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# Activity and Stability of Lipase Preparations from *Penicillium corylophilum*: Potential Use in Biocatalysis

Biocatalysts with lipase activity were produced by *Penicillium corylophilum* under basal and olive oil-induced conditions. In the presence of olive oil, both the extracellular and the mycelium-bound lipase activities were increased significantly. The four biocatalysts maintained a residual lipase activity after incubation in a wide pH range showing high stability in a defined acidic to neutral pH range. Some differences between biocatalyst residual activities were also observed after incubation at various temperatures or in the presence of organic solvents such as methanol, ethanol, acetone, butanol, hexanol, *n*-hexane, and heptane. Enzymes which are stable in the presence of ethanol or butanol could be useful in eco-friendly processes for biodiesel production.

**Keywords:** Biocatalysis, Biodiesel production, Extracellular lipase, Mycelium-bound lipase, *Penicillum corylophilum*

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## 1 Introduction

The use of enzymes creates opportunities for developing a greener, more sustainable, and increasingly modernized industrial chemistry due to the excellent specificity, mild reaction conditions, energy-saving processes, and simplicity involved. At least 75 % of the industrial enzyme market has been occupied by hydrolytic enzymes [1], and BCC (Business Communications Company) research has estimated th e global market for industrial enzymes was about $3.9^billion for 2011. BCC projects this market t ow at a compounded annual growth rate (CAGR) of 9.1^% to reach $6 billion by 2017 [2].

Biocatalysts have gained increasing attention because of their inherent advantages over chemical catalysts. However, the poor operational stability has always prevented their broad application [3]. Among the hydrolytic enzymes, the lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) possess not only the natural ability to hydrolyze triacylglycerol to glycerol and free fatty acids, but also to catalyze esterification, transesterification, and aminolysis in nonaqueous media [4]. Lipases have emerged as one of the leading biocatalysts, with proven potential for contributing to the multi-billion dollar, underexploited lipid technology bioindustry and are already being applied in a multifaceted range of industrial applications, including widespread usage in the food, detergent, energy, chemical and pharmaceutical industries [5]. This has largely been the result of increasing demand for biocatalysts with novel and specific properties, such as specificity and stability in relation to pH, temperature, and organic solvent exposure [6]. Thus, as with any type of catalyst, the stability of lipases is important for their applications in industry.

Several strategies are now available for increasing operational stability such as the use of stabilizing additives, chemical modification of the enzyme structure, derivatization, immobilization, crystallization, and medium engineering [6]. The use of naturally bound lipases is an important immobilization technique. This type of biocatalyst system is potentially cost-effective because the biomass can be directly utilized [7, 8]. In addition, the use of a biomass-bound biocatalyst also allows the elimination of complex procedures involving enzyme isolation, purification, and immobilization, which can often result in loss of activity as well [9].

Microbial lipases have attracted special attention for industry because of their stability, selectivity, and broad substrate specificity [5]. Although many microorganisms are known to be potential producers of lipases, fungal species are preferable, and the genus *Penicillium* is a known fungal producer of lipases. In nature, the genus *Penicillium* is a versatile and opportunistic fungal taxon that is mostly saprophytic [1].

In this context, the objectives of this article were to produce, evaluate, and compare four biocatalysts such as extracellular and mycelium-bound lipase activities from *P. corylophilum* cultivated in absence and presence of a lipid inductor. In addition, the stability of the four biocatalysts was analyzed and the potential application of this enzyme property in biocatalytic processes was discussed.

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## 2 Experimental

### 2.1 Materials

Sucrose was purchased from Biochem SRL (Argentina). Suprapur® $KH_2PO_4$ anhydrous 99.99 %, $MgSO_4{\cdot}7H_2O$, $Na_2B_4O_7{\cdot}10H_2O$, $CuSO_4{\cdot}5H_2O$ anhydrous 99.99 %, NaOH, KCl, and sodium citrate were obtained from Merck (Germany). Acetic acid 99-100 %, $NH_4NO_3$ ACS reagent ≥ 98 %, and *p*-nitrophenyl palmitate were acquired from Sigma-Aldrich (USA). Hexanol, heptane, and *n*-hexane for synthesis, and methanol, absolute ethanol, butanol, and acetone for HPLC were from Merk (USA). Bovine serum albumin (BSA) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Olive oil was bought at a local market.

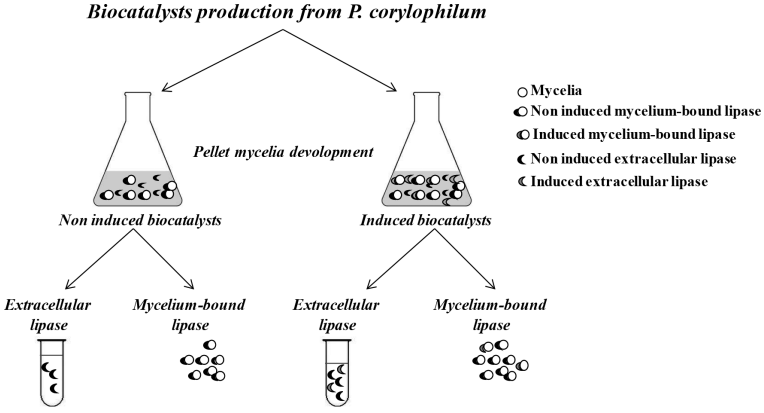


**Figure 1.** Schematic diagram of the production of the four biocatalysts with lipase activity from *Penicillum corylophilum*.

### 2.2 Microorganism and Medium

*Penicillium corylophilum* from CEPESQ culture collection was used throughout this work. It was maintained by monthly transfers to glucose-potato agar slant tubes, incubated at 30 °C, and stored at 4 °C.

The fermentation medium contained (in $g\,L^{-1}$): sucrose, 10.0; $KH_2PO_4$, 1.0; $NH_4NO_3$, 2.0; $MgSO_4{\cdot}7H_2O$, 2.0; $CuSO_4$, 0.06. The initial pH was adjusted to 7.0 using NaOH.

### 2.3 Production of Biocatalysts with Lipase Activity from *P. corylophilum*

Fermentation was carried out at 30 °C in 500-mL shake flasks (250 rpm) containing 100 mL of a fermentation medium. Culture flasks were inoculated with 10 mL of a conidial suspension (~ $10^6$ conidia/mL) from a stock culture. After of incubation, the culture was transferred to another 500-mL shake flask containing either 50 mL 3 % (v/v) olive oil or 50 mL of distilled water and then both were further incubated for six days under the same conditions. With respect to fungal macromorphology, under both culture conditions the mold developed a pelleted form of growth. Mycelium was collected and washed with acetone by filtration at 4 °C for 3 min at 6000 × *g*. These cells were used as the enzyme source.

For determination of dry mycelium, calibration curves were generated with wet and dry mycelium grown either in the medium without olive oil ($R^2$ = 0.972; $y = 2.593x$) or in the medium supplemented with 2 % olive oil ($R^2$ = 0.985; $y = 2.589x$). On the other hand, the microbial culture supernatants were also employed as enzyme sources. A schematic diagram of the experimental setup is presented in Fig. 1. The protein content of the supernatant was determined according to Bradford [10] using BSA (fraction V) as standard. Briefly, the Bradford protein assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

### 2.4 Enzyme Determination

Lipase activity was measured spectrophotometrically at 405 nm with *p*-nitrophenyl palmitate (*p*-NPP) as substrate at 37 °C. About 0.010 g of wet mycelium or 100 µL of a supernatant was added to 1 mL 100 mM phosphate buffer (pH 7.0), containing 2 mM *p*-NPP, 0.1 % (w/v) gum Arabic and 0.4 % (w/v) Triton X-100, according to the method published by Winkler and Stuckman [11]. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per min. The molar absorptivity of *p*-nitrophenol under the given assay conditions was 0.0103 L $\mu mol^{-1} cm^{-1}$. Specific lipase activity was expressed as $mU\,g^{-1}$ of protein (supernatant) or dry biomass (mycelium). Reaction mixtures containing a mycelium-bound enzyme were shaken at 150 rpm.

### 2.5 Effect of Temperature on Biocatalyst Stability

The thermal stability was tested by incubating about 0.010 g of wet mycelium-bound lipase, either induced or non-induced, or 1 mL of extracellular lipase, either induced or non-induced, in 1 mL of 100 mM phosphate buffer (pH 7.0) for 1 h at different temperatures covering the range of 30–55 °C. The remaining enzyme activity was then determined and compared with the control without pre-incubation.

### 2.6 Effect of pH on Biocatalyst Stability

For determination of the pH stability about 0.010 g of wet mycelium-bound lipase, either induced or non-induced, or 1 mL of extracellular lipase, either induced or non-induced, was incubated at 37 °C for 1 h in 1 mL of 100 mM buffers of different pH values, i.e., KCl-HCl, pH 2.0; citrate-phosphate, pH 3.0 and 5.0; phosphate, pH 7.0; borate-HCl, pH 9.0 and 10.0. The remaining enzyme activity was then determined and compared with the control without pre-incubation.



  

### 2.7 Effect of Solvent on Biocatalyst Stability

Assaying of solvent stability was carried out by incubating about 0.010 g of wet mycelium-bound lipase, either induced or non-induced, at 37 °C for 1 h in 1 mL of each organic solvent. The mycelium-bound enzyme was collected by filtration and the residual activity was then quantified.

The extracellular lipase, either induced or non-induced, was diluted at a ratio of 1:1 with each organic solvent. The reaction mixture was incubated at 37 °C for 1 h, and the residual activity was then quantified. In case of obtaining a biphasic system, the aqueous phase was sampled in order to measure the residual enzyme activity.

### 2.8 Statistical Analysis

Statistical analysis was performed using the Minitab (Minitab Inc) software version 14 for Windows. ANOVA was used to evaluate the mean differences among treatments. Subsequent comparisons were performed with Tukey's post-hoc test. Results were presented as the mean ± SD. Differences were accepted as significant when $P < 0.05$.

## 3 Results and Discussion

### 3.1 Lipase Activity of Induced and Non-Induced Biocatalysts

On the third day of incubation in the medium supplemented with olive oil, the extracellular lipase activity increased by four times (163 mU $g^{-1}$) with respect to that determined under non-induced conditions (42 mU $g^{-1}$); see Fig. 2. The non-induced mycelium-bound lipase also showed the highest specific activity (146 mU $g^{-1}$) after three days of cultivation; while, under inducing conditions, this maximum (188 mU $g^{-1}$) was shifted to the following day.

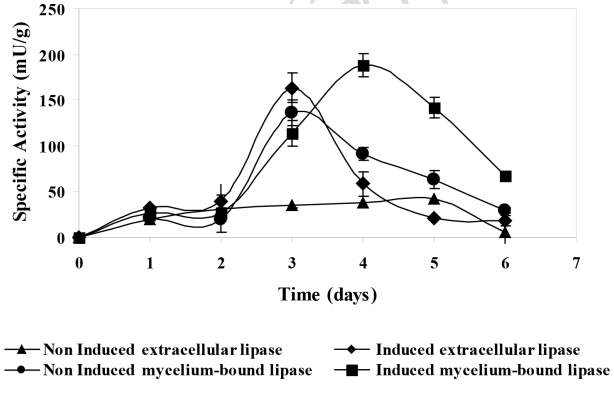


**Figure 2.** Time course of specific extracellular and mycelium-bound lipase activities during fermentation by *Penicillium corylophilum* using a medium without olive oil (non-induced condition) or supplemented with 2 % olive oil (induced condition). Error bars represent the standard deviation calculated from at least three independent experiments.

The extracellular and the mycelium-bound lipase activities produced in the presence of lipids showed different synthesis patterns. Additionally, the production profile of the induced extracellular lipase from *P. corylophilum* differed from that reported for lipase preparations from *Aspergillus niger* MYA 135 produced in the presence of 2 % olive oil. In these works, the highest specific activities were obtained after four days of incubation [7, 12].

### 3.2 Effect of Temperature on Biocatalyst Stability

#### 3.2.1 Non-Induced Biocatalysts

According to Fig. 3, the mycelium-bound lipase was found to be more thermostable than the corresponding extracellular lipase. This effect was especially notable at higher temperatures of 50 °C and 55 °C. After the treatment at 50 °C, the residual activity of mycelium-bound lipase was found to be 2.5 times (75 % residual activity) more stable than the extracellular lipase (30 % residual activity). Similar behavior was observed at 55 °C, where the mycelium-bound lipase was found to be 2.3 times more stable (72 % residual activity) than the extracellular lipase. Razak et al. [13] studied the thermal stability of both extracellular and mycelium-bound lipase from *Rhizopus oryzae*. They observed that the membrane-bound lipase retains about 55 % of its activity at 50 °C while the activity of the extracellular lipase from the same fungus is reduced to 24 %. However, after incubation at 55 °C for 30 min, the membrane-bound lipase from *Rhizopus oryzae* is less stable (~ 60 % of residual activity) than the mycelium-bound lipase from *P. corylophium*. On the other hand, the non-induced extracellular lipase from *P. corylophium* was fairly stable up until 40 °C after 1 h of treatment, with the stability gradually decreasing with higher temperature treatments (Fig. 3). Finally, these results show that the mycelium-bound lipase was more stable, probably due to the protective effect of the cell.

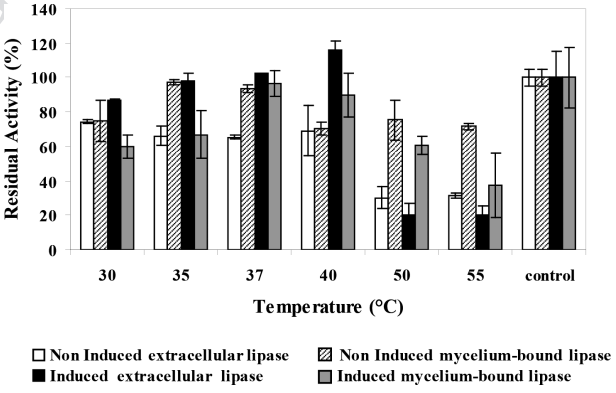


**Figure 3.** Effect of temperature on specific extracellular and mycelium-bound lipase activities from *Penicillium corylophilum* using a medium without olive oil (non-induced condition) or supplemented with 2 % olive oil (induced condition). Remaining activity was compared with a control medium without treatment. Error bars represent the standard deviation calculated from at least three independent experiments.



  

### 3.2.2 Induced Biocatalysts

The induced mycelium-bound lipase was 3.0 and 1.8 times more stable than the induced extracellular lipase at 50 °C and 55 °C, respectively (Fig. 3). Here again, the protective effect of the mycelium can be observed.

Interestingly, the induced extracellular lipase proved to be more stable than the induced mycelium-bound lipase until 40 °C. This effect was not observed under non-induced conditions (Fig. 3). Thus, a discrepancy in terms of stability between induced and non-induced extracellular lipase activities was observed. One possible explanation for these results is that under induced conditions, the enzyme could be protected by the olive oil [14].

## 3.3 Effect of pH on Biocatalyst Stability

### 3.3.1 Non-Induced Biocatalysts

The pH-based stability patterns for non-induced extracellular and mycelium-bound lipase activities were similar maintaining a residual lipase activity in a pH range of 2–10. Both biocatalyst preparations showed the highest stability in the range between pH 3 and 7 ($p$ = 0.0780). In the alkaline pH region, the non-induced extracellular lipase was significantly more stable than the non-induced mycelium bound lipase (pH 9, $p$ = 0.0010 and pH 10, $p$ = 0.0017); see Fig. 4.

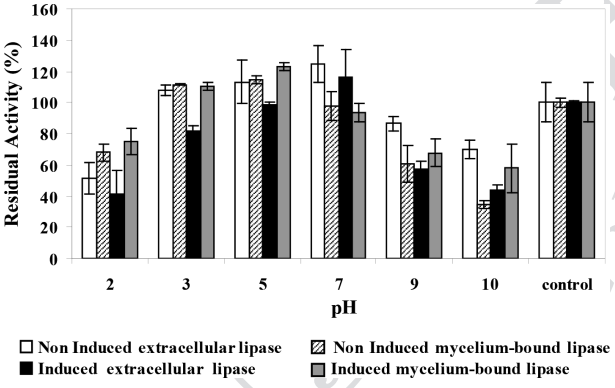


**Figure 4.** Effect of pH on specific extracellular and mycelium-bound lipase activities from *Penicillium corylophilum* using a medium without olive oil (non-induced condition) or supplemented with 2 % olive oil (induced condition). Remaining activity was compared with a control medium without treatment. Error bars represent the standard deviation calculated from at least three independent experiments.

### 3.3.2 Induced Biocatalysts

The induced mycelium-bound lipase from *P. corylophilum* showed the highest stability at pH values of 3 and 5 ($p$ = 0.1130). This pattern of residual activity was different to that reported for the induced mycelium-bound lipase from *Aspergillus niger* MYA 135. In that case, two maxima of residual activity are observed, one at pH 3 and another one at pH 7 [7]. The induced extracellular lipase from *P. corylophilum* was also stable at pH 5 while at pH 7 the enzyme displayed the same residual activity to that obtained in the control without treatment ($p$ = 0.3888); see Fig. 4.

The acidic lipases are potential enzymes for use in the food and flavor industry, where aroma esters are formed under acidic conditions [15]. The four biocatalysts not only maintained a residual activity below pH 3 (pH 2) but also above pH 7 (alkaline region, pH 9 and 10) (Fig. 4). This property may be useful in many sanitation and waste-treatment liquid formulations, where products exhibit activity in a wide pH range. Enzymatic methods are preferred to chemical methods in many sanitation applications such as for grease traps, detergents, prespotters, and industrial cleaning compounds [16].

## 3.4 Effect of Organic Solvents on Biocatalyst Stability

Many enzymes are required to function in nonaqueous media. Thus, much of the current focus in enzyme technology involves enhancement of enzyme activity and stability under nonconventional conditions.

In this work, the non-induced and induced extracellular lipase activities showed high residual activity after treatments with miscible solvents. In fact, high enzyme stability was observed when these biocatalysts were pre-incubated in the presence of ethanol or acetone (Tab. 1). This behavior could be explained considering the fact that the addition of miscible solvents to a solution, such as ethanol or acetone, may cause aggregation of the free enzymes.

Some works reported that such insoluble aggregates display high stability in the presence of organic solvents [17, 18]. This increment in residual activity could be a direct result of the decrease in flexibility, which suppresses the propensity of enzymes towards unfolding and accompanying loss of tertiary structure necessary for activity [19]. On the other hand, the presence of ethanol may contribute to the rearrangement of the secondary structure improving the residual activity as well [20]. The stability of the non-induced and induced extracellular biocatalysts in ethanol is a property that also makes them attractive for biofuel production. At present, commercial biodiesel consists of fatty acid methyl esters (FAME) produced with methanol. However, with the increasing production of bioethanol, this raw material will become the product of choice for synthesis of fatty acid ethyl esters (FAEE) [21]. Since fungi can be grown inexpensively, our results suggest the potential use of these biocatalysts in biodiesel production.

Besides, the non-induced and induced extracellular lipases were also active in the presence of immiscible solvents (Tab. 1). Solvents with high log $P$ and high solvation capacity such as the higher alcohols, e.g., butanol or hexanol, are strong denaturants, causing inactivation [6]. However, the induced extracellular lipase showed 88.2 ± 12.2 % and 86.3 ± 3.2 % of residual activity after treatments with butanol and hexanol, respectively, while the non-induced extracellular lipase pre-incubated with butanol had 52.0 ± 1.2 % of residual activity (Tab. 1).

The non-induced and the induced mycelium-bound lipase activities exhibited high stability in acetone maintaining



  

**Table 1.** Effect of organic solvents on specific extracellular and mycelium-bound lipase activities from *Penicillium corylophilum* using a medium without olive oil (non-induced condition) or supplemented with 2 % olive oil (induced condition). Remaining activity was compared with a control medium without treatment. Results are presented as the mean ± SD.

| | | Residual activity [%][a)] | | | |
|---|---|---|---|---|---|
| | | Non-induced biocatalysts | | Induced biocatalysts | |
| Solvent | log P | Extracellular lipase activity | Mycelium-bound lipase activity | Extracellular lipase activity | Mycelium-bound lipase activity |
| Methanol | –0.77 | 57.2 ± 3.6 | 55.5 ± 7.0 | 89.2 ± 6.0 | 45.3 ± 15.5 |
| Ethanol | –0.31 | 123.4 ± 3.7 | 75.5 ± 13.2 | 119.5 ± 10.8 | 57.1 ± 4.5 |
| Acetone | –0.20 | 135.9 ± 5.0 | 99.5 ± 8.3 | 146.1 ± 2.2 | 95.0 ± 6.0 |
| Butanol | 0.80 | 52.0 ± 1.2 | 57.8 ± 9.4 | 88.2 ± 12.2 | 95.2 ± 2.4 |
| Hexanol | 2.03 | 3[illegible]2.8 ± 8.9 | 58.3 ± 2.7 | 86.3 ± 3.2 | 63.2 ± 13.2 |
| n-Hexane | 3.60 | 40.1 ± 1.1 | 70.1 ± 14.4 | 25.9 ± 12.5 | 76.2 ± 6.6 |
| Heptane | 4.32 | 46.3 ± 6.0 | 76.6 ± 9.8 | 23.2 ± 14.8 | 70.8 ± 14.7 |

a) Lipase activity without solvent pre-incubation served as control and was considered as 100 % of activity.

99.5 ± 8.3 % and 95.0 ± 6.0 % of residual activity, respectively (Tab. 1). Lipases that are active and stable in polar solvents would open new possibilities and opportunities for biocatalysis involving polar substrates [22].

An important difference between the non-induced and induced mycelium-bound lipase activities was in terms of their stabilities in the presence of butanol. Thus, the induced mycelium-bound lipase indicated a higher level of residual activity after treatment with this alcohol (95.2 ± 2.4 % of residual activity) than the non-induced biocatalyst preparation (57.8 ± 9.4 % of residual activity). A lipase with high butanol tolerance is of interest because of this raw material can be produced from a renewable source and could be used for biofuel synthesis via lipase biocatalysis [23–25].

Finally, the extracellular and the mycelium-bound lipase activities also showed different resistance towards hydrophobic solvents such as *n*-hexane and heptane. The non-induced mycelium-bound lipase was more stable than the non-induced extracellular lipase by a factor of 1.75 and 1.67 for *n*-hexane and heptane, respectively. In turn, the induced mycelium-bound lipase was around three times more stable than the induced extracellular lipase for both solvents (Tab. 1). In addition, the non-induced mycelium-bound lipase from *P. corylophilum* was more resistant to heptane than the non-induced mycelium-bound lipase from *Aspergillus niger* MYA 135 [7].

## 4 Conclusions

The presented results suggest that by manipulating the culture conditions of *Penicillium corylophilum* it is possible to produce biocatalysts with different enzymatic properties, which could be applied in a diverse range of industrial processes. Selectively modified enzymes could also be used to decrease the costs of a bioprocess. On the other hand, by combining these potentially cost-effective biocatalysts with the high ethanol or butanol enzyme tolerance, significant reduction could be expected for the cost of biodiesel production. Current research is now being directed towards statistical optimization using an improved biocatalyst for biodiesel production from a variety of triglyceride sources.

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