Parameters necessary to define an immobilized enzyme preparation

Boudrant, Joseph; Woodley, John M.; Fernandez-Lafuente, Roberto

Published in:
Process Biochemistry

Link to article, DOI:
10.1016/j.procbio.2019.11.026

Publication date:
2020

Document Version
Peer reviewed version

Parameters necessary to define an immobilized enzyme preparation.

Joseph Boudrant[1], John M. Woodley[2] and Roberto Fernandez-Lafuente[3,*]

[1]*Laboratoire Réaction et Génie des Procédés, UMR CNRS 7224, University of Lorraine, ENSAIA, 54518, Vandoeuvre Cedex, France*

[2]Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Kgs Lyngby, Denmark

[3]Departamento de Biocatálisis. ICP-CSIC, Campus UAM-CSIC, Madrid, Spain

Corresponding author:

Roberto Fernandez-Lafuente. ICP-CSIC, C/ Marie Curie 2, Campus UAM-CSIC, Cantoblanco, 28049 Madrid, Spain. E-mail, rfl@icp.csic.es

Abstract

Biocatalytic processes continue to find increasing application in industry. Therefore enzyme immobilization has also become of increasing importance as a means of allowing enzyme containment within reactors operating in continuous mode or else separation of enzyme after use in (fed-)batch reactors, as well as potential recycle. Whilst much has been reported in the scientific literature about enzyme immobilization methods, in many cases the protocol leads to losses in enzyme activity. In this review we outline the reasons for loss of activity during immobilization and highlight suitable diagnostic tests to elucidate the precise cause and thereby methods to restore activity. The need for standardized reporting of immobilization methods is also emphasized as a means of benchmarking alternative approaches.

Key words: enzyme immobilization, facts and artifacts, yield determination, enzyme inactivation, enzyme stabilization

1. INTRODUCTION

The demands on the modern chemical industry to fulfil the rules of the green chemistry agenda today promote intense research in the field of biocatalysis (1, 2, 3, 4, 5). Catalysis under mild conditions and high selectivity are amongst the most important reasons for their potential application as sustainable industrial biocatalysts (1, 2, 3, 4, 5). For this reason, the number of biocatalytic applications is increasing in many industrial sectors, from fine and pharmaceutical chemistry to food and chemical production.(6, 7, 8, 9, 10, 11, 12, 13, 14)

However, the cost of enzymes means that in many cases they must be reused multiple times to make the process economically viable, and since they are water soluble molecules, they are often difficult to recover (15). This problem has been solved by the immobilization of enzymes on solid supports, making a heterogeneous catalyst (sometimes using pre-existing supports, (15) sometimes generating an *ex novo* solid during the immobilization (15). Because enzymes are not soluble in organic media, it may be considered that immobilization for use in non-aqueous media may not always be necessary, since enzyme will aggregate (16, 17, 18). However, it should be considered that enzyme aggregates may be not reproducible (they depend on the particular protein, the protein concentration, as well as enzyme purity, etc.), that the size and physical resistance are dependent on the enzyme extract, and that the aggregates could result in substrate diffusional limitations, greatly reducing enzyme activity. In fact, much research has been directed to prepare enzymes soluble in organic media in order to improve enzyme activity (19, 20).

Today, however, in order to establish a suitable immobilization protocol, the researcher must solve several other enzyme limitations, such as stability and activity under conditions far from the physiological ones, enzyme selectivity and specificity (using substrates far from the physiological ones), enzyme purity and sensitivity to inhibition, as well as resistance to chemicals (21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35). In this way, enzyme immobilization has become a powerful tool in biocatalyst design, complementing other strategies routinely used to improve protein properties, like genetic, microbiological or chemical modification tools (36, 37, 38). In some instances, the genetic or chemical modification may even be directly designed in order to improve the way the enzyme is immobilized, rather than to improve soluble enzyme features.

Any immobilization protocol must consider several aspects: the support properties, the active group of the support used to immobilize the enzyme and that of the enzyme molecule, as well as the immobilization protocol itself (45, 46). Only a proper selection of these three aspects allows one to take full advantage of the immobilization process. This has been reviewed in many recent papers, and is therefore not the focus of this manuscript. (47, 48)

Consequently, there is a huge amount of literature on enzyme immobilization (49). In the experiments where most of the enzyme activity is immobilized and where the enzyme is maintained almost intact, it is relatively simple to follow the procedure. Unfortunately, however, in many of the publications the obtained (observed) activity is far from the initial activity. Likewise many times critical information is missing in order to properly understand the reason for this. Indeed, it may be caused by a failure in the enzyme

immobilization itself, or by an inactivation of the enzyme due to the immobilization conditions, or even by an enzyme inactivation due to enzyme-support interactions. Still other reasons will be discussed later in this paper. In fact, each of these causes may have different responses in order to restore the activity of the biocatalyst.

The situation is similar (although not identical) to heterogeneous catalysis using non-proteinaceous material. In heterogeneous catalysis there has also been discussion and debate about how to standardize the reporting of data, what data should be reported and how these data can best be used for process design, optimization and improvement (e.g. 50). More widely the need for benchmarking both catalysis (51) and biocatalysis (52) has been discussed in the scientific literature, where the reporting of measurement protocols has been particularly emphasized.

This discussion is particularly important in the complex field of enzyme immobilization and in this review we will give some indications on the minimum data that are needed to clearly define an enzyme immobilization process and thereby to understand the properties of an immobilized enzyme. Here we will indicate the minimum data that are required to characterize the phenomena occurring during immobilization, and some simple ways to establish what may be occurring. Moreover, this review proposes some terms that can be used to define each of these parameters since currently there is a mixture of terminology that sometimes makes it difficult to understand published data.

2. MINIMUM REQUIRED DATA TO DEFINE AN IMMOBILIZATION PROCESS

When performing an immobilization process, in order to fully understand the effects of the immobilization on enzyme activity, some experimental data should be provided. They can be presented in different ways dependent upon the type of immobilization, but should include the enzyme activity development (or evolution) through the whole immobilization period.

First, it is necessary to know what happens to the activity of the free enzyme under the immobilization conditions. For this, an enzyme solution under identical conditions to those of the immobilization conditions must also be prepared, but preferably without adding support or else by replacing the activated support by a well-identified inert support (this is relatively simple using agarose derivatives, because non-modified agarose will be completely inert) (53). This is always important, but in some cases becomes critical when the enzyme stability is less than satisfactory. In some instances the employed immobilization technique requires the use of alkaline pH values (e.g., immobilization in glyoxyl) (54), or the addition of detergents (e.g., when using lipase to prevent enzyme dimerization) (55, 56, 57, 58, 59). In other examples, the enzyme is in an aggregated form and the only way of having reliable results is by employing a chaotropic agent to break the protein aggregates (60, 61, 62). Ideally, immobilization conditions where the soluble enzyme is fully stable during the whole immobilization process should be selected, adding some stabilizing additives if this is not the case (63). However, in some instances immobilization is so rapid and stabilizing that while the reference solution is inactivated, the decrease in the activity of the immobilized preparation can hardly be measured.

The other data needed are the evolution of the activity in the supernatant. If the enzyme is active and stable under immobilization conditions, this measurement will allow determination of the percentage of enzyme immobilized.

The last data that should be included are the activity evolution in the immobilization suspension; these data will show the effect of enzyme immobilization on activity.

With these three activity curves, the enzyme immobilization course when the enzyme and support are in the immobilization suspension can be fully defined. Nevertheless, some additional data may be necessary to fully understand the immobilization process.

Thus, the activity of the final biocatalyst needs to be assayed after end point protocol and washings. In many cases, frequently in covalent immobilization, an end point of the reaction between the enzyme and the support is used. This is convenient, because it avoids uncontrolled enzyme-support reactions. One point to be considered is that, if this enzyme-support reaction end point is not used, after enzyme immobilization, the reaction between the enzyme and support can continue even under other (milder) conditions and at a slower speed. This continuous enzyme-support reaction may alter the enzyme properties even during storage. For example, using aldehydes as an active group in the support, a reduction step is recommended. This is usually done with sodium borohydride (64), which converts labile imino bonds into stable (more stable than peptide bonds) secondary amino bonds. It is well-known that sodium borohydride may also affect the enzyme activity by reducing some metal bound to the enzyme and may ultimately prove critical for enzyme activity or stability (65). If used at a high concentration, sodium borohydride can even

break peptide bonds (66). Some alternative reagents have been proposed, but are rarely used (67). The case of glutaraldehyde is special, because it loses chemical reactivity after several days and gives very stable enzyme-support bonds (68). In most other cases, the only way to have a chemically inert support is by blocking the residual active groups in the support with a chemically inert compound (69). Using epoxide, mercaptoethanol was initially recommended (70, 71), but it can also break thiol bridges, and high concentrations of any other compound may also affect enzyme stability/activity (72). In this way, the stability of the enzyme in these blocking solutions must also be investigated to understand if negative effects on the enzyme activity can be expected. A good solution to determine these effects on the immobilization end-step conditions on the enzyme activity is to incubate in these solutions an enzyme immobilized preparation (using the enzyme immobilized by another protocol) and check if the enzyme activity changes during incubation. This way, enzyme precipitation or inactivation by exposure to gas bubbles, that can occur using soluble enzyme solution (73), can be avoided and the situation is closer to the reality with an immobilized enzyme.

In case the final observed activity does not fit the balance among all the activity measurements listed above, it is possible that some of the enzyme has been released from the support during washing. This is usually controlled in research which uses physically immobilized enzymes, but it is rarely done when using “covalent” protocols. And it may be a particular problem when using hetero-functional supports (74, 75, 76, 77, 78, 79). This is more importnat considering that in many instances the researcher is not aware if the support used is a hetero-functional one or not. Hetero-functional supports are those bearing the capability of interacting with a protein in several different ways: ion exchange,

hydrophobic interaction, and covalent attachment.(74, 75, 76, 77, 78, 79). In most cases, the covalent attachment is granted if the support has some chemically reactive groups, but this may not always be the case. In fact only in some instances will a covalent bond be achieved (74, 75, 76, 77, 78, 79). The case of aminated supports activated with glutaraldehyde is one such example. Here the enzyme may become immobilized via ion exchange, hydrophobic interaction and covalent reaction (which is the slower one) (80, 81, 82, 83, 84, 85, 86, 87). The study of the activity presented in the washing buffers may be complex, the enzyme will be much diluted and the activity may fall beneath the detection limit. In any case, this possibility should be examined only if the discrepancies between the expected results and the observed results are significant.

3. HOW TO FOLLOW THE ENZYME EVOLUTION IN THE SUPERNATANT: ACTIVITY OR PROTEIN CONTENT?

Many publications present the immobilization course showing the protein in the supernatant. However, this way of presenting an immobilization course may have some problems. If the immobilization yield is 100%, this is not a problem, but if the immobilization yield is less than 100%, this may prove more serious. In fact, in many cases the target enzyme is not fully pure, often accounting for less than 30% of the total protein. In this way, we have an average measurement of the total protein immobilized. It should be also considered that many immobilization protocols can selectively immobilize target proteins. So this will underestimate the actual immobilization yield since other proteins will not be immobilized. For instance, this occurs with immobilization of lipases on

hydrophobic supports at low ionic strength (88), the tagged proteins and affinity domains (89, 90, 91, 92, 93, 94, 95), but this occurs mostly with covalent or physical immobilization protocols even when it is difficult to predict beforehand. Almost no immobilization protocol can immobilize 100% of the proteins contained in a crude protein extract. Moreover, an additional problem is that some non-protein components in the extract can give signals with the protein detection methods usually employed.

In this case, the specific activity of the enzyme increases after immobilization, but it is not because the enzyme conformation is improved. It is because the enzyme is becomes purified: expressed activity may be maintained at or beneath 100%.

Moreover, there are no clues on the effects of the immobilization conditions on enzyme activity, and that can complicate the calculations and the understanding of the reasons for the observed enzyme activity during biocatalyst characterization.

In this way, measuring the activities of the supernatant, reference solution and immobilization solution, a more accurate reality of the immobilization process may be achieved. Indeed, a combination of both activity and protein measurements may be useful in some cases.

4. REQUIRED PARAMETERS TO DEFINE THE IMMOBILIZATION PROCESS

With the data above collected, giving some critical parameters that define the immobilization process becomes possible.

4.1 Immobilization yield.

This term is usually employed to define the percentage of enzyme which is immobilized on or in the support.

However, in some cases, it is used with other meanings, for example as the percentage of enzyme activity that is maintained in the immobilized biocatalyst compared to the offered enzyme activity (frequently in publications where this is the only supplied information.

Whatever the name, this value is important to include in any immobilization report. In order to calculate it, under standard conditions, the residual activity in the supernatant is divided by the activity in the reference suspension at the end of the immobilization period.

The use of the term immobilization yield is under some debate because this really does not reflect the activity yield. Nevertheless, it has been used for many years in hundreds of publications, and if termed the "protein immobilization yield", it could be considered adequate.

4.2 Expressed activity

The expressed activity **(**also called recovered activity or activity recovery), gives the immobilized enzyme activity calculated from the immobilization yield and the activity in the enzyme reference solution. Again, in some papers it shows the percentage of activity that is maintained in the immobilized enzyme compared to the offered enzyme activity, although that is not the standard meaning.

221 This parameter is calculated using the immobilization yield and the activities in the
222 reference and immobilization suspensions, and reflects the effects of the immobilization on
223 the enzyme activity. As explained beneath, it may be more problematic when the reference
224 activity changes during the immobilization protocol.

225 **4.3. The global enzyme activity yield**

226 The global enzyme activity yield is the really pertinent measurement. It relates the
227 initially offered activity and the final activity observed in the immobilized biocatalyst.
228 Many scientific publications only give this value (under different names) and do not show
229 the immobilization time course with the three measures outlined above. In this way, we can
230 consider many different situations where the information supplied by the global activity
231 yield does not reflect the problems occurring in the immobilization process.

232 Figure 1 shows some cases where this parameter alone does not give the required
233 information to understand the process, all with 50% global enzyme activity yield. Figures
234 1a and b show cases where the 50% global enzyme activity yield is the result of only 50%
235 of enzyme activity being immobilized on the support. However, the explanations for this
236 can be very different. For example, in case *a*, the reason for 50% global yield is that the
237 immobilization is too slow. In this case, using a higher ratio of support to enzyme solution,
238 the immobilization yield may be improved, and also an increase in immobilization time (if
239 the groups in the support are stable enough) may improve the immobilization yield.
240 However, in case *b*, a certain percentage of enzyme activity is rapidly immobilized and then
241 the immobilization stopped. There are various explanations for this, which will be
242 discussed later. The case shown by Figure 1 *c*, having identical global enzyme activity yield

to the other cases, exemplifies an immobilization time course where although 100% of the enzyme activity is immobilized, the expressed activity of the immobilized enzyme is only 50%. This result, even though it appears identical to cases a and b, has a completely different explanation (and solution). The cause for this drop in activity will not always relate to enzyme distortion, and will be discussed beneath.

The parameters here are convenient, but it is also highly instructive to give the immobilization time course in order to fully understand what is going on during the process.

In some examples, the expressed activity of the biocatalysts, and therefore global enzyme activity yield may even be higher than the initial activity of the soluble enzyme, that is, the enzyme immobilization starts the process of immobilization with X enzyme activity units and after immobilization 2X, 3X,… (n)X can be obtained. This is exemplified in Figure 2. In some instances this increment may be really significant. Some likely reasons for this have been reviewed recently (96). In short, the reasons may be due to some artefacts (different pH inside and outside the particle, soluble enzyme precipitation while the immobilized enzyme does not precipitate, substrate partition, etc.) (96), higher enzyme stability (using conditions that can affect the enzyme conformation) (97, 98, 99), etc. And in some cases, a real increment in enzyme activity under mild conditions may be found. This is relatively common with redox enzymes, such as when immobilizing an enzyme by the nano-flower formation technique where the metals in the flower may play a role in the electron conductivity (100, 101, 102, 103, 104, 105, 106). There are also examples using lipases, where the open form of the lipase is stabilized after immobilization,

the activity of the immobilized enzyme increases (88, 107, 108). However, in some instances these increases in enzyme activity are merely random conformational changes: the conformational changes to the enzyme which are deleterious in most cases may even prove positive in some exceptional situations, at least for certain substrates, under certain experimental conditions (109, 110, 111). Thus, it is likely that even without immobilizing all the enzyme activity; the final biocatalyst can present activity values over the activity of the soluble enzyme. Again, only a collection of all the parameters and the immobilization time course will give a deep enough explanation of the results. Observing the way the activity changes, one can also deduce if the process is progressive or directly related to the way the enzyme is immobilized. In the latter case, the activity increment should follow the immobilization yield (cases shown in Figures 2 *a* and *b*), while if it is a progressive change, the increment in enzyme activity will also be, up to a maximum (not directly related to the immobilization yield (Figure 2 *c*)).

5. CAUSES FOR REDUCTION OF ACTIVITY DURING AND/OR AFTER IMMOBILIZATION

An immobilization protocol that seriously decreases the expressed activity of the immobilized enzyme may not be considered suitable for a specific enzyme. However, we must also consider some important facts. Usually, as the number of measurements is very high, the activity is followed with some colorimetric synthetic substrates, many times using low substrate concentrations (due to substrate solubility limitations, absorbance problems,

286 etc.), and mild conditions to measure the actual effect of the immobilization on the enzyme
287 activity.

288 In case a decrease in activity is related to an actual enzyme distortion during
289 immobilization, it should be considered if the improvement of other features (e.g., stability)
290 may compensate for this decrease (97). For instance this occurs if the enzyme is going to be
291 used under more severe conditions than usual (standard) conditions, where perhaps enzyme
292 stabilization might allow higher activity (in absolute terms) than the soluble enzyme (98,
293 99). That is, it may be that under that real operational conditions the immobilized enzyme
294 displays a "hyper-activation" compared to the activity of the soluble enzyme, even though
295 the enzyme is distorted and less active under milder conditions (98, 99). Figure 3 shows an
296 example where the immobilized enzyme has only 25% of the activity compared to that of
297 the soluble enzyme, at low temperature. However, when increasing the temperature, the
298 activity of the soluble enzyme increases until a particular point (in the example, 40ºC), and
299 then starts to decrease, while the activity of the immobilized enzyme increases until 70ºC.
300 This means, that at temperatures over 50ºC, the activity of the immobilized enzyme
301 becomes higher than that of the soluble enzyme. Hence some biocatalysts that are
302 apparently unsuitable may become very adequate for the target process if it requires using
303 different and potentially more severe conditions (98, 99, 112, 113). The important point is
304 that if the study has been properly undertaken, then it will be known that this improved
305 activity is due to an improvement of the enzyme stability because under milder conditions
306 for the enzyme, the real negative effect of the immobilization on the enzyme activity has
307 already been determined (97, 98, 99, 112, 113, 114).

In the cases where the recovered enzyme activity is not adequate under operational conditions, and if this is not caused by the effect of the immobilization conditions or the end step on the enzyme activity, the intensity of the enzyme/support multi-interaction should be reduced, for example by decreasing the number of active groups on the support, the immobilization temperature, etc. (115, 116).

However, in some cases this decrease in enzyme activity may not really reflect an enzyme distortion, but rather some other experimental artefact (or other kind of problem). For example, if the substrate is very large (e.g., a protease that hydrolyzes a protein having a size similar to the that of the enzyme itself), the enzyme may be intact with respect to its conformation and the capacity of the active site to catalyze the reaction, but if the active site is oriented towards the support surface due the steric problems, then it will be impossible to catalyze the conversion of such a large substrate (112, 113, 117, 118, 119, 120, 121, 122) (Figure 4). Such steric problems may be dependent on the enzyme loading on the support. If the active site is not fully blocked by the support surface, a reasonable activity against large substrates can be found using a low load of protein, such that each enzyme molecule has a large free space around it (26). Using more enzyme, the other protein molecules can block access of the substrate to the active site, revealing an apparent enzyme inactivation, which in the most drastic cases may result in no observed activity (Figure 4) (26). The use of biocatalysts with different enzyme loadings, and the use of substrates with different molecular sizes can help to understand if the problems are really steric hindrances or rather enzyme distortion.

One common problem when immobilizing an enzyme is an observed decrease of activity, not because the enzyme is distorted or because the enzyme orientation produced a steric hindrance to the reaction with the substrates, but rather because the substrate is more rapidly consumed by the enzyme than it can diffuse into the support particle pores. In this way the substrate concentration decreases along the pore, and the inner enzyme molecules don't have any available substrate to perform the reaction (Figure 5). (123, 124, 125, 126, 127). This is termed substrate diffusion limitation. Conventional stirring of the reaction suspension cannot overcome this because inside the solids only diffusion occurs. An exception to this is found when using ultrasound, which can 'agitate' inside pores, but this is not usually included in a standard enzyme activity determination (128, 129, 130, 131, 132, 133, 134). However, it is important to know if substrate diffusion limitation is the cause for enzyme "inactivation" during the immobilization for two reasons.. First, because the actions to be taken will be different if it is found that enzyme distortion or substrate diffusion limitation is the problem. Second, because in many instances the standard conditions of measurement are far from the operating conditions. For example, even using the same substrate, the concentration of substrate may differ between standard and operating conditions, and perhaps in those conditions the problems could be reduced (e.g. if the substrate concentration is much higher). And if the substrate is different, the enzyme activity may be much lower with the target substrate and the concentrations used consequently much higher, reducing or even eliminating the problem.

There are some easy tricks that may allow a check as to whether the decrease in activity is due to diffusion problems. The simplest one is to mill the immobilized enzyme, since this will reduce the particle size, it will also reduce the substrate diffusion limitation

and consequently the activity will increase (Figure 6). Alternatively, to prevent exposure of enzyme to the milling process, the support can be milled prior to immobilization and it is possible to check if now the effect on the enzyme activity is lower (Figure 6). Other studies that may be interesting are to use immobilized enzyme preparations with different enzyme loadings. Using moderate activity (e.g., under 10 micromols of substrate converted per minute per ml or g of biocatalyst) can negate diffusion problems in most cases. If the activity decreases when increasing the enzyme loading (and if the substrate is small), this can be attributed to an increase in diffusion limitation, and may have a more or less intense effect. Figure 7 shows an example where the diffusional problems are not evident until a significant amount of enzyme per g of support has been immobilized (Figure 7a), while in the Figure 7b, the problem is evident even using a load well beneath the capacity of the support. However, it is not possible to rule out that some protein-protein interactions may also alter the results mainly using the support saturated with enzyme (135, 136). In any case, the use of low loaded biocatalysts may permit visualization of the effect of the immobilization on the individual enzyme molecules.

6. SOME PROBLEMATIC CASES

In some instances, calculation of the different parameters may become a little complicated due to difficulties in giving an actual and accurate measurement. Below, some of these cases are discussed.

6.1. Reference suspension activity decreases during immobilization

If the stability of the enzyme under immobilization conditions is not sufficient to maintain full activity during the entire immobilization time, calculations may become more

374 complex. If the immobilization yield is 100%, the main problem is how to define the
375 expressed activity, since the stability of the immobilized enzyme may be higher than that of
376 the free enzyme. If the immobilization yield is not 100%, calculations may become quite
377 hard to define, in particular if the stability of the free enzyme changes during the
378 immobilization.

379 The immobilization should be described in such a way that the reader can easily
380 understand the cause of the alteration of the enzyme activity during the immobilization.
381 Thus, the main point is to clarify the problems and use all information required to allow
382 easy understanding.

383

384 **6.1.1.** *The immobilization yield is not 100%, the activity of the reference decreases*

385 If there is neither an alteration of the enzyme stability, nor of the specific activity in
386 the supernatant, compared to the reference during the immobilization, the calculations to
387 define the immobilization parameters may be performed as in the general case (of which
388 one is represented in the Figure 8a). However, it should also be mentioned at some point
389 that the activity of the reference enzyme is not 100%. That is, in this case it may be
390 convenient to add an additional parameter, the reference solution residual activity, to
391 clarify that the expressed activity decrease is not only the result of the decrease of activity
392 due to the enzyme-support reaction, but also (partially) due to enzyme inactivation under
393 the immobilization conditions. Usually such inactivation by the immobilization conditions
394 will not be significant provided the final global enzyme activity yield is acceptable, but this
395 may complicate the corresponding calculations.

6.1.2. *The immobilization yield is not 100%, the activity of the reference decreases more rapidly than the activity of the suspension*

In some cases, the enzyme becomes stabilized under the immobilization conditions just after the first immobilization, because it is produced via a multi-point or multi-subunit immobilization (Figure 8b). In this way, using the previous method to define expressed activity, the enzyme will apparently become "hyper-activated", while the global enzyme activity yield will not exceed 100%, as this is not really an improvement of the enzyme activity after immobilization, but a prevention of enzyme inactivation due to the immobilization conditions. In this instance, the best way to treat the results is not to consider the expressed activity but give just immobilization yield and global enzyme activity yield, stating why the expressed activity is not included: this parameter gives the effect of immobilization on the enzyme activity and here that is not possible (because the immobilization conditions also affect the enzyme activity). Thus, in this context it will have no sense. This is very relevant because in some instances the free enzyme could be completely inactivated during the immobilization process, while the suspension maintains reasonable activity (e.g., Figure 8c). In these cases, the global activity yield is the only reliable data, but it should include the immobilization time course in order to understand the immobilization process. This has been reported in some instances using glyoxyl agarose as an immobilization support, since in this support the first immobilization must be performed via at least two points, and it must be performed at alkaline pH, where some enzymes may not be fully stable (137).

6.2. Cases where the enzyme stability or stability depends on the enzyme concentration

This is not such a strange case. For example, multimeric enzyme stability may depend on enzyme concentrations, since a higher enzyme concentration produces a higher percentage of multimeric enzyme forms, usually more stable than the monomeric forms (138, 139) (Figure 9). In this way, as the immobilization progresses and a few enzyme molecules are in the supernatant, it is eventually possible that this diluted multimeric enzyme tends to dissociate giving a lower stability. To discriminate this, reference solutions with the lowest concentration of enzyme that can be considered relevant for the immobilization (e.g., 5 or 10% concentration of the immobilization solution) can be prepared and their stabilities determined under immobilization conditions. This will show if the enzyme really maintains its stability over the entire range of enzyme concentrations during the immobilization time course (Figure 9). If that is not the case, this should be considered in the calculations and the information used to explain the immobilization process should be added. One trick to visualize the relevance of this is to maintain the amount of enzyme per g of support constant, to check the immobilization parameters at different enzyme concentrations (altering the support to enzyme solution ratio). If that phenomenon is not relevant, the final activity of the biocatalyst should be independent of the concentration of enzyme used during the immobilization (Figure 9b). If enzyme dissociation of the diluted enzyme is relevant, the final activity of the immobilized biocatalyst will increase when the concentration of enzyme is increased (Figure 9b).

Moreover, lipases tend to form lipase-lipase dimers via specific interactions between the open forms of the two lipase molecules (Figure 10), and these aggregates have different stability/activity with respect to the monomeric enzymes(140, 141, 142, 143, 144). That is, the concentrated enzyme in the reference will have a specific activity that may be lower than the one in the supernatant of the immobilization suspension, where the enzyme concentrations decrease during the immobilization time course and in this way, the specific activity will increase during the immobilization process (Figure 10). This will reduce the reported enzyme loading of the support and immobilization yield, but will increase the expressed activity. This could make the understanding of the results confusing. The use of detergents during the immobilization and/or during the lipase activity determination may help to solve this situation (Figure 10). It can break the dimers, although they also may have some effects on enzyme activity/stability (145, 146), making necessary a further study of these specific effects.

6.3. Some enzyme release from the support

In this case, global enzyme activity yield is lower than that expected from the immobilization parameters. If we can discard the effects of the end point process (see below), then enzyme release from the support may become a possible explanation. This may be relevant in physical methods of immobilization (e.g. adsorption or entrapment) and in some “covalent” methods, where the support is really hetero-functional (147), and the enzyme is just physically immobilized although the support can, in theory, covalently react with it.

The measurement of activity in the washing solutions, mainly the initial ones, may reveal this problem. The total value of released enzyme may be hard to determine unless all enzyme is washed way, due to the dilution of the enzyme in the washing solution that can make it difficult to give an accurate value of the released enzyme, although enough to indicate that the problem exists. If all enzyme molecules can be released, the immobilization protocol is not a valid one and should be discarded. If some enzymes become strongly attached to the support and the method is considered valid, a disagreement between global enzyme activity recovery, the immobilization yield and expressed activity may be found. In such cases, it should be noted that a percentage of enzyme is lost in the washing steps.

6.4. The end point of the immobilization is not compatible with the activity measurement

In many instances, it is possible to continue measuring the activity of the immobilization suspension during the end point process (e.g., in blocking protocols). This allows determination of the effect of this treatment on the enzyme activity and ensures full control of the process. It is even possible to check the effect of this protocol on soluble enzyme or on enzyme immobilized by another strategy.

However, in some instances the enzyme activity determination is not possible. For example, using sodium borohydride the production of hydrogen bubbles makes it almost impossible to measure anything in a spectrophotometer (and this may also change pH). If in this case, the final activity of the enzyme is much lower than expected from the expressed

activity and immobilization yield, there are two options. In the first, the enzyme is inactivated during the end point step. In the second, the enzyme is not really covalently attached and it is released from the support during the end point process.

The first case may be checked by incubating an immobilized enzyme (immobilized using another strategy) to check if the end point step has (or has not) any effect on enzyme activity.

The second may be harder to check. A small percentage of enzyme release during the washings may be difficult to determine, since the enzyme will be quite diluted, and the activity determination cannot be performed in the first washes, as the end point agent may still interfere with the enzyme activity determination. It is possible to think as the real explanation when other likely explanations considering other causes for the low global enzyme activity yield are discarded.

6.5. Immobilized enzyme is released from the support during activity determination

This is quite common using physical methods of enzyme immobilization. This can happen for example, if the enzyme activity of ion exchanged enzymes is determined under conditions where the enzyme is released (different pH value, higher ionic strength) (148, 149), or lipases immobilized via interfacial activation on hydrophobic supports are measured in the presence of detergents (or detergent like substrates) (150). Indeed it may occur even in some covalent immobilization procedures, e.g., using glyoxyl supports that at neutral pH (or in the presence of aminated substrates) will be unable to retain the immobilized enzyme on the support (151). This means that until somehow the immobilization is made irreversible, the measured activities are those of the soluble

503 enzyme: without steric hindrance, no diffusion limitation and no enzyme distortion. This
504 way, an "immobilization time course" like the one shown in Figure 11a will be obtained
505 (when the actual situation is that shown in figure 11b). However in an irreversible
506 immobilization all these problems arise. This may be simply via reduction of imino bonds
507 using sodium borohydride, use of glutaraldehyde to crosslink enzyme molecules (or
508 enzymes and support) etc. In those moments, the enzyme activity may decrease because
509 now it is really immobilized during the activity determination, and the researcher can
510 wrongly conclude that the problem is the step that makes irreversible the enzyme
511 immobilization, when what is really responsible is the enzyme-support interaction that was
512 not seen previously.

513 **6.6. Negative effect of some reagents released from the activated support on the**
514 **enzyme stability/activity**

515 In some cases, the support itself can release compounds used in the activation (e.g.,
516 if after support activation the washings are not sufficient), and in this way the enzyme in
517 the supernatant of the immobilization suspension may become inactivated by reaction with
518 these reagents. Consequently, immobilization yield may be overestimated, while expressed
519 activity will be underestimated. More intense support washing is the obvious solution (an
520 immobilized enzyme biocatalyst should not release any contaminant to the reaction media),
521 but to detect the problem, a comparison of the reference solution and one prepared using as
522 medium a supernatant after incubating the support under identical conditions to that of the
523 immobilization may prove useful. In any case, if the release of compounds is not possible
524 to avoid, then the immobilization method should be discarded.

6.7. The enzyme needs to be physically or chemically modified before being immobilized

There are many examples, but one very clear one is the immobilization of enzymes on aminated supports via the carbodiimide route, where the enzyme is at least modified using carbodiimide (152, 153, 154). In these cases, the effect of each enzyme modification on the enzyme activity should be shown, and the reference solution should be prepared using the native and also the modified enzyme, to check if the changes in enzyme activity with time are due to the enzyme's natural instability under immobilization conditions, or the result of the chemical modification. When presenting the results, all these facts should be made clear. Moreover, the global enzyme activity yield should show the results starting with the non-modified enzyme, and the data used in the determination of the other parameters should be clearly stated.

6.8. There is not an actual immobilization time course

This is not such a rare occurrence. For example, using the immobilization of enzymes by the strategy of crosslinked enzyme aggregates (CLEAs) (155, 156, 157, 158) this situation can arise. Here, the first step is enzyme aggregation. Many authors show the effect of this aggregation on enzyme activity, but to do that, they re-dissolve the enzyme aggregate before measuring enzyme activity. This information is very valuable, but it only shows the irreversible inactivation caused by the aggregation agent, meaning that diffusion limitations, steric problems or enzyme distortion in the aggregate are not determined. Next, the aggregates need to be chemically crosslinked, usually using glutaraldehyde. This step shows simultaneously the effect of the chemical modification and also of the diffusion

limitations, steric problems or enzyme distortion that now cannot revert because the enzyme immobilization becomes irreversible. One specific problem is when crosslinking fails, and individual enzyme molecules (or small enzyme aggregates) are released from the CLEA and washed way. This may be determined by measuring the activity of the suspension and supernatants in each washing step. The solution may be to add media rich in primary amino groups, proteins or polymers, amination of the enzyme or alternatively use other cross-linkers (159, 160, 161, 162, 163, 164, 165, 166, 167, 168).

6.9. The support is able to adsorb the substrate and/or the product

In some instances the support is able to adsorb a certain amount of substrate or product. If it is the substrate, the concentration of substrate available for the enzyme will be lower, that way perhaps producing an apparent decrease in enzyme activity if the measurements are not done under substrate saturation conditions (Figure 12). If this is exaggerated, it may even be possible that the suspension has less activity than the supernatant, if the immobilization is not very rapid and the activity of the first supernatant measured is significant, and this will already give some clues to the problem (Figure 12a). However, in other instances perhaps not so clear, the observed (solid line) and actual (pointed line) results may be very different (Figure 12b). This can be investigated by adding soluble enzyme to a mixture of support and substrate. Here, if the activity is much lower than in absence of the support, the likeliest explanation is that substrate has been adsorbed on the support. This will produce an apparently lower expressed activity than the real one. The easiest way to solve this problem is to increase the concentration of substrate, but in some instances this strategy is not possible because the substrate concentrations are

already close to maximum solubility. To avoid this, another possibility is to alter the medium conditions during the activity determination in such a way that the substrate can hardly be adsorbed on the support. For example, using a hydrophobic support and a hydrophobic substrate, this can be prevented using detergent or an organic co-solvent. However, we must consider that this can also lead to enzyme release during activity determination, as stated above. The effect of this problem is an incremental increase in apparent K_M after enzyme immobilization.

If the problem is the product adsorption, again an apparent low expressed activity will be observed. A simple way to confirm this is by adding the support after the determination of the activity using the soluble enzyme. If the absorbance decreases, one can confirm that the problem is the adsorption of the final product on the support. The solution will be to use conditions where the product does not become adsorbed on the support. The effect of this will be a decrease in the apparent maximum activity, which may also affect the apparent K_M, since when the product saturates the support, the activity will increase.

These problems will not have real relevance during operation, since the support will be rapidly saturated by the great excess of substrate/product, but may perhaps interfere in the first minutes of operation in a flow reactor, or in the first cycle of a batch process.

6. 10. Immobilization is very rapid but is not total: less than 100% immobilization yield

589 In some instances, immobilization is very rapid but is not complete (Figure 13). The
590 most obvious reason for this result is that an amount of enzyme that is over the capacity of
591 the support has been used. However, in some cases this low immobilization yield may be
592 based on other reasons.

593 For example, it is possible that the utilized enzyme extract contains several enzymes
594 able to catalyze the reaction and only some of these enzymes become immobilized on the
595 support. The case of a lipase extract contaminated by another non-lipase esterase when
596 immobilized on hydrophobic supports at low ionic strength, (following the activity using *p*-
597 nitrophenyl esters), is perhaps one of the most best illustrations (169, 170, 171, 172). Only
598 the lipase will be immobilized on these supports under these conditions, and the esterase
599 will remain in the supernatant (Figure 13). To check this (or similar situations), when using
600 different amounts of enzyme per g of support, a similar immobilization yield should be
601 always obtained until reaching an enzyme loading that can fully saturate the support surface
602 with the target enzyme. Beyond that point the lipase immobilization yield should start to
603 decrease. When presenting these results, an immobilization yield under 100% at all enzyme
604 concentrations should be seen. However, it should be noted that this is a positive result.: the
605 enzyme has effectively been purified during the immobilization process (Figure 14). This
606 lipase immobilization peculiarity has been used even to separate different lipases having
607 different affinities on different supports (173).

608 In other cases, the reason for not all of the enzyme being immobilized may be
609 simply because of different extents of glycosylation between different enzymes (e.g. to

610 immobilize hyper-glycosylated enzymes may prove very difficult), this will give observed
611 behavior similar to the case above.

612 In other cases, the problem may be the presence of some large soluble protein
613 aggregates that can close the pores and that way prevent enzyme immobilization. This can
614 be overcome by adding moderate amounts of chaotropic agents to break the aggregates
615 (Figure 15).

616 To distinguish between the different possibilities, clean supports must be offered to
617 the supernatant of the previous immobilization suspension. If some enzyme is immobilized,
618 an overestimate of the loading capacity of the support may be the problem. If this problem
619 remains when using different enzyme loadings, a likely explanation is that something in the
620 extract is blocking the enzyme immobilization.

621 If no more enzyme activity is immobilized in this experiment, it is clear that the
622 remaining enzymes that express this percentage of activity cannot become immobilized on
623 the used support, and they must be different from the enzyme that has been immobilized.

624 **6.11. The enzyme extract contains an inhibitor that is adsorbed by the support**

625 Many liquid commercial enzyme extracts are supplemented with some stabilization
626 formulations, and in some instances some of these components may interfere with the
627 activity determination measurement (e.g. inhibition of the enzyme, since the compounds
628 become adsorbed to the support matrix). This can lead to some curious "immobilization
629 time courses" (Figure 16). The concentration of such an inhibitor in the supernatant will
630 progressively decrease , and in this way the observed activity of the enzyme in the

supernatant will increase, while the activity of the reference is maintained during the entire immobilization time course. If the adsorbed inhibitor is unable to interact with the immobilized enzyme, also the immobilized enzyme will apparently increase activity during immobilization (Figure 16 a). That way, the expressed activity will be over 100%, but the calculation of immobilization yield will be nearly impossible (in the figure it shows a "negative yield"), and the global enzyme activity yield will gave a confusing number. Here, the combination of activity and protein concentration data may be helpful.

Results may be wrongly too negative for the global enzyme activity yield. A theoretical case may be the use of a hydrophobic support able to immobilize an inhibitor by hydrophobic interaction. Figure 16b shows an example where the inhibitor is released during the activity determination (e.g., some solvent or detergent is added). In this way, it can also interact with the enzyme in the supernatant and can show an almost real immobilization time course. After immobilization, the immobilized biocatalyst is washed with water (that may not be eliminated by the inhibitor), and now we determine the activity at a high concentration of enzyme because these are the desired operating conditions: the enzyme will be exposed to more inhibitor, and that can produce a falsely low activity.

In these cases, the problem is to ensure what is the cause of the results.

To discriminate this, the supernatant of the immobilization suspension may be taken, offered to a new batch of clean support, and check to see if the problem remains or if now the enzyme activity in the supernatant is maintained. Using inactivated immobilized enzyme may also help to reveal if a component added to the support after immobilization has altered the enzyme activity. A more satisfying way to do the experiment may be to

dialyze the enzyme extract and check if this has some effect on enzyme activity and the immobilization time course (although may be the amount of inhibitor is insufficient or else cannot be dialyzed). In case dialysis can remove the inhibitor, it would be better to use the dialyzed extract to perform the immobilization, even though this can produce some problems for enzyme inactivation during immobilization.

7. IMPLICATIONS OF DEFINITION OF ENZYME IMMOBILIZED BIOCATALYSTS FOR PROCESS APPLICATIONS

Today there are two important drivers for the increased application of immobilized enzymes in biocatalysis. The first concerns the need to retain and easily separate enzymes run in continuous flow systems (15). This has attracted great interest in the pharmaceutical industry where it is the ability of enzymes to selectively and accurately produce high quality product that is exploited. Flow systems can capitalize upon this, since the residence time of material in the reactor is very carefully controlled, and this necessitates also enzyme immobilization for enzyme retention. In such cases knowledge about the observed activity is extremely important since it helps explain deviations from the expected results when operating in such a different hydrodynamic environment to that where standard enzyme tests may be performed. Additionally a feature of flow reactors is that very rapid changes in conditions can be made (due to the high surface area) which may also give interesting results. The second driver for the increased application of immobilized enzymes is in the low-priced chemical (and biofuel) industry. Here reuse of the enzyme is essential or else continuous operation required. In either case immobilization is a key tool, in order to

675 separate or retain enzyme. Here too understanding the immobilization protocol and its
676 effects are essential, in this case since the economy is vital to the viability of the final
677 process. Even small improvements in observed activity can have a marked benefit to the
678 process. Likewise the amount of activity which can be loaded in the reactor can become of
679 great importance (related also to enzyme purity and loading on the immobilization support).

680

681 **Acknowledgments:**

682 This research was funded by MICIU, grant number CTQ2017-86170-R

683

684 **References**

685 **References**

686 1) Sheldon, R.A. Fundamentals of green chemistry: Efficiency in reaction design (2012)
687 *Chemical Society Reviews*, 41 (2012) 1437-1451. DOI: 10.1039/c1cs15219j

688 2) Pollard, D.J., Woodley, J.M. Biocatalysis for pharmaceutical intermediates: the future is
689 now, *Trends in Biotechnology*, 25 (2007) 66-73. DOI: 10.1016/j.tibtech.2006.12.005

690 3) Woodley, J.M., New opportunities for biocatalysis: making pharmaceutical processes
691 greener, *Trends in Biotechnology*, 26 (2008) 321-327. DOI: 10.1016/j.tibtech.2008.03.004

692 4) Sheldon, R.A., Woodley, J.M., Role of Biocatalysis in Sustainable Chemistry, *Chemical*
693 *Reviews*, 118 (2018) 801-838. DOI: 10.1021/acs.chemrev.7b00203

694 5) Sheldon, R.A., Brady, D., Broadening the Scope of Biocatalysis in Sustainable Organic
695 Synthesis, *ChemSusChem*, 12 (2019) 2859-2881. DOI: 10.1002/cssc.201900351).

696 6) Thompson, M.P., Peñafiel, I., Cosgrove, S.C., Turner, N.J. Biocatalysis Using
697 Immobilized Enzymes in Continuous Flow for the Synthesis of Fine Chemicals, *Organic*
698 *Process Research and Development*, 23 (2019) 9-18. , DOI: 10.1021/acs.oprd.8b00305

699 7) Bezbradica, D., Ćrovic, M., Tanasković, S.J., Luković, N., Carević, M., Milivojević, A.,
700 Knezević-Jugović, Z., Enzymatic syntheses of esters - Green chemistry for valuable food,
701 fuel and fine chemicals, *Current Organic Chemistry*, 21 (2017) 104-138. DOI:
702 10.2174/1385272821666161081233 26

8) Schoemaker, H.E., Mink, D., WubboLts, M.G., Dispelling the myths - Biocatalysis in industrial synthesis, *Science*, 299 (2003) 1694-1697. DOI: 10.1126/science.1079237

9) Serra, S., Fuganti, C., Brenna, E., Biocatalytic preparation of natural flavours and fragrances, *Trends in Biotechnology*, 23 (2005) 193-198. DOI: 10.1016/j.tibtech.2005.02.003

10) Aravindan, R., Anbumathi, P., Viruthagiri, T., Lipase applications in food industry, *Indian Journal of Biotechnology*, 6 (2007) 141-158.

11) Tan, T., Lu, J., Nie, K., Deng, L., Wang, F., Biodiesel production with immobilized lipase: A review, *Biotechnology Advances*, 28 (2010) 628-634. DOI: 10.1016/j.biotechadv.2010.05.012

12) Gog, A., Roman, M., Toşa, M., Paizs, C., Irimie, F.D. Biodiesel production using enzymatic transesterification - Current state and perspectives, *Renewable Energy*, 39 (2012) 10-16. DOI: 10.1016/j.renene.2011.08.007

13) Volpato, G., Rodrigues, R.C., Fernandez-Lafuente, R., Use of enzymes in the production of semi-synthetic penicillins and cephalosporins: Drawbacks and perspectives, *Current Medicinal Chemistry*, 17 (2010) 3855-3873. DOI: 10.2174/092986710793205435

14) Fei, Q., Guarnieri, M.T., Tao, L., Laurens, L.M.L., Dowe, N., Pienkos, P.T. Bioconversion of natural gas to liquid fuel: Opportunities and challenges, *Biotechnology Advances*, 32 (2014) 596-614. DOI: 10.1016/j.biotechadv.2014.03.011

15) Sheldon, R.A., van Pelt, S., Enzyme immobilisation in biocatalysis: Why, what and how, *Chemical Society Reviews*, 42 (2013) 6223-6235. DOI: 10.1039/c3cs60075k

16) Klibanov, A.M., Improving enzymes by using them in organic solvents, *Nature*, 409 (1989) 241-246. DOI: 10.1038/35051719

17) Dordick, J.S., Enzymatic catalysis in monophasic organic solvents, *Enzyme and Microbial Technology*, 11 (1989) 194-211. DOI: 10.1016/0141-0229(89)90094-X

18) Klibanov, A.M., Enzymatic catalysis in anhydrous organic solvents, *Trends in Biochemical Sciences*, 14 (1989) 141-144. DOI: 10.1016/0968-0004(89)90146-1.

19) Inada, Y., Takahashi, K., Yoshimoto, T., Ajima, A., Matsushima, A., Saito, Y.Application of polyethylene glycol-modified enzymes in biotechnological processes: organic solvent-soluble enzymes, *Trends in Biotechnology*, 4 (1986) 190-194. DOI: 10.1016/0167-7799(86)90244-1

20) Luisi, P.L., Laane, C., Solubilization of enzymes in apolar solvents via reverse micelles, *Trends in Biotechnology*, 4 (1986) 153-161. DOI: 10.1016/0167-7799(86)90166-6

21) Ren, S., Li, C., Jiao, X., Jia, S., Jiang, Y., Bilal, M., Cui, J., Recent progress in multienzymes co-immobilization and multienzyme system applications, *Chemical Engineering Journal*, 373 (2019) 1254-1278. DOI: 10.1016/j.cej.2019.05.141

22) Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R., Strategies for the one-step immobilization-purification of

743 enzymes as industrial biocatalysts, *Biotechnology Advances*, 33 (2015) 435-456.
744 DOI: 10.1016/j.biotechadv.2015.03.006

745 23) Bilal, M., Zhao, Y., Noreen, S., Shah, S.Z.H., Bharagava, R.N., Iqbal, H.M.N.,
746 Modifying bio-catalytic properties of enzymes for efficient biocatalysis: a review
747 from immobilization strategies viewpoint, *Biocatalysis and Biotransformation*, 37
748 (2019) 159-182. DOI: 10.1080/10242422.2018.156474

749 24) Hwang, E.T., Lee, S., Multienzymatic Cascade Reactions via Enzyme Complex
750 by Immobilization, *ACS Catalysis*, 9 (2019) 4402-4425. DOI:
751 10.1021/acscatal.8b04921

752 25) Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M., Fernandez-
753 Lafuente, R., Improvement of enzyme activity, stability and selectivity via
754 immobilization techniques, *Enzyme and Microbial Technology*, 40 (2007) 1451-
755 1463. DOI: 10.1016/j.enzmictec.2007.01.018

756 26) Rodrigues, R.C., Ortiz, C., Berenguer-Murcia, A., Torres, R., Fernández-
757 Lafuente, R., Modifying enzyme activity and selectivity by immobilization,
758 *Chemical Society Reviews*, 42 (2013) 6290-6307. DOI: 10.1039/c2cs35231a

759 27) Garcia-Galan, C., Berenguer-Murcia, A., Fernandez-Lafuente, R., Rodrigues,
760 R.C., Potential of different enzyme immobilization strategies to improve enzyme
761 performance, *Advanced Synthesis and Catalysis*, 353 (2011) 2885-2904.

762 28) Santos, J.C.S.D., Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Rodrigues, R.C.,
763 Fernandez-Lafuente, R., Importance of the Support Properties for Immobilization

764 or Purification of Enzymes, *ChemCatChem*, 7 (2015) 2413-2432. DOI:
765 10.1002/cctc.201500310

766 29) Iyer, P.V., Ananthanarayan, L., Enzyme stability and stabilization-Aqueous and
767 non-aqueous environment, *Process Biochemistry*, 43 (2008) 1019-1032. DOI:
768 10.1016/j.procbio.2008.06.004

769 30) Brady, D., Jordaan, J. , Advances in enzyme immobilisation, *Biotechnology*
770 *Letters*, 31 (2009) 1639-1650. DOI: 10.1007/s10529-009-0076-4

771 31) Cao, L., Immobilized enzymes: Science or art? *Current Opinion in Chemical*
772 *Biology*, 9 (2005) 217-226. DOI: 10.1016/j.cbpa.2005.02.014

773 32) Cao, L., van Langen, L., Sheldon, R.A., Immobilised enzymes: Carrier-bound or
774 carrier-free?, *Current Opinion in Biotechnology*, 14 (2003) 387-394. DOI:
775 10.1016/S0958-1669(03)00096-X

776 33) Bilal, M., Zhao, Y., Noreen, S., Shah, S.Z.H., Bharagava, R.N., Iqbal, H.M.N.,
777 Modifying bio-catalytic properties of enzymes for efficient biocatalysis: a review
778 from immobilization strategies viewpoint, *Biocatalysis and Biotransformations*,
779 37 (3) (2019) 159-182. , DOI: 10.1080/10242422.2018.1564744

780 34) Ren, S., Li, C., Jiao, X., Jia, S., Jiang, Y., Bilal, M., Cui, J. , Recent progress in
781 multienzymes co-immobilization and multienzyme system applications, Chemical
782 Engineering Journal, 373 (2019) 1254-1278. DOI: 10.1016/j.cej.2019.05.141

35) Hwang, E.T., Lee, S., Multienzymatic Cascade Reactions via Enzyme Complex by Immobilization, *ACS Catalysis*, 9 (2019) 4402-4425. DOI: 10.1021/acscatal.8b04921.

36) Bilal, M., Iqbal, H.M.N., Tailoring Multipurpose Biocatalysts via Protein Engineering Approaches: A Review, *Catalysis Letters*, 149 (2019) 2204-2217. DOI: 10.1007/s10562-019-02821-8

37) Shemsi, A.M., Khanday, F.A., Qurashi, A., Khalil, A., Guerriero, G., Siddiqui, K.S., Site-directed chemically-modified magnetic enzymes: fabrication, improvements, biotechnological applications and future prospects, *Biotechnology Advances*, 37 (2019) 357-381. DOI: 10.1016/j.biotechadv.2019.02.002

38) Bernal, C., Rodríguez, K., Martínez, R., Integrating enzyme immobilization and protein engineering: An alternative path for the development of novel and improved industrial biocatalysts, *Biotechnology Advances*, 36 (2018) 1470-1480. DOI: 10.1016/j.biotechadv.2018.06.002.

39) Rodrigues, R.C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., Coupling chemical modification and immobilization to improve the catalytic performance of enzymes, *Advanced Synthesis and Catalysis*, 353 (2011) 2216-2238. DOI: 10.1002/adsc.201100163

40) Rodrigues, R.C., Godoy, C.A., Volpato, G., Ayub, M.A.Z., Fernandez-Lafuente, R., Guisan, J.M., Immobilization-stabilization of the lipase from Thermomyces

803 lanuginosus: Critical role of chemical amination, *Process Biochemistry*, 44 (2009)
804 963-968. DOI: 10.1016/j.procbio.2009.04.015

805 41) Rueda, N., dos Santos, J.C.S., Ortiz, C., Torres, R., Barbosa, O., Rodrigues, R.C.,
806 Berenguer-Murcia, Á., Fernandez-Lafuente, R., Chemical Modification in the
807 Design of Immobilized Enzyme Biocatalysts: Drawbacks and Opportunities,
808 (2016) *Chemical Record*, 16 (2016) 1436-1455. DOI: 10.1002/tcr.201600007

809 42) Rodrigues, R.C., Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R.,
810 Fernandez-Lafuente, R., Amination of enzymes to improve biocatalyst
811 performance: Coupling genetic modification and physicochemical tools, (2014)
812 *RSC Advances*, 4 (2014) 38350-38374. DOI: 10.1039/c4ra04625k

813 43) Cowan, D.A., Fernandez-Lafuente, R., Enhancing the functional properties of
814 thermophilic enzymes by chemical modification and immobilization, *Enzyme and*
815 *Microbial Technology*, 49 (2011) 326-346. DOI:
816 10.1016/j.enzmictec.2011.06.023

817 44) Hernandez, K., Fernandez-Lafuente, R., Control of protein immobilization:
818 Coupling immobilization and site-directed mutagenesis to improve biocatalyst or
819 biosensor performance, *Enzyme and Microbial Technology*, 48 (2011) 107-122.
820 DOI: 10.1016/j.enzmictec.2010.10.003

821 45) Barbosa, O., Torres, R., Ortiz, C., Berenguer-Murcia, A., Rodrigues, R.C.,
822 Fernandez-Lafuente, R., Heterofunctional supports in enzyme immobilization:

From traditional immobilization protocols to opportunities in tuning enzyme properties, *Biomacromolecules*, 14 (2013) 2433-2462. DOI: 10.1021/bm400762h

46) Virgen-Ortíz, J.J., Peirce, S., Tacias-Pascacio, V.G., Cortes-Corberan, V., Marzocchella, A., Russo, M.E., Fernandez-Lafuente, R. Reuse of anion exchangers as supports for enzyme immobilization: Reinforcement of the enzyme-support multiinteraction after enzyme inactivation. *Process Biochemistry*, 51 (2016) 1391-1396. DOI: 10.1016/j.procbio.2016.06.020

47) Barbosa, O., Torres, R., Ortiz, C., Berenguer-Murcia, A., Rodrigues, R.C., Fernandez-Lafuente, R., Heterofunctional supports in enzyme immobilization: From traditional immobilization protocols to opportunities in tuning enzyme properties, *Biomacromolecules*, 14 (2013) 2433-2462. DOI: 10.1021/bm400762h

48) Virgen-Ortíz, J.J., Pedrero, S.G., Fernandez-Lopez, L., Lopez-Carrobles, N., Gorines, B.C., Otero, C., Fernandez-Lafuente, R. Desorption of lipases immobilized on octyl-agarose beads and coated with ionic polymers after thermal inactivation. Stronger adsorption of polymers/unfolded protein composites. *Molecules*, 22 (2017) art. no. 91. DOI: 10.3390/molecules22010091

49) Gonçalves, M.C.P., Kieckbusch, T.G., Perna, R.F., Fujimoto, J.T., Morales, S.A.V., Romanelli, J.P., Trends on enzyme immobilization researches based on bibliometric analysis, *Process Biochemistry*, 76 (2019) 95-110. DOI: 10.1016/j.procbio.2018.09.016 .

50) Kozuch, S., Martin, J.M.L., 'Turning Over' Definitions in Catalytic Cycles. *ACS Catalysis*, 2 (2012) 2787-2794.

51) Bligaard, T., Bullock, R.M., Campbell, C.T., Chen, J.G., Gates, B.C., Gorte, R.J., Jones, C.W., Jones, W.D., Kitchen, J.R., Scott, S.L. 2016. Towards Benchmarking in Catalysis Science: Best practices, Challenges and Opportunities. *ACS Catalysis*, 6 (2016) 2590-2602.

52) Dias Gomes, M., Woodley, J.M. 2019. Considerations when Measuring Biocatalyst Performance. *Molecules*, 24 (2019) art. no 3573.

53) Zucca, P., Fernandez-Lafuente, R., Sanjust, E., Agarose and its derivatives as supports for enzyme immobilization, *Molecules*, 21 (2016) art. no. 1577. DOI: 10.3390/molecules21111577.

54) Mateo, C., Abian, O., Bernedo, M., Cuenca, E., Fuentes, M., Fernandez-Lorente, G., Palomo, J.M., Grazu, V., Pessela, B.C.C., Giacomini, C., Irazoqui, G., Villarino, A., Ovsejevi, K., Batista-Viera, F., Fernandez-Lafuente, R., Guisán, J.M., Some special features of glyoxyl supports to immobilize proteins, *Enzyme and Microbial Technology*, 37 (2005) 456-462. DOI: 10.1016/j.enzmictec.2005.03.020,

55) Palomo, J.M., Fuentes, M., Fernández-Lorente, G., Mateo, C., Guisan, J.M., Fernández-Lafuente, R., General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality, *Biomacromolecules*, 4 (2003) 1-6. , DOI: 10.1021/bm025729+

56) Wilson, L., Palomo, J.M., Fernández-Lorente, G., Illanes, A., Guisán, J.M., Fernández-Lafuente, R., Effect of lipase-lipase interactions in the activity, stability and specificity of a lipase from *Alcaligenes* sp., *Enzyme and Microbial Technology*, 39 (2006) 259-264. DOI: 10.1016/j.enzmictec.2005.10.015.

57) Fernández-Lorente, G., Palomo, J.M., Fuentes, M., Mateo, C., Guisán, J.M., Fernández-Lafuente, R., Self-assembly of *Pseudomonas fluorescens* lipase into bimolecular aggregates dramatically affects functional properties, *Biotechnology and Bioengineering*, 82 (2003) 232-237. DOI: 10.1002/bit.10560

58) Palomo, J.M., Ortiz, C., Fuentes, M., Fernandez-Lorente, G., Guisan, J.M., Fernandez-Lafuente, R., Use of immobilized lipases for lipase purification via specific lipase-lipase interactions, *Journal of Chromatography* A, 1038 (2004) 267-273. DOI: 10.1016/j.chroma.2004.03.058

59) Palomo, J.M., Ortiz, C., Fernández-Lorente, G., Fuentes, M., Guisán, J.M., Fernández-Lafuente, R., Lipase-lipase interactions as a new tool to immobilize and modulate the lipase properties, *Enzyme and Microbial Technology*, 36 (2005) 447-454. DOI: 10.1016/j.enzmictec.2004.09.013.

60) Pessela, B.C.C., Mateo, C., Carrascosa, A.V., Vian, A., García, J.L., Rivas, G., Alfonso, C., Guisan, J.M., Fernández-Lafuente, R., One-step purification, covalent immobilization, and additional stabilization of a thermophilic poly-his-tagged β-galactosidase from Thermus sp. strain T2 by using novel heterofunctional chelate - Epoxy sepabeads, *Biomacromolecules*, 4 (2003) 107-113. DOI: 10.1021/bm020086j

886 61) Rocchietti, S., Ubiali, D., Terreni, M., Albertini, A.M., Fernández-Lafuente, R.,
887 Guisán, J.M., Pregnolato, M., Immobilization and stabilization of recombinant
888 multimeric uridine and purine nucleoside phosphorylases from *Bacillus subtilis*,
889 *Biomacromolecules*, 5 (2004) 2195-2200. DOI: 10.1021/bm049765f

890 62) Ubiali, D., Rocchietti, S., Scaramozzino, F., Terreni, M., Albertini, A.M.,
891 Fernández-Lafuente, R., Guisán, J.M., Pregnolato, M., Synthesis of 2′-
892 deoxynucleosides by transglycosylation with new immobilized and stabilized
893 uridine phosphorylase and purine nucleoside phosphorylase, *Advanced Synthesis*
894 *and Catalysis*, 346 (2004) 1361-1366. DOI: 10.1002/adsc.200404019.

895 63) Bommarius, A.S., Paye, M.F., Stabilizing biocatalysts, *Chemical Society Reviews*,
896 42 (2013) 6534-6565. DOI: 10.1039/c3cs60137d.

897 64) Mateo, C., Palomo, J.M., Fuentes, M., Betancor, L., Grazu, V., López-Gallego, F.,
898 Pessela, B.C.C., Hidalgo, A., Fernández-Lorente, G., Fernández-Lafuente, R.,
899 Guisán, J. M., Glyoxyl agarose: A fully inert and hydrophilic support for
900 immobilization and high stabilization of proteins, *Enzyme and Microbial*
901 *Technology*, 39 (2006) 274-280. DOI: 10.1016/j.enzmictec.2005.10.014 .

902 65) Blanco, R.M., Guisán, J. M.Stabilization of enzymes by multipoint covalent
903 attachment to agarose-aldehyde gels. Borohydride reduction of trypsin-agarose
904 derivatives, *Enzyme and Microbial Technology*, 11 (1989) 360-366. DOI:
905 10.1016/0141-0229(89)90020-3.

66) Guisán, J.M. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. *Enzyme and Microbial Technology*, 10 (1988) 375-382. DOI: 10.1016/0141-0229(88)90018-X.

67) Orrego, A.H., Romero-Fernández, M., Millán-Linares, M.C., Yust, M.M., Guisán, J.M., Rocha-Martin, J., Stabilization of enzymes by multipoint covalent attachment on aldehyde-supports: 2-picoline borane as an alternative reducing agent, *Catalysts*, 8, (2018) art. no. 333. DOI: 10.3390/catal8080333.

68) Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R.Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization, *RSC Advances*, 4 (2014) (2014) 1583-1600. DOI: 10.1039/c3ra45991h.

69) Virgen-Ortíz, J.J., Dos Santos, J.C.S., Berenguer-Murcia, Á., Barbosa, O., Rodrigues, R.C., Fernandez-Lafuente, R. Polyethylenimine: A very useful ionic polymer in the design of immobilized enzyme biocatalysts. *Journal of Materials Chemistry B*, 5 (2017) 7461-7490. DOI: 10.1039/c7tb01639e

70) Katchalski-Katzir, E., Kraemer, D.M., Eupergit® C, a carrier for immobilization of enzymes of industrial potential, *Journal of Molecular Catalysis - B Enzymatic*, 10 (2000) 157-176. DOI: 10.1016/S1381-1177(00)00124-7,

71) Boller, T., Meier, C., Menzler, S., EUPERGIT oxirane acrylic beads: How to make enzymes fit for biocatalysis, *Organic Process Research and Development*, 6 (2002) 509-519. DOI: 10.1021/op015506w

927 72) Mateo, C., Abian, O., Fernández-Lorente, G., Pedroche, J., Fernández-Lafuente,
928 R., Guisan, J.M., Tam, A., Daminati, M., Epoxy Sepabeads: A novel epoxy
929 support for stabilization of industrial enzymes via very intense multipoint covalent
930 attachment, *Biotechnology Progress*, 18 (2002) 629-634. DOI:
931 10.1021/bp010171n.

932 73) Garcia-Galan, C., Berenguer-Murcia, A., Fernandez-Lafuente, R., Rodrigues,
933 R.C., Potential of different enzyme immobilization strategies to improve enzyme
934 performance, *Advanced Synthesis and Catalysis*, 353 (2011) 2885-2904. DOI:
935 10.1002/adsc.201100534,

936 74) Barbosa, O., Torres, R., Ortiz, C., Berenguer-Murcia, A., Rodrigues, R.C.,
937 Fernandez-Lafuente, R., Heterofunctional supports in enzyme immobilization:
938 From traditional immobilization protocols to opportunities in tuning enzyme
939 properties, *Biomacromolecules*, 14 (2013) 2433-2462. DOI: 10.1021/bm400762h,

940 75) Torres, R., Mateo, C., Fernández-Lorente, G., Ortiz, C., Fuentes, M., Palomo,
941 J.M., Guisan, J.M., Fernández-Lafuente, R., A novel heterofunctional epoxy-
942 amino sepabeads for a new enzyme immobilization protocol: Immobilization-
943 stabilization of β-galactosidase from Aspergillus oryzae, *Biotechnology Progress*,
944 19 (2003) 1056-1060. DOI: 10.1021/bp025771g,

945 76) Rueda, N., Dos Santos, J.C.S., Torres, R., Ortiz, C., Barbosa, O., Fernandez-
946 Lafuente, R., Improved performance of lipases immobilized on heterofunctional
947 octyl-glyoxyl agarose beads, (2015) *RSC Advances*, 5 (2015) 11212-11222. DOI:
948 10.1039/c4ra13338b

77) Mateo, C., Bolivar, J.M., Godoy, C.A., Rocha-Martin, J., Pessela, B.C., Curiel, J.A., Muñoz, R., Guisan, J.M., Fernández-Lorente, G.Improvement of enzyme properties with a two-step immobilizaton process on novel heterofunctional supports, *Biomacromolecules*, 11 (11) (2010) 3112-3117. DOI: 10.1021/bm100916r,

78) Albuquerque, T.L.D., Rueda, N., Dos Santos, J.C.S., Barbosa, O., Ortiz, C., Binay, B., Özdemir, E., Gonçalves, L.R.B., Fernandez-Lafuente, R., Easy stabilization of interfacially activated lipases using heterofunctional divinyl sulfone activated-octyl agarose beads. Modulation of the immobilized enzymes by altering their nanoenvironment, *Process Biochemistry*, 51 (2016) 865-874. DOI: 10.1016/j.procbio.2016.04.002.

79) Zaak, H., Sassi, M., Fernandez-Lafuente, R., A new heterofunctional amino-vinyl sulfone support to immobilize enzymes: Application to the stabilization of β-galactosidase from *Aspergillus oryzae*, Process Biochemistry, 64 (2018) 200-205. DOI: 10.1016/j.procbio.2017.09.020.

80) Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R., Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization, *RSC Advances*, 4 (2014) 1583-1600. DOI: 10.1039/c3ra45991h.

81) Betancor, L., López-Gallego, F., Hidalgo, A., Alonso-Morales, N., Dellamora-Ortiz, G., CesarMateo, G.D., Fernández-Lafuente, R., Guisán, J.M., Different mechanisms of protein immobilization on glutaraldehyde activated supports:

971 Effect of support activation and immobilization conditions, *Enzyme and Microbial*
972 *Technology*, 39 (2006) 877-882. , DOI: 10.1016/j.enzmictec.2006.01.014.

973 82) de Andrades, D., Graebin, N.G., Kadowaki, M.K., Ayub, M.A.Z., Fernandez-
974 Lafuente, R., Rodrigues, R.C., Immobilization and stabilization of different β-
975 glucosidases using the glutaraldehyde chemistry: Optimal protocol depends on the
976 enzyme, *International Journal of Biological Macromolecules*, 129 (2019) 672-
977 678. DOI: 10.1016/j.ijbiomac.2019.02.057.

978 83) Ait Braham, S., Hussain, F., Morellon-Sterling, R., Kamal, S., Kornecki, J.F.,
979 Barbosa, O., Kati, D.E., Fernandez-Lafuente, R., Cooperativity of covalent
980 attachment and ion exchange on alcalase immobilization using glutaraldehyde
981 chemistry: Enzyme stabilization and improved proteolytic activity, *Biotechnology*
982 *Progress*, 35 (2019) art. no. e2768. DOI: 10.1002/btpr.2768

983 84) Siar, E.-H., Arana-Peña, S., Barbosa, O., Zidoune, M.N., Fernandez-Lafuente, R.,
984 Immobilization/stabilization of ficin extract on glutaraldehyde-activated agarose
985 beads. Variables that control the final stability and activity in protein hydrolyses,
986 *Catalysts*, 8 (2018) art. no. 149. DOI: 10.3390/catal8040149

987 85) Vazquez-Ortega, P.G., Alcaraz-Fructuoso, M.T., Rojas-Contreras, J.A., López-
988 Miranda, J., Fernandez-Lafuente, R., Stabilization of dimeric β-glucosidase from
989 Aspergillus niger via glutaraldehyde immobilization under different conditions,
990 *Enzyme and Microbial Technology*, 110 (2018) 38-45. DOI:
991 10.1016/j.enzmictec.2017.12.007

86) Zaak, H., Peirce, S., de Albuquerque, T.L., Sassi, M., Fernandez-Lafuente, R., Exploiting the versatility of aminated supports activated with glutaraldehyde to immobilize β-galactosidase from *Aspergillus oryzae*, *Catalysts*, 7 (2017) art. no. 250. DOI: 10.3390/catal7090250

87) Barbosa, O., Torres, R., Ortiz, C., Fernandez-Lafuente, R. Versatility of glutaraldehyde to immobilize lipases: Effect of the immobilization protocol on the properties of lipase B from Candida antarctica, *Process Biochemistry*, 47 (2012) 1220-1227. DOI: 10.1016/j.procbio.2012.04.019.

88) Rodrigues, R.C., Virgen-Ortíz, J.J., dos Santos, J.C.S., Berenguer-Murcia, Á., Alcantara, A.R., Barbosa, O., Ortiz, C., Fernandez-Lafuente, R., Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions, *Biotechnology Advances*, 37 (2019) 746-770. DOI: 10.1016/j.biotechadv.2019.04.003,

89) Qin, Z., Lin, S., Qiu, Y., Chen, Q., Zhang, Y., Zhou, J., Zhao, L., One-step immobilization-purification of enzymes by carbohydrate-binding module family 56 tag fusion, *Food Chemistry*, 299 (2019) art. no. 125037. DOI: 10.1016/j.foodchem.2019.125037

90) Zhou, L., Li, J., Gao, J., Liu, H., Xue, S., Ma, L., Cao, G., Huang, Z., Jiang, Y., Facile Oriented Immobilization and Purification of His-Tagged Organophosphohydrolase on Viruslike Mesoporous Silica Nanoparticles for Organophosphate Bioremediation, *ACS Sustainable Chemistry and Engineering*, 6 (2018) 13588-13598., DOI: 10.1021/acssuschemeng.8b04018

91) Zhou, Y., Yuan, S., Liu, Q., Yan, D., Wang, Y., Gao, L., Han, J., Shi, H., Synchronized purification and immobilization of his-tagged β-glucosidase via Fe3O4/PMG core/shell magnetic nanoparticles, *Scientific Reports*, 7 (2017) art. no. 41741. DOI: 10.1038/srep41741

92) Cao, G., Gao, J., Zhou, L., Huang, Z., He, Y., Zhu, M., Jiang, Y., Fabrication of Ni2+-nitrilotriacetic acid functionalized magnetic mesoporous silica nanoflowers for one pot purification and immobilization of His-tagged Ω-transaminase, *Biochemical Engineering Journal*, 128 (2017) 116-125. DOI: 10.1016/j.bej.2017.09.01,

93) Döbber, J., Pohl, M., New Options for Biocatalysis: Merging Purification and Immobilization through Innovative Binding Tags, Chemie-Ingenieur-Technik, 88 (2016) 1245. DOI: 10.1002/cite.201650087

94) Pessela, B.C.C., Mateo, C., Carrascosa, A.V., Vian, A., García, J.L., Rivas, G., Alfonso, C., Guisan, J.M., Fernández-Lafuente, R., One-step purification, covalent immobilization, and additional stabilization of a thermophilic poly-his-tagged β-galactosidase from *Thermus* sp. strain T2 by using novel heterofunctional chelate - Epoxy sepabeads, *Biomacromolecules*, 4 (2003) 107-113. DOI: 10.1021/bm020086j

95) Mateo, C., Fernández-Lorente, G., Cortés, E., Garcia, J.L., Fernández-Lafuente, R., Guisan, J.M., One-step purification, covalent immobilization, and additional stabilization of poly-his-tagged proteins using novel heterofunctional chelate-

epoxy supports, *Biotechnology and Bioengineering*, 76 (2001) 269-276. DOI: 10.1002/bit.10019

96) Rodrigues, R.C., Ortiz, C., Berenguer-Murcia, A., Torres, R., Fernández-Lafuente, R., Modifying enzyme activity and selectivity by immobilization, *Chemical Society Reviews*, 42 (2013) 6290-6307. DOI: 10.1039/c2cs35231a.

97) Hernandez, K., Fernandez-Lafuente, R. Control of protein immobilization: Coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. *Enzyme and Microbial Technology*, 48 (2011) 107-122. DOI: 10.1016/j.enzmictec.2010.10.003.

98) Dal Magro, L., Kornecki, J.F., Klein, M.P., Rodrigues, R.C., Fernandez-Lafuente, R., Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range, *Enzyme and Microbial Technology*, 132 (2020) art. no. 109397, DOI: 10.1016/j.enzmictec.2019.109397

99) Dal Magro, L., Kornecki, J.F., Klein, M.P., Rodrigues, R.C., Fernandez-Lafuente, R., Optimized immobilization of polygalacturonase from *Aspergillus niger* following different protocols: Improved stability and activity under drastic conditions, *International Journal of Biological Macromolecules*, 138 (2019) 234-243. DOI: 10.1016/j.ijbiomac.2019.07.092,

100) Bilal, M., Asgher, M., Shah, S.Z.H., Iqbal, H.M.N., Engineering enzyme-coupled hybrid nanoflowers: The quest for optimum performance to meet

biocatalytic challenges and opportunities, *International Journal of Biological Macromolecules*, 135 (2019) 677-690. DOI: 10.1016/j.ijbiomac.2019.05.206

101) Bilal, M., Iqbal, H.M.N., Chemical, physical, and biological coordination: An interplay between materials and enzymes as potential platforms for immobilization, *Coordination Chemistry Reviews*, 388 (2019) 1-23. DOI: 10.1016/j.ccr.2019.02.024

102) Tran, T.D., Kim, M.I., Organic-Inorganic Hybrid Nanoflowers as Potent Materials for Biosensing and Biocatalytic Applications, *Biochip Journal*, 12 (2018) 268-279. DOI: 10.1007/s13206-018-2409-7

103) Celik, C., Tasdemir, D., Demirbas, A., Kati, A., Gul, O.T., Cimen, B., Ocsoy, I., Formation of functional nanobiocatalysts with a novel and encouraging immobilization approach and their versatile bioanalytical applications, *RSC Advances*, 8 (2018) 25298-25303. DOI: 10.1039/c8ra03250e

104) Altinkaynak, C., Tavlasoglu, S., Özdemir, N., Ocsoy, I., A new generation approach in enzyme immobilization: Organic-inorganic hybrid nanoflowers with enhanced catalytic activity and stability, *Enzyme and Microbial Technology*, 93-94 (2016) 105-112. DOI: 10.1016/j.enzmictec.2016.06.011

105) Wu, X., Hou, M., Ge, J., Metal-organic frameworks and inorganic nanoflowers: A type of emerging inorganic crystal nanocarrier for enzyme immobilization, *Catalysis Science and Technology*, 5 (2015) 5077-5085. DOI: 10.1039/c5cy01181g

106) Lee, S.W., Cheon, S.A., Kim, M.I., Park, T.J., Organic-inorganic hybrid nanoflowers: Types, characteristics, and future prospects, *Journal of Nanobiotechnology*, 13 (2015) art. no. 54, . DOI: 10.1186/s12951-015-0118-0.

107) Rodrigues, R.C., Virgen-Ortíz, J.J., dos Santos, J.C.S., Berenguer-Murcia, Á., Alcantara, A.R., Barbosa, O., Ortiz, C., Fernandez-Lafuente, R., Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions, *Biotechnology Advances*, 37 (2019) 746-770. DOI: 10.1016/j.biotechadv.2019.04.003

108) Manoel, E.A., dos Santos, J.C.S., Freire, D.M.G., Rueda, N., Fernandez-Lafuente, R., Immobilization of lipases on hydrophobic supports involves the open form of the enzyme, *Enzyme and Microbial Technology*, 71 (2015) 53-57. DOI: 10.1016/j.enzmictec.2015.02.001.

109) Zaak, H., Sassi, M., Fernandez-Lafuente, R., A new heterofunctional amino-vinyl sulfone support to immobilize enzymes: Application to the stabilization of β-galactosidase from *Aspergillus oryzae*, *Process Biochemistry*, 64 (2018) 200-205. DOI: 10.1016/j.procbio.2017.09.020

110) Dos Santos, J.C.S., Rueda, N., Barbosa, O., Millán-Linares, M.D.C., Pedroche, J., Del Mar Yuste, M., Gonçalves, L.R.B., Fernandez-Lafuente, R., Bovine trypsin immobilization on agarose activated with divinylsulfone: Improved activity and stability via multipoint covalent attachment, *Journal of Molecular Catalysis B: Enzymatic*, 117 (2015) 38-44. Cited 26 times. DOI: 10.1016/j.molcatb.2015.04.008

111) Vazquez-Ortega, P.G., Alcaraz-Fructuoso, M.T., Rojas-Contreras, J.A., López-Miranda, J., Fernandez-Lafuente, R., Stabilization of dimeric β-glucosidase from Aspergillus niger via glutaraldehyde immobilization under different conditions. *Enzyme and Microbial Technology*, 110 (2018) 38-45. DOI: 10.1016/j.enzmictec.2017.12.007.

112) Siar, E.-H., Arana-Peña, S., Barbosa, O., Zidoune, M.N., Fernandez-Lafuente, R. Immobilization/stabilization of ficin extract on glutaraldehyde-activated agarose beads. Variables that control the final stability and activity in protein hydrolyses, *Catalysts*, 8 (2018) art. no. 149. DOI: 10.3390/catal8040149

113) Siar, E.-H., Zaak, H., Kornecki, J.F., Zidoune, M.N., Barbosa, O., Fernandez-Lafuente, R. Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins, *Process Biochemistry*, 58 (2017) 98-104. DOI: 10.1016/j.procbio.2017.04.009.

114) Ranjbari, N., Razzaghi, M., Fernandez-Lafuente, R., Shojaei, F., Satari, M., Homaei, A. Improved features of a highly stable protease from Penaeus vannamei by immobilization on glutaraldehyde activated graphene oxide nanosheets, *International Journal of Biological Macromolecules*, 130 (2019) 564-572. DOI: 10.1016/j.ijbiomac.2019.02.163.

115) Pedroche, J., del Mar Yust, M., Mateo, C., Fernández-Lafuente, R., Girón-Calle, J., Alaiz, M., Vioque, J., Guisán, J.M., Millán, F., Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of

proteins on glyoxyl-agarose supports: Correlation between enzyme-support linkages and thermal stability, *Enzyme and Microbial Technology*, 40 (2007) 1160-1166. DOI: 10.1016/j.enzmictec.2006.08.023.

116) Guisán, J.M., Alvaro, G., Fernandez-Lafuente, R., Rosell, C.M., Garcia, J.L., Tagliani, A. Stabilization of heterodimeric enzyme by multipoint covalent immobilization: Penicillin G acylase from Kluyvera citrophila. *Biotechnology and Bioengineering*, 42 (1993) 455-464. DOI: 10.1002/bit.260420408

117) Siar, E.-H., Morellon-Sterling, R., Zidoune, M.N., Fernandez-Lafuente, R., Amination of ficin extract to improve its immobilization on glyoxyl-agarose: Improved stability and activity versus casein, *International Journal of Biological Macromolecules*, 133 (2019) 412-419. DOI: 10.1016/j.ijbiomac.2019.04.123

118) Tavano, O.L., Berenguer-Murcia, A., Secundo, F., Fernandez-Lafuente, R., Biotechnological Applications of Proteases in Food Technology, *Comprehensive Reviews in Food Science and Food Safety*, 17 (2018) 412-436. DOI: 10.1111/1541-4337.12326

119) Tardioli, P.W., Pedroche, J., Giordano, R.L.C., Fernández-Lafuente, R., Guisán, J.M. Hydrolysis of proteins by immobilized-stabilized Alcalase-glyoxyl agarose. *Biotechnology Progress*, 19 (2003) 352-360. DOI: 10.1021/bp025588

120) Tardioli, P.W., Fernández-Lafuente, R., Guisán, J.M., Giordano, R.L.C. Design of new immobilized-stabilized carboxypeptidase a derivative for

production of aromatic free hydrolysates of proteins. *Biotechnology Progress*, 19 (2003) 565-574. DOI: 10.1021/bp025636.

121) Sousa Jr., R., Lopes, G.P., Tardioli, P.W., Giordano, R.L.C., Almeida, P.I.F., Giordano, R.C. Kinetic model for whey protein hydrolysis by alcalase multipoint-immobilized on agarose gel particles, *Brazilian Journal of Chemical Engineering*, 21 (2004) 147-153.

122) Amaral-Fonseca, M., Kopp, W., Giordano, R.L.C., Fernández-Lafuente, R., Tardioli, P.W. Preparation of magnetic cross-linked amyloglucosidase aggregates: Solving some activity problems, *Catalysts*, 8 (2018) art. no. 496, DOI: 10.3390/catal8110496.

123) Pronk, S.; Lindahl, E.; Kasson, P.M. Dynamic heterogeneity controls diffusion and viscosity near biological interfaces. *Nature Communications*, 5 (2014) 3034.

124) Regan, D.L.; Lilly, M.D.; Dunnill, P. Influence of intraparticle diffuisional limitation on the observed kinetics of immobilized enzymes and on catalyst design. *Biotechnology and Bioengineering*, 16 (1974) 1081–1093.

125) Shen, L.; Chen, Z. Critical review of the impact of tortuosity on diffusion, *Chemical Engineering and Science*, 62 (2007) 3748–3755.

126) Bolivar, J.M.; Consolati, T.; Mayr, T.; Nidetzky, B. Shine a light on immobilized enzymes: real-time sensing in solid supported biocatalysts. *Trends in Biotechnololgy*, 31 (2013) 194–203.

127) Bolivar, J.M.; Eisl, I.; Nidetzky, B. Advanced characterization of immobilized enzymes as heterogeneous biocatalysts. *Catalysis Today*, 80 (2016) 66–80.

128) Bhalerao, M.S., Kulkarni, V.M., Patwardhan, A.V., Ultrasound-assisted chemoenzymatic epoxidation of soybean oil by using lipase as biocatalyst, *Ultrasonics Sonochemistry*, 40 (2018) 912-920. DOI: 10.1016/j.ultsonch.2017.08.042

129) Rokhina, E.V., Lens, P., Virkutyte, J., Low-frequency ultrasound in biotechnology: state of the art, *Trends in Biotechnology*, 27 (2009) 298-306. DOI: 10.1016/j.tibtech.2009.02.001

130) Veljković, V.B., Avramović, J.M., Stamenković, O.S., Biodiesel production by ultrasound-assisted transesterification: State of the art and the perspectives, *Renewable and Sustainable Energy Reviews*, 16 (2012) 1193-1209. DOI: 10.1016/j.rser.2011.11.022

131) Subhedar, P.B., Gogate, P.R., Intensification of enzymatic hydrolysis of lignocellulose using ultrasound for efficient bioethanol production: A review, *Industrial and Engineering Chemistry Research*, 52 (2013) 11816-11828. DOI: 10.1021/ie401286z

132) Martins, A.B., Schein, M.F., Friedrich, J.L.R., Fernandez-Lafuente, R., Ayub, M.A.Z., Rodrigues, R.C., Ultrasound-assisted butyl acetate synthesis catalyzed by Novozym 435: Enhanced activity and operational stability,

1182 *Ultrasonics Sonochemistry*, 20 (2013) 1155-1160. DOI:
1183 10.1016/j.ultsonch.2013.01.018

1184 133) Paludo, N., Alves, J.S., Altmann, C., Ayub, M.A.Z., Fernandez-Lafuente,
1185 R., Rodrigues, R.C. The combined use of ultrasound and molecular sieves
1186 improves the synthesis of ethyl butyrate catalyzed by immobilized Thermomyces
1187 lanuginosus lipase, *Ultrasonics Sonochemistry*, 22 (2015) 89-94. DOI:
1188 10.1016/j.ultsonch.2014.05.004

1189 134) Patil, U., Mokashe, N., Shaha, J., Arthekar, S., Jagatap, H., Ultrasound-
1190 assisted improvements in biocatalytic activity and production of organic-solvent
1191 stable protease from Bacillus circulans MTCC 7942, *Ultrasonics Sonochemistry*,
1192 40 (2018) 201-205. DOI: 10.1016/j.ultsonch.2017.07.012

1193 135) Fernandez-Lopez, L., Pedrero, S.G., Lopez-Carrobles, N., Gorines, B.C.,
1194 Virgen-Ortíz, J.J., Fernandez-Lafuente, R., Effect of protein load on stability of
1195 immobilized enzymes, *Enzyme and Microbial Technology*, 98 (2017) 18-25. DOI:
1196 10.1016/j.enzmictec.2016.12.002

1197 136) Zaak, H., Siar, E.-H., Kornecki, J.F., Fernandez-Lopez, L., Pedrero, S.G.,
1198 Virgen-Ortíz, J.J., Fernandez-Lafuente, R., Effect of immobilization rate and
1199 enzyme crowding on enzyme stability under different conditions. The case of
1200 lipase from Thermomyces lanuginosus immobilized on octyl agarose beads,
1201 *Process Biochemistry*, 56 (2017) 117-123. DOI: 10.1016/j.procbio.2017.02.024. I

137) Mateo, C., Palomo, J.M., Fuentes, M., Betancor, L., Grazu, V., López-Gallego, F., Pessela, B.C.C., Hidalgo, A., Fernández-Lorente, G., Fernández-Lafuente, R., Guisán, J.M., Glyoxyl agarose: A fully inert and hydrophilic support for immobilization and high stabilization of proteins, *Enzyme and Microbial Technology*, 39 (2006) 274-280. DOI: 10.1016/j.enzmictec.2005.10.014

138) Fernandez-Lafuente, R., Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation, *Enzyme and Microbial Technology*, 45 (2009) 405-418. DOI: 10.1016/j.enzmictec.2009.08.009

139) Poltorak, O.M., Chukhray, E.S., Torshin, I.Y., Dissociative thermal inactivation, stability, and activity of oligomeric enzymes, *Biochemistry (Moscow)*, 63 (1998) 303-311.

140) Palomo, J.M., Fuentes, M., Fernández-Lorente, G., Mateo, C., Guisan, J.M., Fernández-Lafuente, R., General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality, *Biomacromolecules*, 4 (2003) 1-6. DOI: 10.1021/bm025729+

141) Wilson, L., Palomo, J.M., Fernández-Lorente, G., Illanes, A., Guisán, J.M., Fernández-Lafuente, R., Effect of lipase-lipase interactions in the activity, stability and specificity of a lipase from Alcaligenes sp., *Enzyme and Microbial Technology*, 39 (2006) 259-264. DOI: 10.1016/j.enzmictec.2005.10.015

142) Fernández-Lorente, G., Palomo, J.M., Fuentes, M., Mateo, C., Guisán, J.M., Fernández-Lafuente, R., Self-assembly of Pseudomonas fluorescens lipase into

1223 bimolecular aggregates dramatically affects functional properties, *Biotechnology*
1224 *and Bioengineering*, 82 (2003) 232-237. DOI: 10.1002/bit.10560

1225 143) Palomo, J.M., Ortiz, C., Fuentes, M., Fernandez-Lorente, G., Guisan, J.M.,
1226 Fernandez-Lafuente, R., Use of immobilized lipases for lipase purification via
1227 specific lipase-lipase interactions, *Journal of Chromatography A*, 1038 (2004)
1228 267-273. DOI: 10.1016/j.chroma.2004.03.058

1229 144) Palomo, J.M., Ortiz, C., Fernández-Lorente, G., Fuentes, M., Guisán, J.M.,
1230 Fernández-Lafuente, R., Lipase-lipase interactions as a new tool to immobilize
1231 and modulate the lipase properties, *Enzyme and Microbial Technology*, 36 (2005)
1232 447-454. DOI: 10.1016/j.enzmictec.2004.09.013.

1233 145) Fernandez-Lorente, G., Palomo, J.M., Cabrera, Z., Fernandez-Lafuente, R.,
1234 Guisán, J.M., Improved catalytic properties of immobilized lipases by the
1235 presence of very low concentrations of detergents in the reaction medium,
1236 *Biotechnology and Bioengineering*, 97 (2007) 242-250. DOI: 10.1002/bit.21230

1237 146) Mogensen, J.E., Sehgal, P., Otzen, D.E., Activation, inhibition, and
1238 destabilization of *Thermomyces lanuginosus* lipase by detergents, *Biochemistry*,
1239 44 (5) (2005) 1719-1730. DOI: 10.1021/bi0479757,

1240 147) Barbosa, O., Torres, R., Ortiz, C., Berenguer-Murcia, A., Rodrigues, R.C.,
1241 Fernandez-Lafuente, R., Heterofunctional supports in enzyme immobilization:
1242 From traditional immobilization protocols to opportunities in tuning enzyme
1243 properties, *Biomacromolecules*, 14 (2013) 2433-2462. DOI: 10.1021/bm400762h,

148) Dal Magro, L., Kornecki, J.F., Klein, M.P., Rodrigues, R.C., Fernandez-Lafuente, R., Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range, *Enzyme and Microbial Technology*, 132 (2020) art. no. 109397. DOI: 10.1016/j.enzmictec.2019.109397

149) Dal Magro, L., Kornecki, J.F., Klein, M.P., Rodrigues, R.C., Fernandez-Lafuente, R., Optimized immobilization of polygalacturonase from Aspergillus niger following different protocols: Improved stability and activity under drastic conditions, *International Journal of Biological Macromolecules*, 138 (2019) 234-243. DOI: 10.1016/j.ijbiomac.2019.07.092,

150) Virgen-Ortíz, J.J., Tacias-Pascacio, V.G., Hirata, D.B., Torrestiana-Sanchez, B., Rosales-Quintero, A., Fernandez-Lafuente, R., Relevance of substrates and products on the desorption of lipases physically adsorbed on hydrophobic supports, *Enzyme and Microbial Technology*, 96 (2017) 30-35. DOI: 10.1016/j.enzmictec.2016.09.010.

151) Mateo, C., Abian, O., Bernedo, M., Cuenca, E., Fuentes, M., Fernandez-Lorente, G., Palomo, J.M., Grazu, V., Pessela, B.C.C., Giacomini, C., Irazoqui, G., Villarino, A., Ovsejevi, K., Batista-Viera, F., Fernandez-Lafuente, R., Guisán, J.M., Some special features of glyoxyl supports to immobilize proteins, *Enzyme and Microbial Technology*, 37 (2005) 456-462. DOI: 10.1016/j.enzmictec.2005.03.020.

152) Fischer, M.J., Amine coupling through EDC/NHS: a practical approach, *Methods in molecular biology* (Clifton, N.J.), 627 (2010) 55-73. DOI: 10.1007/978-1-60761-670-2_3

153) Jordan, J., Kumar, C.S.S.R., Theegala, C., Preparation and characterization of cellulase-bound magnetite nanoparticles, *Journal of Molecular Catalysis B: Enzymatic*, 68 (2011) 139-146. DOI: 10.1016/j.molcatb.2010.09.010

154) Sun, J., Yang, L., Jiang, M., shi, Y., Xu, B., Ma, H.-L., Stability and activity of immobilized trypsin on carboxymethyl chitosan-functionalized magnetic nanoparticles cross-linked with carbodiimide and glutaraldehyde, *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 1054 (2017) 57-63.

155) L. Cao, L., Van Rantwijk, F., Sheldon, R.A., Cross-linked enzyme aggregates: A simple and effective method for the immobilization of penicillin acylase, *Organic Letters*, 2 (10) (2000) 1361-1364.

156) Sheldon, R.A., Cross-linked enzyme aggregates (CLEA®s): Stable and recyclable biocatalysts, *Biochemical Society Transactions*, 35 (2007) 1583-1587. DOI: 10.1042/BST0351583

157) Sheldon, R.A., Cross-linked enzyme aggregates as industrial biocatalysts, *Organic Process Research and Development*, 15 (2011) 213-223. DOI: 10.1021/op100289f

158) Šulek, F., Fernández, D.P., Knez, Z., Habulin, M., Sheldon, R.A. Immobilization of horseradish peroxidase as crosslinked enzyme aggregates (CLEAs), *Process Biochemistry*, 46 (2011) 765-769. DOI: 10.1016/j.procbio.2010.12.001

159) Wilson, L., Fernández-Lorente, G., Fernández-Lafuente, R., Illanes, A., Guisán, J.M., Palomo, J.M., CLEAs of lipases and poly-ionic polymers: A simple way of preparing stable biocatalysts with improved properties, *Enzyme and Microbial Technology*, 39 (2006) 750-755. DOI: 10.1016/j.enzmictec.2005.12.011

160) Cruz, J., Barbosa, O., Rodrigues, R.C., Fernandez-Lafuente, R., Torres, R., Ortiz, C., Optimized preparation of CALB-CLEAs by response surface methodology: The necessity to employ a feeder to have an effective crosslinking, *Journal of Molecular Catalysis B: Enzymatic*, 80 (2012) 7-14. DOI: 10.1016/j.molcatb.2012.04.013

161) Galvis, M., Barbosa, O., Ruiz, M., Cruz, J., Ortiz, C., Torres, R., Fernandez-Lafuente, R., Chemical amination of lipase B from *Candida antarctica* is an efficient solution for the preparation of crosslinked enzyme aggregates, *Process Biochemistry*, 47 (2012) 2373-2378. DOI: 10.1016/j.procbio.2012.09.018

162) Amaral-Fonseca, M., Kopp, W., Giordano, R.L.C., Fernández-Lafuente, R., Tardioli, P.W., Preparation of magnetic cross-linked amyloglucosidase aggregates: Solving some activity problems, Catalysts, 8 (2018) art. no. 496. DOI: 10.3390/catal8110496

163) Rojas, M.J., Amaral-Fonseca, M., Zanin, G.M., Fernandez-Lafuente, R., Giordano, R.L.C., Tardioli, P.W. Preparation of crosslinked enzyme aggregates of a thermostable cyclodextrin glucosyltransferase from thermoanaerobacter sp. Critical effect of the crosslinking agent, *Catalysts*, 9 (2019) art. no. 120. DOI: 10.3390/catal9020120

164) Tükel, S.S., Hürrem, F., Yildirim, D., Alptekin, Ö., Preparation of crosslinked enzyme aggregates (CLEA) of catalase and its characterization, *Journal of Molecular Catalysis B: Enzymatic*, 97 (2013) 252-257. DOI: 10.1016/j.molcatb.2013.09.007

165) Sahutoglu, A.S., Akgul, C., Immobilisation of *Aspergillus oryzae* α-amylase and *Aspergillus niger* glucoamylase enzymes as cross-linked enzyme aggregates, *Chemical Papers*, 69 (2015) 433-439. DOI: 10.1515/chempap-2015-0031

166) Zhang, Q., Zha, X., Zhou, N., Tian, Y., Preparation of crosslinked enzyme aggregates (CLEAs) of acid urease with urethanase activity and their application, *Journal of Basic Microbiology*, 56 (2016) 422-431. DOI: 10.1002/jobm.201500498

167) Guimarães, J.R., de Lima Camargo Giordano, R., Fernandez-Lafuente, R., Tardioli, P.W., Evaluation of strategies to produce highly porous cross-linked aggregates of porcine pancreas lipase with magnetic properties, *Molecules*, 23 (2018) art. no. 2993, . DOI: 10.3390/molecules23112993

168) Tirunagari, H., Basetty, S., Rode, H.B., Fadnavis, N.W., Crosslinked enzyme aggregates (CLEA) of phytase with soymilk proteins, *Journal of Biotechnology*, 282 (2018) 67-69.DOI: 10.1016/j.jbiotec.2018.07.003

169) Rodrigues, R.C., Virgen-Ortíz, J.J., dos Santos, J.C.S., Berenguer-Murcia, Á., Alcantara, A.R., Barbosa, O., Ortiz, C., Fernandez-Lafuente, R., Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions, *Biotechnology Advances*, 37 (2019) 746-770. DOI: 10.1016/j.biotechadv.2019.04.003

170) Fernández-Lorente, G., Ortiz, C., Segura, R.L., Fernández-Lafuente, R., Guisán, J.M., Palomo, J.M. Purification of different lipases from Aspergillus niger by using a highly selective adsorption on hydrophobic supports, *Biotechnology and Bioengineering*, 92 (2005) 773-779. DOI: 10.1002/bit.20656

171) Palomo, J.M., Fernández-Lorente, G., Mateo, C., Fuentes, M., Guisan, J.M., Fernández-Lafuente, R., Enzymatic production of (3S,4R)-(-)-4-(4′-fluorophenyl)-6-oxo-piperidin-3-carboxylic acid using a commercial preparation of lipase A from *Candida antarctica*: The role of a contaminant esterase, *Tetrahedron Asymmetry*, 13 (24) (2002) 2653-2659. DOI: 10.1016/S0957-4166(02)00754-1

172) Segura, R.L., Palomo, J.M., Mateo, C., Cortes, A., Terreni, M., Fernández-Lafuente, R., Guisan, J.M., Different properties of the lipases contained in porcine pancreatic lipase extracts as enantioselective biocatalysts, *Biotechnology Progress*, 20 (2004) 825-829. DOI: 10.1021/bp034363p

173) Segura, R.L., Betancor, L., Palomo, J.M., Hidalgo, A., Fernández-Lorente, G., Terreni, M., Mateo, C., Cortés, A., Fernández-Lafuente, R., Guisán, J.M. Purification and identification of different lipases contained in PPL commercial extracts: A minor contaminant is the main responsible of most esterasic activity , *Enzyme and Microbial Technology*, 39 (2006) 817-823. DOI: 10.1016/j.enzmictec.2006.01.007

Figure legends

Figure 1. Examples of enzyme immobilization where the global enzyme activity yield is 50%. In case A the immobilization is too slow to immobilize all the protein in the given time? That way immobilization yield is 50% and expressed activity is 100%. In case B, the immobilization is rapid but only involve to 50% of the enzyme activity, the expressed activity???is maintained, again immobilization yield is 50% and expressed activity is 100%. In case C, the immobilization yield is 100%, but the expressed activity is 50%. Reference solution: triangles; Immobilization suspension: solid squares, Supernatant of the immobilization suspension: empty squares.

Figure 2. Examples of immobilization where the global enzyme activity yield is over 100% due to enzyme hyperactivation. In cases A and B, the enzyme increases the activity immediately after immobilization. In case C, it increases the activity progressively after all enzyme has been already immobilized. Reference solution: triangles; Immobilization suspension: solid squares, Supernatant of the immobilization suspension: empty squares.

Figure 3. Temperature/activity profile of free enzyme (squares) and immobilized/stabilized enzyme (triangles). Activity is given as absolute activity per mg of free or immobilized enzyme.

Figure 4. Effect of enzyme orientation and support loading on the activity of immobilized enzymes versus large substrates.

Figure 5. Schematic representation of the substrate diffusion limitations.

Figure 6. Decreasing the substrate diffusion limitation by reducing the size of the biocatalyst: milling the biocatalysts or use of a milled support to immobilize the enzyme.

Figure 7. Effect of enzyme loading on enzyme activity: The higher the enzyme loading, the higher the diffusional problems.

Figure 8. Immobilization time courses where the immobilization yield is lower than 100% and the activity of the reference solution decreases during the immobilization time. Case A is a standard immobilization, cases B and C shows are examples where the enzyme becomes more stable after immobilization and maintains more activity at the end of the immobilization than the reference solution. Reference solution: triangles; Immobilization suspension: solid squares, supernatant of the immobilization suspension: empty squares.

Figure 9. A. Effect of enzyme concentration on the maintenance of the activity of a multimeric enzyme under immobilization conditions. Triangles: concentration used to immobilize the enzyme, circles: enzyme at 50% concentration, squares: enzyme at 10% concentration. **B. Expected effect of enzyme concentration on final biocatalysts activity maintaining the enzyme loading.** Triangles; diluted enzyme tends to become inactivated via subunit dissociation, squares; inactivation of multimeric enzyme via subunit dissociation is not a problem.

Figure 10. Effect on activity/enzyme concentration profile of the formation of lipase-lipase dimers. The use of detergent may solve the problem.

Figure 11. The obtained (A) and actual (B) immobilization courses when the enzyme is released from the support during enzyme activity determination. Reference solution: triangles, immobilization suspension: solid squares, supernatant of the immobilization suspension: empty squares.

Figure 12. Observed immobilization time courses when the substrate becomes massively (A) or just partially (B) adsorbed on the support. Reference solution: triangles; observed immobilization suspension: solid line, solid squares, real immobilization suspension: pointed line, solid squares, supernatant of the immobilization suspension: empty squares.

Figure 13. Immobilization coupled to purification of lipases from esterases via immobilization on hydrophobic supports at low ion strength. Immobilization yield is not 100%, but it is likely that really 100% of the lipase has been immobilized and the enzyme has been purified.

Figure 14. Immobilization yield/enzyme loading expected is some catalytically active component of the enzyme extract is not immobilized on the support.

Figure 15. The problems of enzyme aggregation in enzyme immobilization.

Figure 16. Enzyme immobilization time courses when the enzyme extract have an inhibitor that is immobilized on the support. (A) The adsorbed inhibitor is unable to interact with the enzyme. (B). The adsorbed inhibitor is released during the activity measurement and can interact with the enzyme. The figure shows the activity of: Reference solution: triangles; observed immobilization suspension: solid line, solid squares, real immobilization suspension: pointed line, solid squares; observed supernatant of the immobilization suspension: empty squares, solid line; real supernatant of the immobilization suspension; empty squares, dotted line.