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The effects of the chemical modification on immobilized lipase features are affected by the enzyme crowding in the support

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Abstract

In this article, we have analyzed the interactions between enzyme crowding on a given support and its chemical modification (ethylenediamine modification via the carbodiimide route and picryl sulfonic (TNBS) modification of the primary amino groups) on the enzyme activity and stability. Lipase from *Thermomyces lanuginosus* (TLL) and lipase B from *Candida antarctica* (CALB) were immobilized on octyl-agarose beads at two very different enzyme loadings, one of them exceeding the capacity of the support, one well under this capacity. Chemical modifications of the highly loaded and lowly loaded biocatalysts gave very different results in terms of activity and stability, which could increase or decrease enzyme activity depending on the enzyme support loading. For example, both lowly loaded biocatalysts increased their activity after modification while the effect was the opposite for the highly loaded biocatalysts. Additionally, the modification with TNBS of highly loaded CALB biocatalyst increased its stability while decrease the activity.

KEYWORDS
modulation of enzyme activity, modulation of enzyme stability, protein–protein interactions

1 | INTRODUCTION

Enzyme biocatalysis has attracted a growing interest because these biocatalysts fulfill most requirements of an ecologically ideal process: enzymes are highly active under mild conditions and in aqueous media exhibiting high selectivity and specificity.[1–4] However, the biological origin of enzymes induces some features that are necessary for the response of the microorganisms under very specific conditions, but that are problematic in an industrial reactor where reaction conditions are significantly harsher. That way, enzymes are soluble biocatalysts, with short stability, and their activity, selectivity and specificity have been optimized by nature for their physiological reactions under physiological conditions.[5] As industrial catalysts, enzymes should be heterogeneous biocatalysts (to facilitate enzyme recovery and reaction control), stable, active, selective, and specific for the applied targeted substrate and reaction under industrially relevant conditions.[1–4] Fortunately, there are many tools that enable to bridge this gap, such as metagenomics, that enables to utilize most biodiversity,[6–9] directed evolution, that permits to mimic natural selection but focusing on the enzyme and the feature that the researcher has selected in an accelerated way,[10–12] directed mutagenesis,[13–16] that enables to build new enzymes, even enzymes bearing several different enzymes[17] (e.g., plurizymes),[18–21] and some physicochemical tools. Among the last ones, chemical modification of the enzyme surface can reach diverse objectives, such as altering the enzyme surface features, the mobility of a specific section of the protein or introducing intra or

intermolecular crosslinkings.[22–27] Moreover, enzyme immobilization has revealed itself as a powerful tool to solve the problem of enzyme solubility.[28–31] Moreover, it is also able to solve many other enzyme limitations if properly utilized, such as enzyme stabilization (for different reasons, e.g., multipoint or multisubunit immobilization),[32–34] or coupling enzyme immobilization to purification.[35] In an empiric approach, immobilization, able to induce enzyme distortions, can also alter enzyme selectivity or specificity.[36–41]

In many instances, chemical modification of enzymes and immobilization can be used in an integrated way, with synergetic effects.[42–45] For example, the free enzyme may be enriched in nucleophiles to make getting an intense multipoint covalent immobilization simpler.[42–45] On the other hand, the use of previously immobilized enzymes can facilitate their chemical modification: reaction control becomes very simple, the enzyme cannot aggregate during modification (intermedium or final states). If the enzyme has been stabilized by immobilization, it can resist modification better.[42–45] In some instances, only by using both strategies is it likely to reach the desired result, as for example in the case of stabilization of complex multimeric enzymes, where the immobilization of all enzyme subunits on the support surface becomes unfeasible (e.g., tetraedric enzymes) and only the use of a crosslinking step (e.g., using a polymer) can prevent the release of enzyme subunits to the reaction media.[46,47]

Lipases are frequently utilized at both academic and industrial levels,[48–51] with applications as detergents (as fat cleaning),[52–55] food and feed industries (modifying oils and fats),[56–61] fine chemistry (resolution of racemic mixtures, regio- or enantio- selective acylations, etc.),[62–67] polymer science (production or degradation of polymeric materials),[68–71] or energy (production of biodiesel).[72–77] They have a specific catalytic feature: they can act on interfaces. This is because the so-called interfacial activation mechanism, where a large and hydrophobic pocket, containing the active center that can be closed and isolated from the medium by a peptide chain called lid or flap, becomes adsorbed to hydrophobic drops of oils, becoming stabilized. This permits the lipase to attack the triglycerides drops.[78–86]

This peculiar mechanism of action promotes the immobilization of lipases on hydrophobic supports at low ionic strength,[87] enabling the one step immobilization, stabilization, purification and hyperactivation of lipases, making this method the preferred one for lipases.[88] Lipases immobilized very rapidly on this support (forming a crown on the outermost area of the agarose particle).[89] It has been shown that using an excess of enzyme, it is possible to have biocatalysts where protein–protein interactions alter enzyme stability.[90] This has been found using the lipase B from *Candida antarctica* (CALB) or the standard lipase from *Thermomyces lanuginosus* (TLL).[91,92]

On the other hand, the chemical modification of these enzymes has been found to become a tool to alter enzyme features. The full amination of the external carboxylic groups of TLL and CALB has proved to be a potent tool to alter enzyme features,[93–101] as well as the modification of the primary amino groups of the enzyme with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (also called picryl sulfonic acid) permits the global enzyme hydrophobization.[97–101]

In general, there are few studies on how enzyme crowding can alter the effects of the chemical modification on the alteration of the enzyme features.[102–104] Recently it has been shown how the effect of chemical modification can be altered by the buffers where they are incubated.[105] That way also the protein–protein interactions may produce a change in these effects. Bearing in mind that the protein–protein interactions are considered to be the reason for the different biocatalyst stability of lowly loaded and overcrowded enzyme preparations,[90–92] it is possible to imagine that while using lowly loaded biocatalysts, the main effect of the modification is the intrinsic effect on the enzyme structure stability. Using overloaded catalysts, the alteration of the enzyme–enzyme intermolecular interactions may also alter the effects of the modification. At industrial level, the use of highly loaded biocatalysts will be the target, to maximize volumetric activity, while in most cases the studies on chemical modification are performed using lowly loaded biocatalysts, as a way of preventing the effects of the substrate diffusional limitations on the intrinsic enzyme features.[29] If the effect of the chemical modification is very different, it may have a high importance when designing a chemical modification of an enzyme for an industrial application. For example, it has been recently shown that the load in the biocatalyst can alter the prospects of getting an intense intermolecular crosslinking.[106]

Thus, in this article, we intend to study if the effects of the chemical modification on agarose-octyl-TLL and CALB can be somehow modified by the loading of the enzyme.

2 | MATERIALS AND METHODS

2.1 | Materials

Liquid formulations of CALB (24.77 mg/mL) and TLL (38.49 mg/mL) were obtained by the kind donation of Novozymes (Madrid, Spain) as liquid solution formulations. Ethylenediamine (EDA), *p*-nitrophenyl-butyrate (*p*-NPB), picryl sulfonic acid solution (TNBS) and ethylcarbodiimide hydrochloride (ECD) were purchased from Sigma-Aldrich (Madrid, Spain). Octyl-Sepharose® 4BCL beads were attained from GE Healthcare. Bradford method was employed for the quantification of proteins concentration.[107] The other compounds were of analytical grade

2.2 | Methods

2.2.1 | Determination of enzyme activity with p-NPB

A cuvette was filled with 2.5 mL of 25 mM sodium phosphate at pH 7.0. Then 50 μL of *p*-NPB solution (final concentration of 50 mM dissolved in acetonitrile) was added. To start the reaction, samples of 50 μL of the free or immobilized enzyme were incorporated under magnetic stirring. A Jasco V-730 spectrophotometer (Jasco, Madrid, Spain) was utilized in these studies. The increase of absorbance that

occurs during 90 seconds due to the release of *p*-nitrophenol, caused by the hydrolysis of *p*-NPB, was measured at 348 nm (isosbestic point of *p*-nitrophenol, ε is 5150 M^{-1} cm^{-1} under these conditions).[108] The activity of the enzymes can be expressed in activity units (U), and they were defined as micromoles of *p*-NP that are produced per minute.

2.2.2 | Immobilization of the lipases on octyl-sepharose® beads

CALB and TLL were immobilized on octyl-sepharose® beads using two different enzyme loadings, one that ensures a full coating of the supports surface with the enzyme going over the capacity of the support (high enzyme concentration), 24 mg of protein per gram of support was employed; and the other one using a low amount of enzyme, selecting one where the enzyme molecules can be dispersed on thr support but that also the biocatalyst presents a reliable activity (low enzyme concentration), 1 mg of protein per gram of support were used.[91,92] The immobilization was performed using 1 gram of support per 10 mL of enzyme solution employed 5 mM of sodium phosphate as buffer at 25°C and a pH of 7.0. Samples at different times of the reference suspension (where octyl agarose was changed by inert agarose), suspension and supernatant were taken, and their activities were quantified employing *p*-NPB as substrate. This way, immobilization yield and expressed activities could be calculated.[109] After an hour, the supports containing the enzymes were washed with distilled water to eliminate the excess of free lipase, vacuum filtered and stored at 4–6°C.

2.2.3 | Amination of immobilized CALB and TLL

After the enzymes were immobilized on the octyl-sepharose® supports, these derivatives were introduced into a solution of 2 M EDA at pH 4.75 and 25°C, in a 1/10 (g support/ mL solution) ratio. After this, solid ECD was added to reach a concentration of 10 mM. The suspension was kept for 2 h under constant mild stirring. Then, the modified immobilized enzymes were vacuum filtered, washed to eliminate the remaining EDA and ECD and stored at 6°C temperature in a fridge. With these conditions, all the exposed carboxylic groups were modified.[110–112]

2.2.4 | Modification of primary amino groups of immobilized lipases with TNBS

With a 1/10 (g support/mL) ratio, the enzymatic supports were suspended in a solution composed of 17.5 mM TNBS in 100 mM sodium pH of 8.0, at room temperature.[98,99,113] The suspension was mildly stirred for an hour and afterwards, washed, vacuum filtered and stored at a low temperature.

2.2.5 | Thermal inactivation of the different lipase biocatalysts

CALB and TLL biocatalyst were resuspended in 10 mM Tris–HCl at pH 7.0 in different ratios depending on the enzyme loading (1/30 (g support /mL) for the highly loaded biocatalysts, 1/10 for the lowly loaded biocatalyst). Then, these suspensions were incubated in a warm bath at specific temperatures depending on the enzyme; CALB was incubated at 76°C and TLL at 72°C. At different times, 50 µL of suspension samples were acquired to measure the loss of activity that occurs due to the temperature inactivation using *p*-NPB as substrate. Residual activities were calculated as the percentage of the biocatalysts initial activity.

3 | RESULTS

3.1 | Effect of chemical modification on the activity of the different immobilized enzymes

The enzymes were immobilized on octyl agarose beads at 1 mg/g (lowly loaded) and offering an amount of enzyme over that of the capacity of the support (highly loaded) (Table 1).[91,92] While lowly loaded TLL biocatalyst increased the activity upon immobilization,

TABLE 1 Immobilization parameters of the highly and lowly loaded CALB and TLL-octyl agarose beads biocatalysts.

Enzyme source	Support loading (mg/g)	Immobilization yield, (%)	Expressed activity, (%)
TLL	1	≥95	230 ± 4
	24	50 ± 2	105 ± 2
CALB	1	≥95	100 ± 3
	24	80 ± 4	80 ± 2

Note: The immobilization was performed for 2 h and then activities of the supernatant and suspension of the immobilization suspension and that of the reference suspension (that maintained 100% of the initial activity) were determined. Immobilization yield was defined as the percentage of enzyme activity that is immobilized on the support. Expressed activity was defined as the percentage of the observed activity from the expected activity from the immobilization yield. Other specifications may be found in Section 2.2.

the highly loaded biocatalyst gave a marginal increase in activity, very likely due to diffusional limitations.[29] The increase of activity after immobilization using the lowly loaded TLL should be related to the stabilization of the open form of the lipase.[87] In the case of CALB, the expressed activity was 100% or 80% for the lowly and highly loaded biocatalysts, respectively. This may be related to the small size of the CALB lid, which cannot fully isolate the active center from the medium.[114]

Next, all biocatalysts were chemically modified. Starting with TLL (Table 2) and the modification with TNBS, the lowly loaded biocatalyst maintained its catalytic activity almost unaltered, while the highly loaded biocatalyst slightly decreased its activity after modification (by a 10%). The differences between highly and lowly loaded biocatalyst are clearer when the enzyme was aminated, while the lowly loaded biocatalyst increased the activity by 20%, the highly loaded biocatalyst decreased the activity by a similar magnitude. That is, the chemical modification could increase or decrease the enzyme activity depending on the enzyme loading, suggesting that the enzyme–enzyme interactions can play a significant role on the final effects of the modification. The TNBS modification produces an increase on the hydrophobicity of the enzyme surface, it can change repulsive ionic interactions by positive hydrophobic interactions and this can generate some conformational changes on the enzyme that can (negatively or positively) alter its activity.[115]

Amination should produce an increase of repulsive ionic interaction, as carboxylic groups are changed by amino groups.[45] Table 3 shows the results obtained using CALB biocatalysts. The lowly loaded biocatalysts increased the enzyme activity after modification with TNBS (by 2.2 fold) and amination (by a 1.3 fold), while both modifications promoted a significant decrease on the activity of the highly loaded biocatalyst (by 2.2 fold in the case of TNBS modification and 2.6 fold for the aminated biocatalyst). In this case, the effect of enzyme crowding is much clearer than in the case of TLL, confirming that the enzyme–enzyme interactions strongly alter the effects of chemical modifications on enzyme activity, very likely by altering the enzyme structure. CALB seemed to be more sensitive to these changes than TLL (it has been described that for CALB enzyme crowding is negative, while this effect is not so clear for TLL).[92] Nevertheless, it should be highlighted that these changes in activity take place with this specific substrate. It may be expected that changing the substrate the results may be fully different, as it has been described in many instances for other lipase modifications.[94–98]

TABLE 2 Effect of the chemical modification on the activity of highly and lowly loaded TLL biocatalysts.

Biocatalysts	Chemical modification	Biocatalyst activity, (U/g)
Lowly loaded	None	71.1 ± 2.3
	TNBS	73.9 ± 1.8
	Amination	86.1 ± 2.8
Highly loaded	None	141.8 ± 4.1
	TNBS	127.1 ± 1.2
	Amination	115.6 ± 5.1

Note: The chemical modifications were performed as described in Section 2.2. Other specifications may be found in Section 2.2.

TABLE 3 Effect of the chemical modification on the activity of highly and lowly loaded CALB biocatalysts.

Biocatalysts	Chemical modification	Biocatalyst activity, (U/g)
Lowly loaded	None	8.6 ± 0.5
	TNBS	19.5 ± 1.4
	Amination	11.36 ± 1.2
Highly loaded	None	87.1 ± 7.2
	TNBS	39.6 ± 2.2
	Amination	33.31 ± 2.1

Note: The chemical modifications were performed as described in Section 2.2. Other specifications may be found in Section 2.2.

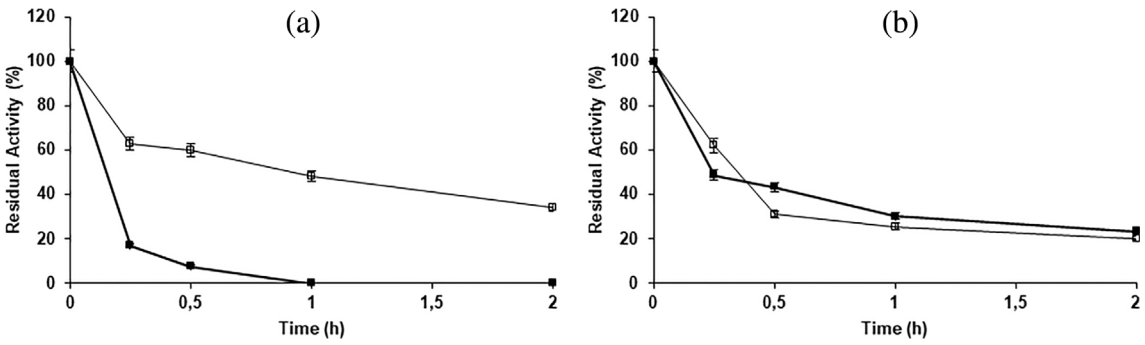

FIGURE 1 Effect of the TNBS modification on the thermal inactivation courses of 1 mg/g (a) and 24 mg/g (b) octyl-TLL biocatalysts. The inactivation was performed using 10 mM of Tris–HCl buffer at pH 7.0 and 72°C; Empty symbols: No modified biocatalyst and solid symbols: TNBS modified biocatalyst. Other specifications were described in Section 2.2.

3.2 | Effect of the chemical modification on the stability of the different immobilized enzymes

Next, the effect of the chemical modifications on the TLL stability have been analyzed. Figure 1 shows that the TNBS modification of the lowly biocatalysts produced a drastic destabilization of the biocatalysts, while it presented no effect on the enzyme stability when using the highly loaded biocatalyst. This suggested that while the TLL surface hydrophobization had an intrinsic negative effect for the stability of the enzyme, the changes in the enzyme–enzyme interactions (that should change ionic repulsion/attraction interactions by positive hydrophobic interactions) between those produced due to the modification were positive. In the case of amination, the effects were even clearer (Figure 2). While the stability of the lowly loaded biocatalyst decreased, the effect of the amination was positive for

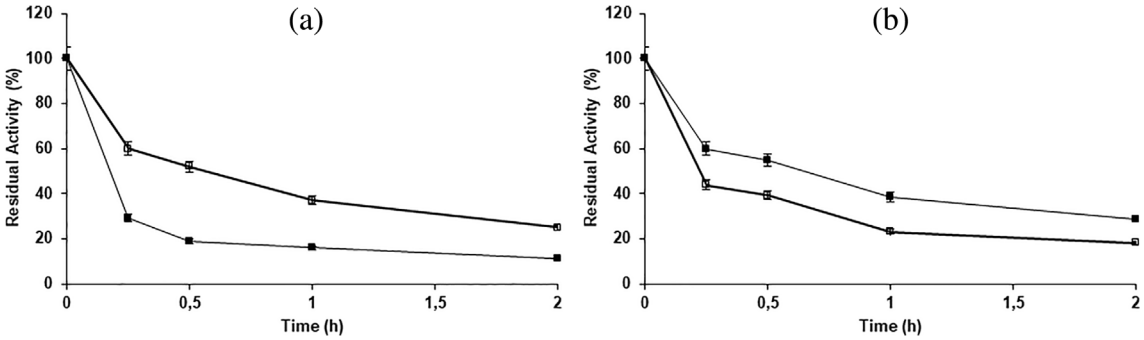

FIGURE 2 Effect of the chemical amination on the thermal inactivation courses of 1 mg/g (a) and 24 mg/g (b) octyl-TLL biocatalysts. The inactivation was performed using 10 mM of Tris–HCl buffer at pH 7.0 and 72°C; Empty symbols: No modified biocatalyst and solid symbols: Chemically aminated biocatalyst. Other specifications were described in Section 2.2.

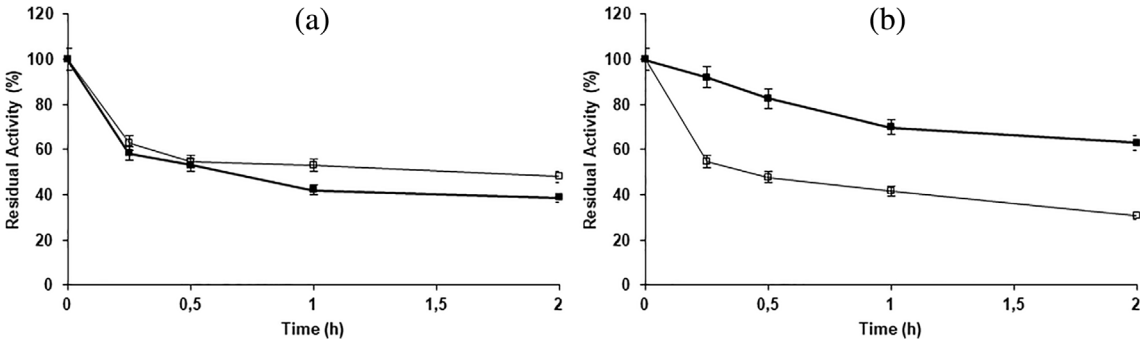

FIGURE 3 Effect of the TNBS modification on the thermal inactivation courses of 1 mg/g (a) and 24 mg/g (b) octyl-CALB biocatalysts. The inactivation was performed using 10 mM of Tris–HCl buffer at pH 7.0 and 76°C; Empty symbols: No modified biocatalyst and solid symbols: TNBS modified biocatalyst. Other specifications were described in Section 2.2.

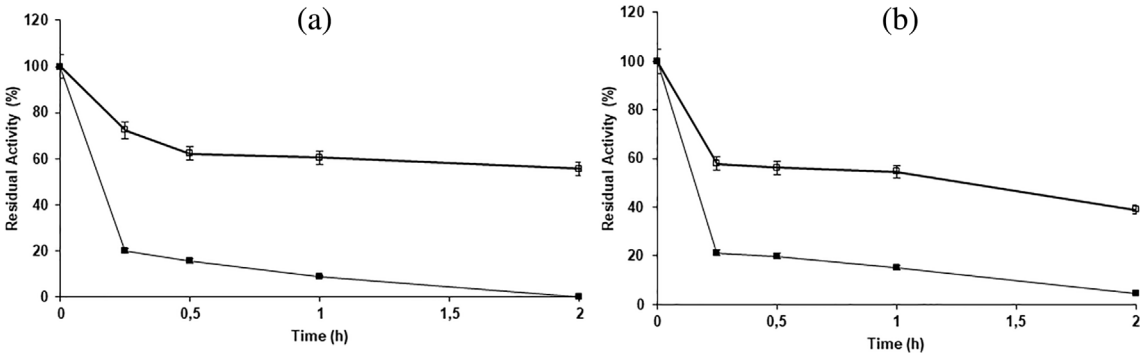

FIGURE 4 Effect of the chemical amination on the thermal inactivation courses of 1 mg/g (a) and 24 mg/g (b) octyl-CALB biocatalysts. The inactivation was performed using 10 mM of Tris–HCl buffer at pH 7.0 and 76°C. Empty symbols: No modified biocatalyst and solid symbols: Chemically aminated biocatalyst. Other specifications were described in Section 2.2.

the stability of highly loaded biocatalyst. That is, it appears that the increase in ionic repulsions generated by the amination of the carboxylic groups generated a negative effect for the intrinsic stability of the enzyme structure, but these ionic repulsions (that may reduce the enzyme–enzyme-interactions) are positive for enzyme stability using highly loaded biocatalyst. In fact, the highly loaded TLL biocatalyst was slightly less stable than the lowly loaded biocatalyst. That way, the final effects seem to be the addition of very different causes, some perhaps unknown.

Next, we moved to the CALB biocatalysts, The TNBS modification produced a high stabilization of the highly loaded CALB biocatalyst while the stability of the lowly loaded biocatalyst stability was marginally impoverished (Figure 3). If the enzyme was aminated, the effect was always negative for CALB stability, but this negative effect was higher for the lowly loaded biocatalyst, that passed from being more stable than the highly loaded biocatalysts to be less stable (Figure 4). That way, it seemed that there is also a strong co-interaction on enzyme stability between modification of the enzyme surface and the nature of the buffers when using CALB biocatalysts.

4 | CONCLUSIONS

The chemical modification that occurs due to the amination or hydrophobization of immobilized enzymes surfaces can greatly alter their activity and stability. These effects are closely related to the existence of protein–protein interactions. That way, the crowding of the enzyme on the support can greatly affect the final result in terms of enzyme activity/stability. The same modification can stabilize/destabilize an immobilized enzyme or decrease/increase enzyme activity depending on the support loading of the enzyme. The causes for these co-interactions deserve to be further investigated, as very likely some unknown causes can be under the final global effects found in the experiments. Now it seems a combination of positive/negative effects on the intrinsic enzyme features and positive/negative effects on the protein–protein intermolecular interactions. This means that the impact of the chemical modification on the enzyme features must be evaluated on exactly the biocatalysts that we intend to use. Extrapolation from one loading to other seems unrealistic. In the examples utilized in this article, TNBS modification of overcrowded octyl-CALB gave the highest stability/stabilization, compensating the negative effects of the enzyme overcrowding, For TLL, the best results were obtained using the aminated overcrowded biocatalyst.

AUTHOR CONTRIBUTIONS

Pedro Abellanas-Perez: Formal analysis; investigation; writing – review and editing. **Diego Carballares**: Research; preparing first version of the paper; editing final version. **Javier Rocha-Martin:** Conceptualization; formal analysis; supervision; writing – original draft; writing – review and editing. **Roberto Fernandez-Lafuente:** Conceptualization; formal analysis; funding acquisition; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study

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