agitation of 180 rpm at 37°C, and aliquots were removed for 0h and 72h. These aliquots were centrifuged at 4,000 rpm for 20 minutes at 25ºC to obtain the cell-free broth. This stage and the following were performed in triplicate.

Purification of the Enzyme

In the purification step, 15 mL of the enzymatic extract was removed, then 6.354g of ammonium sulfate was added for 60% saturation and 4.236g of ammonium sulfate for 40% saturation. The reaction medium was placed in a magnetic stirrer until the salt was dissolved entirely [5].

Immobilization in Chitosan

For the immobilization, 0.18 g of chitosan was solubilized in 6 mL of 1% acetic acid and precipitated in 1M NaOH solution (12 mL). The solution of the acid medium was dripped with a syringe in the alkaline solution and kept under gentle agitation of 90 rpm in an orbital agitator for 24 hours at room temperature [6].

Then, two substances were tested for activation, one with Quaternary ammonium and the other with Citral; the activation occurred for 1 hour at 90 rpm. After activation, 10 mL of enzymatic solution was added. The solution was kept in agitation at 90 rpm for 4 hours at 4°C so that the enzyme could bind to the support.

Quantification of Hydrolytic Lipase Activity by Titration

The reaction mixture, composed of gum arabic 6% and tributyrin, along with the enzymes (free or immobilized), was incubated at 37º C for 30 min at 160 rpm. Then, the reaction was interrupted with a 1:1:1 (v/v/v) solution of acetone:ethanol: water and titrated with a standardized 0.05 M NaOH solution. The white reaction was prepared using the same

method described above, except for adding the enzyme. [7]

Transesterification

For transesterification, 50 g of soybean oil and 20 g of methanol (25.25 mL) were used, having as chemical catalysts 0.5 g of NaOH and as biological catalysts 0.1 mL and 0.5 mL of free enzyme and 0.1g and 0.5g of immobilized enzyme [8].

For both, it was counting the time of 1 hour after the change of color of the reaction; after the determined time, the solution was placed in a separation funnel so that the separation by the density of biodiesel and glycerin occurred, washing steps, and finally,y the biodiesel was filtered with filter paper.

## Results and Discussion

Fermentation

Among the culture media tested was the one that obtained the highest enzymatic activity with 1.11 U/mL. In contrast, the TSB medium obtained a deficient activity, being considered as zero, comparing the media with the addition of oil (MMS and TSB 1%); the synthetic mineral medium showed the highest enzymatic activity (0.72 U/mL) (Figure 2).

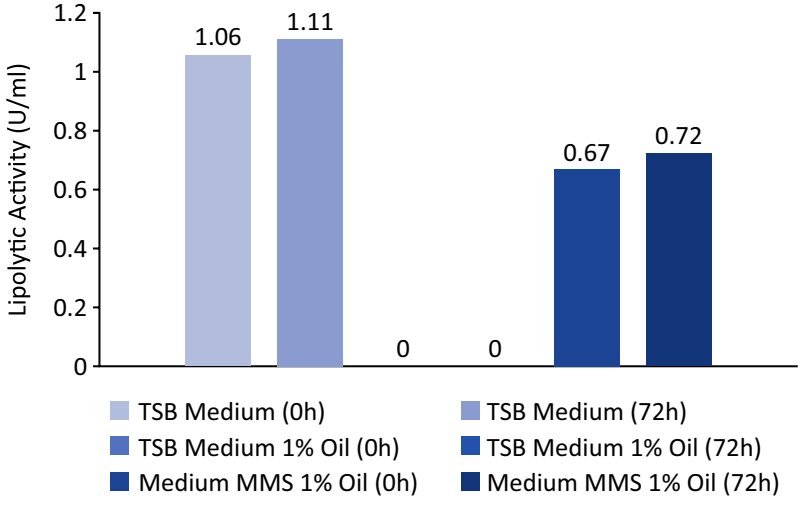
The production of lipase by other species of *Bacillus* is reported in the literature, as the use of *Bacillus licheniformis* that using as a carbon source orange flour obtained a variation of 1.19 to 4.44 IU/mL in its enzymatic activity [5], while the cultivation of *Bacillus megaterium* in modified LB medium obtained a maximum activity of 0.07 IU/mL [6]. These results show that the production of lipases can be improved depending on the growing conditions, allowing more space for research in the area.

Precipitation of Lipase

Precipitation with ammonium sulfate is based on differential precipitation in high concentrations of ionic salts. Two concentrations of ammonium sulfate saturation in the enzyme, 60% and 40%, were tested. They presented better purification to saturation with a 60% concentration of $(NH4)_2SO_4$ (Figure 3).

Similar results are found in other studies, where the use of an 80% concentration of ammonium sulfate obtained an increase in the specific enzymatic activity of the crude extract after purification, going from 0.29 to 17.74U/mg of

**Figure 2.** Comparison between the enzymatic activity of TSB Medium, TSB Medium 1% oil, and Synthetic Mineral Medium.



specific activity from vegetable lipase of soybean oil [7], in another research used ammonium sulfate for purification of the enzyme *Bacillus* sp. lipase having an increase in enzymatic activity from 6.531 U/mL to 67.899 U/mL [8].

Enzyme Immobilisation

Two reagents were tested for chitosan activation: quaternary ammonium ($NH_4^+$) with two concentrations, 1% and 0.5%, and Citral. The activation with ammonium quaternary was ineffective, while the activation with Citral was successful, presenting an average activity of 3.89 U/g, with a yield of 74.5% (Figure 4).

The activation of chitosan occurs through a nucleophilic attack between the amino group of chitosan and the carbonyl group of compounds such as glutaraldehyde, formaldehyde, and methane [9]. The presence of the aldehyde group in Citral indicates the positive result of immobilization due to the possible interaction between this compound's carbonyl group and chitosan's amino group.

**Figure 3.** Lipolytic activity precipitation with ammonium sulfate.

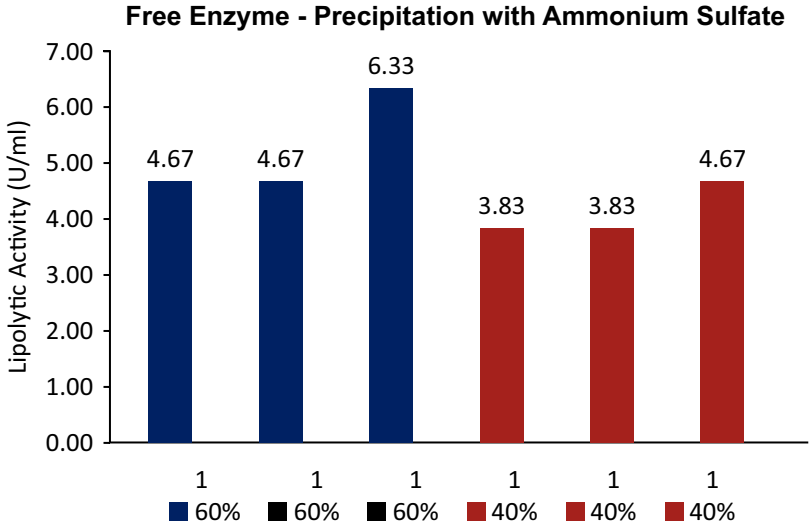
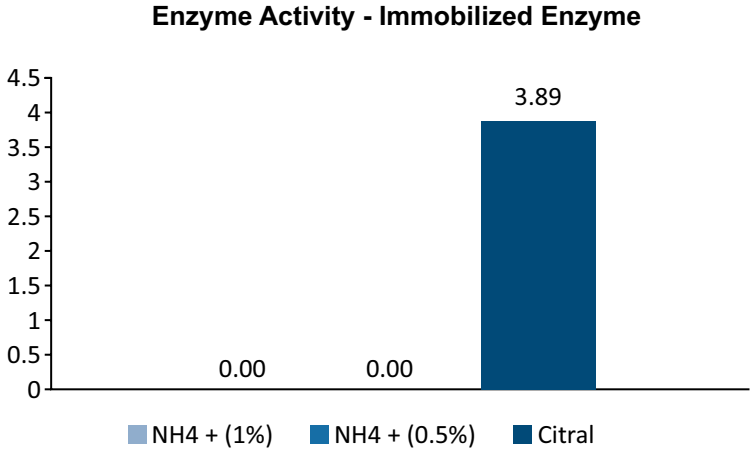



**Figure 4.** Comparison between the lipolytic activity of $NH_4^+$ and citral.



Quantification of Lipolytic Activity

After purification, the extract with a 60% concentration showed the highest activity, having an average of 5.22 U/mL, while with 40%, it presented only 4.11 U/mL. The enzyme immobilized with $NH_4^+$ presented a shallow activity, considered in this work as zero, for the concentration of 60% of ammonium sulfate; the enzyme immobilized with Citral presented an average activity of 3.89 U/mL (Table 1).

Biodiesel Synthesis

The density of biodiesel is linked to its overall molecular structure; biodiesel shows higher density if most lipids have long carbon chai; biodiesel combustion quality is also linked to the number of unsaturated lipids comprising the mixture [10]. Therefore, titration, acidity index, free fatty acids, and ester content were carried out to estimate the quality of the biodiesel generated with enzymatic transesterification (Table 2).

As none of the produced samples reached 96.5% of esters described by the ANP, it is impossible to classify them correctly as biodiesel. However, it is possible to analyze the processes that obtained the values most similar to the standard requested. The results show that the biodiesel produced using immobilized enzymes is close to reaching the standard parameters described by the ANP. When using 0.1 g of immobilized enzymes, the product showed the best acidity index, density, and the highest levels of esters.

## Conclusion

TSB without supplementation was identified as the best medium for the growth of *Bacillus subtilis*. We observed that purification with ammonium sulfate with 60% concentration helped recover the enzymes and retained the highest enzymatic activity. Citral was the most efficient agent for lipase immobilization. Free enzymes showed a higher enzymatic activity than the immobilized ones. However,

**Table 1.** Data enzymatic activities: free enzyme and immobilized enzyme.

| Immobilized Enzyme (U/g) | | | Free Enzyme (U/mL) | |
|---|---|---|---|---|
| $NH_4^+$ | | Citral | 60% | 40% |
| 1% | 5% | 3.89 | 5.22 | 4.11 |
| 0 | 0 | | | |

**Table 2.** Biodiesel quality analysis.

| Quality Parameters | Values Found | | | | | ANP |
|---|---|---|---|---|---|---|
| | 1* | 2* | 3* | 4* | 5* | |
| Acidity Index (mgKOH/g) | 1.40 | 0.67 | 0.61 | 0.53 | 0.44 | 0.50 |
| Free Fatty Acids | 0.70 | 0.33 | 0.31 | 0.26 | 0.22 | NC* |
| Ester Content (%) | 2.8 | 1.4 | 2.8 | 11.2 | 12.6 | 96,5 |
| Density (g/mL) | 0.87 | 0.80 | 0.95 | 0.85 | 0.98 | 0.85 a 0.90 |

1* = Biodiesel with chemical catalyst; 2* = Biodiesel with enzymatic catalyst (0.1mL); 3* = Biodiesel with enzymatic catalyst (0.5); 4* = Biodiesel with immobilized enzymatic catalyst (0.1g); 5* = Biodiesel with immobilized enzymatic catalyst (0.5g); NC* = Not listed.

the transesterification product was closer to ANP standards when using 0.1g of immobilized enzymes. Therefore, the lipase enzyme obtained from the *Bacillus subtilis* could undergo transesterification during biodiesel production. The present study demonstrated the potential of applying enzymes to replace the chemical transesterification process during biodiesel production. It is a valuable contribution to the biofuel industry and should receive special attention or further research to improve yields and large-scale applications.

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