Pretreatment of an Induced Mycelium-Bound Lipase from Aspergillus niger *MYA 135 Improves Its Hydrolytic and Synthetic Activity*

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Abstract Whole-cell enzymes have been used as biocatalysts in a variety of reactions, such as free fatty acid production and the synthesis of fatty acid esters. In the present study, enzyme pretreatments with PEG, MES, Tween 80, Saponine, $MgCl_2 \cdot H_2O$, $CaCl_2$ and different pH values were evaluated by using the Plackett–Burman statistical design to improve both the hydrolytic and synthetic activity of an induced mycelium-bound lipase from *Aspergillus niger* MYA 135. Interestingly, the preincubation at pH 4 had a significant effect on both the hydrolytic and transesterification activity, demonstrating the influence of the correct ionisation state on these activities. Meanwhile, the enzyme pretreatment with $MgCl_2$ for in situ water activity control positively affected the esterification catalyst. Thus, compared with the control without pretreatment, the hydrolytic and the transesterification activities increased to 60.1 and 60.8 %, respectively, and with respect to the esterification reaction, the conversion was improved 2.33 times. Based on these results, by applying a simple pretreatment to the biocatalyst, the catalyst's activity toward hydrolysis and synthesis was enhanced.

Keywords Plackett–Burman · Mycelium-bound lipase · Hydrolysis · Synthesis · *Aspergillus niger*

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1 Introduction

Lipases or triacylglycerol acylhydrolases (E.C. 3.1.1.3) are enzymes belonging to the hydrolases group, which have a remarkable catalytic activity toward insoluble triacylglycerols to generate mono- and diacylglycerols, glycerol and free fatty acids. In addition to their natural function, lipases can catalyse esterification, interesterification and transesterification reactions in aqueous and non-aqueous media [1, 2].

Lipases have emerged as one of the leading biocatalysts, with proven potential for contributing to the multi-billion dollar and under-exploited lipid technology bio-industry and are already being used over a multifaceted range of industrial applications, including widespread use in the food, detergent, energy, chemical and pharmaceutical industries [3].

Lipases of microbial origin represent the most widely used class of enzymes in biotechnological applications and organic chemistry. For example, lipases have been researched as esterification/transesterification catalysts for the production of biodiesel [4]. In addition, biodiesel production through the hydroesterification route catalysed by lipases has also been explored [5].

Fungi are one of the most important sources of lipases for industrial applications, and lipases are currently being produced extracellularly via submerged or solid-state fermentations by several fungal species [6].

An alternative source of lipases is organisms that produce intracellular enzymes expressed on their cell walls or membranes. Such lipases are employed as "whole-cell" biocatalysts, instead of extracellular enzymes, that require extraction and purification from culture media. If organisms that produce lipases can be readily and inexpensively cultured in large quantities, such as whole-cell systems,

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they could become promising and inexpensive biocatalysts [7]. Thus, among the established whole-cell biocatalyst systems, filamentous fungi have arisen as the most robust whole-cell biocatalyst for industrial applications [8].

In a previous study, the synthesis of several esters using "whole-cell" lipase from *Aspergillus niger* MYA 135 was evaluated. Induced mycelium-bound lipase activity has selected for its reactivity in esterification and transesterification reactions with long-chain fatty acid esters in the presence of ethanol. Ethyl palmitate, a component of biodiesel, was synthesised. Thus, combining a versatile whole-cell biocatalyst system with its high tolerance to ethanol could represent a significant reduction in the cost of biodiesel production [9].

Knowing the variables that affect the activity of mycelium-bound lipase in hydrolysis and synthesis reactions in organic and aqueous solvents should contribute to increasing the catalytic activity of this biocatalyst. The regulation of pH memory, water activity or the surfactant effect on the biocatalyst may be necessary if the standardisation of enzymatic preparation should prove difficult and if the enzyme exhibits poor activity. The adjustment of an enzyme's microenvironment can contribute to its increased stability. Therefore, enzymatic preparations used for large-scale biodiesel production may require this treatment [10].

In the present study, enzyme pretreatments were evaluated by using the Plackett–Burman statistical design to improve both the hydrolytic and the synthetic activity of an induced mycelium-bound lipase from *A. niger* MYA 135.

2 Experimental Section

2.1 Microorganism and Maintenance

Aspergillus niger ATCC MYA 135, formerly *A. niger* 419 from the PROIMI culture collection, was used throughout this project. It was maintained by monthly transfer onto glucose–potato agar slants, incubated at 30 °C and stored at 4 °C.

2.2 Fermentation Medium

The fermentation medium was as follows (in g/l): 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 with NaOH.

2.3 Enzyme Production

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 (approximately 10^6 conidia/ml) from a stock culture. After 24 h of incubation, the culture was transferred to another 500 ml shake flask containing 50 ml 3 % (v/v) olive oil and incubated for 4 days under the same conditions. The mold developed in pelleted growth form. Mycelium was collected, washed with acetone and centrifuged at 6,000×*g* at 4 °C for 3 min; cells were used as the enzyme source. A calibration curve was generated with both wet and dry mycelium grown in medium supplemented with olive oil ($R^2 = 0.982$; $y = 2.5899$x).

2.4 Enzyme Assaying

2.4.1 Hydrolysis Reaction

Hydrolytic activity was measured with *p*-NPP (*p*-nitrophenyl palmitate; C_{16}). A reaction was prepared by adding approximately 0.010 g of wet mycelium to 1 ml of 100 mM phosphate buffer (pH 7.0) containing 2 mM *p*-NP derivative, 0.1 % (w/v) Arabic gum and 0.4 % (w/v) Triton X-100 [11]. The molar extinction coefficient of *p*-nitrophenol (*p*-NP) under the given assay conditions was 0.0103 $\mu M^{-1}\ cm^{-1}$. One unit of enzyme activity was defined as the amount of biocatalyst that released 1 µmol of *p*-NP per min. Specific activity was expressed as milliunits per gram of dry mycelium weight.

2.4.2 Transesterification Reaction

Enzymatic transesterification was carried out as follows: *p*-NPP (*p*-nitrophenyl palmitate; C_{16}) dissolved in acetone was added at a final concentration of 2 mM; 100 µl of ethanol and approximately 0.010 g of wet mycelium were added to 800 µl of *n*-hexane. The reaction mixture was shaken (150 rpm) for 1 h at 37 °C. The *p*-NP was extracted from *n*-hexane with 1 ml 0.25 M Na_2CO_3. The absorbance of the supernatant containing *p*-NP was determined at 405 nm. A reaction mixture without alcohol served as a hydrolysis control. In the absence of a biocatalyst, no reaction was observed. The molar extinction coefficient of *p*-NP under these assay conditions was 0.0205 $\mu M^{-1}\ cm^{-1}$. One unit of transesterification activity was defined as the amount of biocatalyst that released 1 µmol of *p*-NP per min. Specific transesterification activity was expressed as milliunits per gram of dry mycelium weight.

2.4.3 Esterification Reaction

Enzymatic esterification was carried out in 10 ml of *n*-hexane. Palmitic acid was used at a final concentration of 2 mM. To the reaction mixture, 1,000 µl of ethanol was added. Approximately 0.10 g of wet mycelium was used. The reaction mixture was shaken (150 rpm) for 24 h at

Table 1 Plackett–Burman experimental design used to evaluate 7 variables with coded values and 24 runs for induced mycelium-bound lipase from *A. niger* MYA 135 with respect to hydrolysis (Y1), transesterification (Y2) and esterification (Y3) reactions. X1: PEG; X2: pH; X3: MES; X4: Tween 80; X5: Saponine; X6: $MgCl_2$; X7: $CaCl_2$

Duplicates for sample	Run	X1 PEG	X2 pH	X3 MES	X4 T80	X5 Sap	X6 $MgCl_2$	X7 $CaCl_2$	Hydrolysis (mU/g) (Y1)	Transesterification (mU/g) (Y2)	Esterification (%) conversion (Y3)
T5	21	−1	1	1	1	−1	1	1	106.3	39.4	48.8
T10	22	−1	1	1	−1	1	−1	−1	79.5	56.0	37.7
T1	1	1	1	−1	1	1	−1	1	94.8	40.4	11.1
T11	15	−1	−1	1	1	1	−1	1	88.1	46.3	15.5
T4	4	−1	−1	−1	−1	−1	−1	−1	126.0	40.6	33.3
T2	2	1	−1	−1	−1	1	1	1	163.4	53.5	35.5
T4	12	−1	−1	−1	−1	−1	−1	−1	158.4	34.9	22.2
T13	23	1	−1	1	1	−1	1	−1	73.3	49.1	33.3
T9	18	−1	−1	−1	1	1	1	−1	87.3	76.3	35.5
T8	14	−1	1	−1	−1	−1	1	1	115.9	20.2	35.5
T3	17	1	1	1	−1	1	1	−1	136.6	26.9	33.3
T6	7	1	−1	1	−1	−1	−1	1	137.3	46.6	22.2
T3	3	1	1	1	−1	1	1	−1	124.8	28.1	31.1
T9	9	−1	−1	−1	1	1	1	−1	95.9	62.9	35.5
T13	13	1	−1	1	1	−1	1	−1	74.8	67.6	28.8
T5	5	−1	1	1	1	−1	1	1	105.6	40.5	44.4
T16	16	1	1	−1	1	−1	−1	−1	74.8	33.0	48.8
T2	20	1	−1	−1	−1	1	1	1	130.8	38.3	37.7
T8	8	−1	1	−1	−1	−1	1	1	110.8	22.4	40.0
T6	6	1	−1	1	−1	−1	−1	1	127.1	59.5	22.2
T1	24	1	1	−1	1	1	−1	1	96.3	30.9	13.3
T16	19	1	1	−1	1	−1	−1	−1	73.3	45.5	35.5
T11	11	−1	−1	1	1	1	−1	1	104.9	54.1	20.0
T10	10	−1	1	1	−1	1	−1	−1	76.4	59.2	35.5
Control									99.8	41.3	21.0
Control									83.5	43.4	19.0

37 °C. The ester content was quantified by the alkalimetric titration of unreacted acid with 0.1 N NaOH, directly measuring the pH with a pH-meter. The conversion (%) in ester synthesis was based on acid consumed.

2.5 Experimental Design

Screening designs are commonly used when little is known about a system or process. The Plackett–Burman design analyses input data, ranks the variables in order of magnitude of effect, and designates signs to the effects to indicate whether an increase in factor value is advantageous or not [12].

The Plackett–Burman statistical design was used in the present study to evaluate the main effects of seven independent variables. A 7-factor 24-run (12 mixtures with respective random duplicated) Plackett–Burman statistical design at two levels was generated using MINITAB 14 statistical experiment design software (Table 1). The dependent and levels of independent variables evaluated in this study are listed in Table 2. The final concentrations of the variables evaluated were X1 (PEG 20.000, 1:10), X2 (pH 4, citrate phosphate buffer 100 mM and pH 7, phosphate buffer 100 mM), X3 (MES, 100 mM), X4 (Tween 80, 1 % w/v), X5 (Saponine, 10 % w/v), X6 ($MgCl_2.H_2O$, 100 mM) and X7 ($CaCl_2$, 100 mM). The responses estimated in the present work were Y1 (hydrolysis activity), Y2 (transesterification activity) and Y3 (esterification activity).

A statistical analysis was performed with data obtained after the incubation of 0.1 g of the biocatalyst for 1 h in 10 ml of each pretreatment reaction mixture at 37 °C and 150 rpm. Then, the mycelium was recovered by centrifugation and hydrolysis; transesterification and esterification reactions were evaluated. In each case, a control without pretreatment was used ($control_{wt}$).

Both *t* test and *p* value statistical parameters were used to confirm the significance of the factors studied. Small

Table 2 Effect of variable and statistical analysis of induced mycelium-bound lipase from *A. niger* MYA 135 using Plackett–Burman design for hydrolysis, transesterification and esterification reactions

Code	Variable	Level		Hydrolysis			Transesterification			Esterification		
				Effect (*E*)	Statistical Significance		Effect(*E*)	Statistical significance		Effect(*E*)	Statistical significance	
		−1	+1		Test *t*	*p* value		Test *t*	*p* value		Test *t*	*p* value
X1	PEG	Absence	Presence	7.81	1.17	0.260	−2.78	−0.59	0.562	−4.26	−1.28	0.22
X2	pH	4	7	**−20.71**	−3.10	**0.007**	−15.60	**−3.32**	**0.004**	6.11	1.84	0.08
X3	MES	Absence	Presence	−3.13	−0.47	0.646	6.20	1.32	0.205	−0.93	−0.28	0.79
X4	Tween 80	Absence	Presence	**−26.78**	−4.01	**0.001**	8.32	1.77	0.096	−1.29	−0.39	0.70
X5	Saponine	Absence	Presence	−5.02	−0.75	0.463	6.13	1.31	0.210	−6.11	−1.84	0.08
X6	$MgCl_2$	Absence	Presence	**14.91**	2.23	**0.040**	−1.82	−0.39	0.704	**10.18**	3.06	**0.007**
X7	$CaCl_2$	Absence	Presence	10.33	1.55	0.142	−7.33	−1.56	0.138	−5.37	−1.61	0.13

This result are in bold for facilitate the result interpretation

p values were associated with larger *t* values because they imply that the effects (or coefficients) were much greater than the standard error. Thus, $p < 0.05$ suggested significance at the 0.05 level. This also corresponded to a 95 % confidence level for the test of the hypothesis that the effects (or coefficients) in question were equal to zero.

3 Results and Discussion

The main purpose of this work was to evaluate enzyme pretreatments to improve both the hydrolytic and synthetic activity of an induced mycelium-bound lipase from *A. niger* MYA 135.

The experimental conditions and the results for lipase activity in the Plackett–Burman design are shown in Scheme 1; Table 1, respectively. The statistical analysis was performed with data obtained after 1 h of incubation of the biocatalyst with each pretreatment. An enzyme activity control in the pretreatment mixture reaction was prepared after the exposure of the biocatalyst. Residual lipase activity was not detected, indicating no release of the enzyme into the pretreatment mixture reaction. The estimated effects for each variable were determined and are reported in Table 2. The main effect was estimated by evaluating the difference in the process performance caused by a change from the low (−1) to the high (+1) level of the corresponding factor. The process performance was measured by the lipase activity response. The *t* test and *p* value parameters are shown in Table 2.

3.1 Hydrolysis Reaction

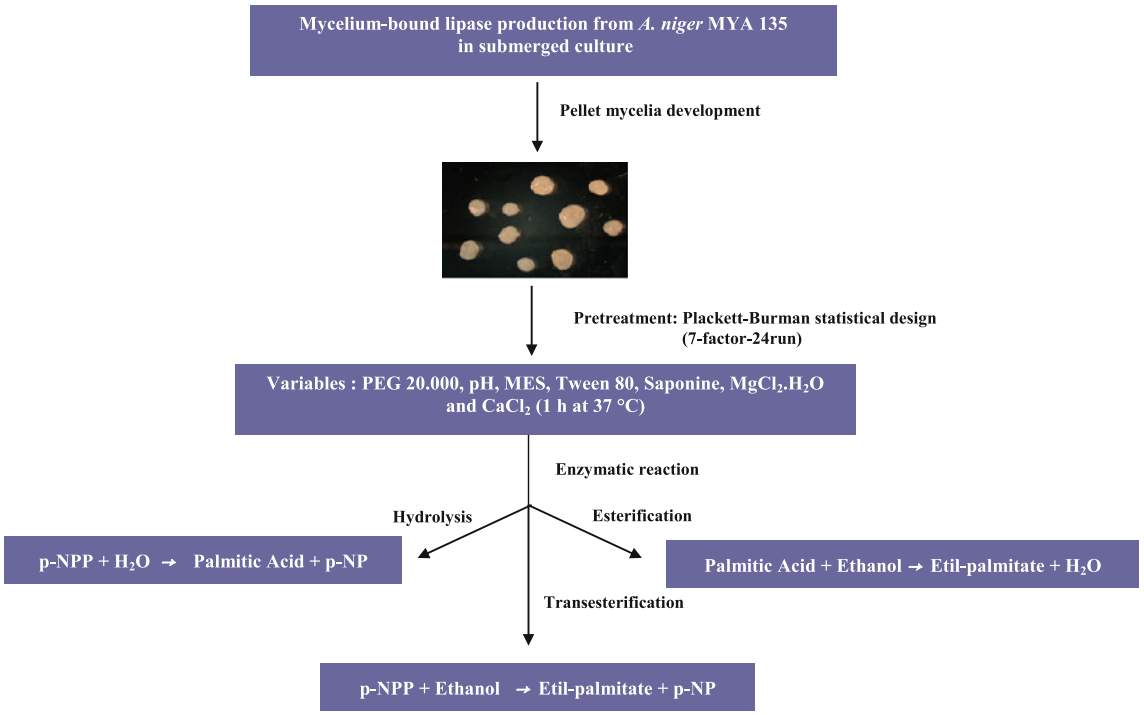
A significant increase in lipase hydrolytic activity after 1 h of pretreatment, for most of the experiments, is shown in Table 1. The highest hydrolytic specific activity observed after the pretreatment was 147.1 ± 23.1 mU/g against 91.6 ± 11.5 mU/g from the $control_{wt}$ ($p = 0.0066$). It can be seen that the most relevant variables concerning the hydrolytic lipase activity were pH ($p = 0.007$), Tween 80 ($p = 0.001$) and $MgCl_2$ ($p = 0.040$). Moreover, pH 4 and $MgCl_2$ were not only statistically significant in lipase activity but also exhibited a positive influence, showing an increase in hydrolytic activity when both were present (Table 2). However, the presence of Tween 80 showed a negative influence (Table 2).

3.2 Transesterification Reaction

A significant increase in lipase transesterification activity after 1 h of pretreatment, for most of the experiments, is shown in Table 1. The highest transesterification activity observed was 69.6 ± 9.4 mU/g against 42.3 ± 1.5 mU/g from the $control_{wt}$ ($p = 0.0068$). The statistically significant variable associated with the increase in transesterification activity was the pH ($p = 0.004$) (Table 2). The transesterification activity of the biocatalyst was enhanced when exposed to pH 4 (*E* −15.60) (Table 2).

3.3 Esterification Reaction

A significant increase in lipase esterification activity after 1 h of pretreatment, for most of the experiments, is shown in Table 1. The highest esterification activity observed was 46.7 ± 3.14 % against 20.0 ± 1.0 % from the $control_{wt}$ ($p = 0.0235$). The statistically significant variable associated with the increase in esterification activity was $MgCl_2$ ($p = 0.007$) (Table 2). The esterification activity of the biocatalyst was enhanced when it was exposed to $MgCl_2$ (*E* 10.18) (Table 2).



Scheme 1 Schematic representation of the performance of mycelium-bound lipase from *A. niger* MYA 135 as a biocatalyst after pretreatment using a Plackett–Burman design

3.4 Effect of pH on Mycelium-Bound Lipase Activity

The hydrolytic activity of the biocatalyst was increased to 1.60 times that of the control$_{wt}$ when it was previously exposed to pH 4. This result is in agreement with that of a previous work in which the optimal pH found for an induced mycelium-bound lipase from *A. niger* MYA 135 was pH 4 [13]. This effect could be attributed to the conformational modulation of the biocatalyst associated with pH due to the flexible and sensitive conformation of the enzyme, which is responsible for the significant variation in hydrolytic activity with the tuning of the biocatalyst's microenvironment [14]. On the other hand, this increase in the mycelium-bound lipase activity could also be due to the influence of environmental modifications on hyphal morphological patterns in the mycelia of *A. niger*. The pH could affect cell membrane function, cell morphology and structure, solubility or the ionic state of substrates [15]. In a previous study, changes in the *A. niger* MYA 135 morphology were directly related to the mycelium-bound enzyme activity (NAGASe activity) [16]. Other authors have also indicated that morphological changes significantly affect the lipase activity of *Rhizopus chinensis* and suggested that the presence of aggregated mycelia results in high lipase activity [17].

The transesterification activity of the biocatalyst was enhanced when it was previously exposed to pH 4. The activity obtained after pretreatment was 1.64 times that of the control$_{wt}$. One of the most influential parameters affecting enzymatic activity in aqueous solution is pH, but it has no meaning in organic solvents. Instead, it was found that enzymes in such media have something called pH memory: their catalytic activity reflects the pH of the last aqueous solution to which they were exposed [18]. This phenomenon is because protein ionogenic groups retain their latest ionisation state in aqueous solution and when subsequently placed in organic solvents. Consequently, the enzymatic activity in such media can be much enhanced if the enzymes are exposed to aqueous solutions at the pH optimal for catalysis [18, 19].

In this study, the ionisation state of the biocatalyst in organic solvents was optimised and hence the enzymatic activity maximised by adding appropriate buffer pairs of acids and their conjugated bases. Interestingly, the biocatalyst was able to enhance the transesterification activity only by controlling the pH value. A different behaviour was observed in the mycelium-bound lipase from *R. chinensis*. The memory of this lipase at acidic (under 6) pHs resulted in a huge activity loss [20].

With respect to the esterification reaction, the pH did not show a significant effect ($p = 0.08$). This behaviour allows the biocatalyst to be used in both acid and neutral pH, unlike other mycelium-bound lipases that show esterification activity, for example, only at neutral pH (7.5) [21].

The application of enzymes bearing appropriate charges on their polar groups (obtained through preincubation in

buffer solutions with suitable pH) in esterification/transesterification reactions carried out in organic solvents could contribute to the higher catalytic activity of these enzymes.

3.5 Effect of $MgCl_2$ and $CaCl_2$ on Mycelium-Bound Lipase Activity

The hydrolytic activity was slightly enhanced when the biocatalyst was previously exposed to $MgCl_2$. This behaviour could be attributed to the formation of insoluble Mg salts of fatty acids released in the hydrolysis, thus avoiding product inhibition. Mg^{2+} ions are proposed to stimulate lipase-catalysed hydrolysis, not only by possibly removing fatty acids from the oil–water interface but also by activating the lipase [22, 23]. In fact, Sharon et al. [22] proposed that Mg^{2+} ions stimulate castor oil's hydrolysis more efficiently than Ca^{2+} ions. Different results were observed for *R. chinensis* with respect to hydrolysis lipase activity. An inhibitory effect was observed in the presence of Mg^{2+}, and no significant effect after 1 h of incubation was observed in the presence of the cation Ca^{2+} [20].

Water plays a crucial role in the enzyme structure and function of proteins and thus determines the activity and stability of enzymes. Water dynamics and the salt-activation mechanism of enzymes in organic solvents can directly influence enzyme activity in a positive or negative manner by changing the activity of water (aw) in the reaction system [24]. Salt hydrates tend to affect the ionisation state of acidic residues in proteins and hence enzymatic activity. In fact, salt hydrates are able to affect the pH memory of enzymes obtained from different aqueous pHs [25]. Thus, the effect of salt hydrates was evaluated on the biocatalyst of *A. niger* MYA 135.

The ester conversion by esterification was enhanced when the biocatalyst was previously exposed to $MgCl_2$. The extent of ester conversion observed after the pretreatment 2.33 times that of the $control_{wt}$. However, with respect to the transesterification reaction, the pretreatment of the mycelium-bound lipase from *A. niger* MYA 135 with $MgCl_2$ did not show a significant effect ($p = 0.704$). This result indicates that the control over the activity of water by salt hydrates such as $MgCl_2$ is more significant in the esterification reaction because water is a product of this reaction. During esterification, water is able to act like substrate, competing with the alcohol in the reaction [26]. In this case, $MgCl_2$ could absorb the water formed during the condensation reaction and maintain the water activity at the level that stimulates ethyl palmitate synthesis. Moreover, it is interesting that mycelium-bound lipase from *A. niger* MYA 135 showed higher ester conversion by esterification when the biocatalyst was pretreated with $MgCl_2$, becoming effective in enhancing ester conversion compared with the control. Salt hydrates are typically used to improve the esterification activity of lipases. For example, the lipase from *R. arrhizus* showed higher ester conversion when the activity of water was controlled using $MgCl_2$ in the reaction [27]. In this study, the biocatalyst showed higher activity when pretreated with the salt hydrate and then used in the esterification reaction, indicating that $MgCl_2$ could be an important component during esterification. Meanwhile, the alternative of using the salt hydrate in the pretreatment of the biocatalysts prior to the esterification reaction is interesting.

3.6 Effect of Surfactant and MES on the Mycelium-Bound Lipase Activity

The presence of Tween 80 exhibited a negative and significant influence on the hydrolysis activity ($p = 0.001$). In fact, the highest hydrolysis activity values (between 113.3 ± 3.6 and 147.1 ± 23.1 mU/g) were observed in all pretreatments in which Tween 80 was absent, and no significant difference was observed between these values ($p = 0.1743$). Surfactants, such as Tween 80, are used as enzyme activators due to their ability to improve substrate solubility. However, in some cases, the presence of a surfactant leads to significant inhibition [28]. This could be attributed to the excessive adsorption of the surfactant on an enzyme surface, resulting in the diffusional limitation of the substrate during reaction [28]. It should be note that Tween 80 could be a substrate of the reaction and might compete with the *p*-NPP in the hydrolysis reaction. In fact, an inhibition effect on the hydrolytic activity of the enzyme was observed by other researchers. Glogauer et al. [29] reported that Tween 80 inhibited the activity of the lipase LipC12 from a metagenomic library. This effect was due to the long acyl ester chains of this detergent, which make it a substrate for LipC12 and therefore was a competitive inhibitor in the assay. Thus, it could be considered that the inhibitor effect of Tween 80 on mycelium-bound lipase from *A. niger* MYA 135 might be due to the competition between the substrates in the hydrolysis reaction (Tween 80 and *p*-NPP). Furthermore, in the condition assay, there were no statistically significances in the effect of PEG ($p = 0.260$) and saponine ($p = 0.463$) on the biocatalyst with respect to the hydrolysis reaction. The biocatalyst behaviour suggests that the exposure of the enzyme to some surfactants is not indispensable to the hydrolysis activity of the enzyme.

Generally, enzymes experience a decrease in their activity in organic solvents due to their reduced structural flexibility. The addition of small quantities of water to enzyme suspensions in anhydrous solvents [30] or raising the thermodynamic activity of water by other means can increase the enzymatic activity of such systems by several orders of magnitude. To a certain extent, this activating

effect of water can be mimicked by other solvents capable of forming multiple hydrogen bonds, such as PEG (Zaks and Klivanov [30]) or MES salt [31]. In this condition assay, the additives used did not show a significant effect on the transesterification or esterification activity of the biocatalyst (Table 2). In a previous study, the transesterification capacity of the mycelium-bound lipase from *A. niger* MYA 135 was studied (Romero et al. [13]). In that case, neither the solvent nor the biocatalysts were dried before use. No significant correlation between the amount of biocatalyst water in the reaction mixtures (from 0.11 to 1.47 %) and the transesterification activity was found ($r = -0.066$, $p = 0.629$). Thus, the biocatalyst only requires a suitable ionisation state to enhance its synthesis activity in organic solvents.

4 Conclusions

In the three reactions assayed (hydrolysis, transesterification and esterification), the most relevant effect on the activity enzyme was the ionisation state of the biocatalyst. With respect to both hydrolysis and transesterification activities, the biocatalyst was influenced by acid pH. Meanwhile, in the esterification reaction, an enhancement in the activity of the biocatalyst was observed when the activity water was controlled using salt. The behaviour of proteins was strongly dependent on the protonation state of their ionisable groups.

From an ionisation point of view, the ionisation constant of ionisable groups is greatly affected by the solvent. Organic solvents affect not only the ionisation state of active site groups of enzymes but also electrostatic interactions within proteins. In this case, the pretreatment of the biocatalyst at the optimal pH (in this case an acid pH) or with a salt hydrate ($MgCl_2$) allowed the ionisation state of the mycelium-bound lipase to be maintained when the biocatalyst was then used in an organic solvent, improving the synthesis activity.

Current studies are directed toward statistical optimisation using an improved biocatalyst and assays for coupling esterification and transesterification reactions and controlling the ionisation state of biocatalysts to support biodiesel production from different triglyceride sources.

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