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Published in:

Biotechnology Letters, ***(2015)***

http://dx.doi.org/10.1007/s10529-015-1835-z

Micro-scale procedure for enzyme immobilization screening and operational stability assays

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Abstract

A simple and inexpensive methodology, based on the use of micro-centrifuge filter tubes, is proposed for establishing the best enzyme immobilization conditions. The immobilized biocatalyst is located inside the filter holder during the whole protocol, thus facilitating the incubations, filtrations and washings. This procedure minimizes the amount of enzyme and solid carrier needed, and allows exploring different immobilization parameters (pH, buffer concentration, enzyme/carrier ratio, incubation time, etc.) in a fast manner. The handling of immobilized enzymes using micro-centrifuge filter tubes can also be applied to assess the apparent activity of the biocatalysts, as well as their reuse in successive batch reaction cycles. To illustrate the usefulness of the proposed methodology, the determination of the optimum pH for the immobilization of an inulinase (Fructozyme L) on two anion-exchange polymethacrylate resins (Sepabeads EC-EA and Sepabeads EC-HA) was exemplified.

Keywords: Biotransformations; Enzyme immobilization; Enzyme carriers; Immobilized biocatalysts; Inulinase; Operational stability

Introduction

The design of efficient methods for the immobilization of enzymes represents one of the main bottlenecks for the development of many industrial-scale biocatalytic processes (Sheldon 2007). Immobilized biocatalysts can be separated from the reaction medium and reused, and usually exhibit enhanced enzyme resistance against inactivation by extreme pHs, high temperatures or organic solvents (Cao 2005b; Torres-Salas et al. 2011; Vasquez et al. 2014). Enzyme immobilization is also often a critical step for the implementation of stable nanobiodevices (*e.g.* biosensors and biofuel cells) (Ge et al. 2009) and solid-phase protein chemistry tools (Bautista-Barrufet et al. 2014; Oliver-Calixte et al. 2014).

The selection of a proper support and the optimization of the immobilization conditions (pH, buffer, temperature, enzyme/support ratio) are always cumbersome. Numerous organic and inorganic carriers for enzyme binding are currently available, showing different textural and chemical properties (particle size, hydrophilicity, pore size, isoelectric point, reactive groups, spacer arms, etc.), which allows to choose the support "*a la carte*" depending on the enzyme and its required application (Tobis & Tiller 2014; Zhou et al. 2014).

The immobilization protocols are commonly developed on empirical basis: different carriers and experimental conditions are assayed until a satisfactory immobilized biocatalyst is obtained (Alcalde et al. 1999; Cao 2005a; Gonçalves et al. 2013). The design of immobilized biocatalysts may be considered non-rational because it results from the screening of several parameters; in consequence, many industrial biotransformations might not be operating under optimal conditions. In this context, novel *in silico* analyses (e.g. predicting the location of certain protein residues or domains involved in the binding with the support) combined with experimental research are making significant advances (Basso et al. 2010; Torres-Salas et al. 2011; Weber et al. 2010).

The multi-screening assays to establish optimal immobilization conditions require the expense of a large amount of carrier, enzyme and reagents (buffers, washing solutions, etc.). In order to minimize the consumption of these components, and especially of the most valuable (the enzyme and, in many cases, the support), we present in this work a simple and inexpensive methodology based on the use of micro-centrifuge filter tubes, which allow to carry out the screening experiments in a scale of milligrams of carrier and microliters of enzyme with great reproducibility. Such methodology is also applicable to assess the apparent activity of the resulting immobilized biocatalysts, as well as to simulate their operational stability under batch conditions.

Materials and Methods

Materials

Inulinase from *Aspergillus niger* (Fructozyme-L) was kindly donated by Novozymes A/S. Sucrose, glucose and fructose were from Merck. Sepabeads EC-EA and EC-HA (approx. 0.8 meq ml^{-1} wet resin) were kindly donated by Resindion Srl. The micro-centrifuge filter tubes (with 0.45 µm cellulose acetate membranes) can be purchased from National Scientific (USA), Merck Millipore (Germany), Corning (USA), GE Healthcare Life Sciences (Sweden), Chrom Tech (USA) and others. All reagents and chemicals were of analytical reagent grade.

Activity assay and protein determination

β-Fructofuranosidase activity was determined by the 3,5-dinitrosalicylic acid (DNS) assay for reducing sugars adapted to 96-well plates (Ghazi et al. 2005) using 100 mg/ml sucrose as substrate. One unit of activity (U) corresponded to the release of one µmol of reducing sugars per minute. Total protein concentration was determined by the Bradford assay using Bio-Rad dye reagent (Bradford 1976).

Enzyme immobilization micro-scale procedure

50 mg of the carrier (amino-activated resin Sepabeads EC-EA or EC-HA) were weighed inside a micro-centrifuge filter tube and then washed twice with 100 µl of immobilization buffer to equilibrate the support. The washing step included the mixture of the carrier with the buffer by closing the microfilter tube and inverting 6 times, followed by a centrifuge spin for the separation of the washing buffer from the solid particles, which remained inside the filter holder. After washing, the amino-activated resin was mixed (inside the filter holder) with 50 µl of the enzyme solution (Fructozyme-L) and 250 µl of the immobilization buffer. In this study we used 10 mM Britton–Robinson buffer varying the pH from 2.0 to 12.0 (Britton & Robinson 1931). After 24 h of incubation at room temperature in a roller mixer (J.P. Selecta), the filter tubes were centrifuged at 5000 x *g* and the filtrate was collected and stored. The resulting biocatalyst was washed three times, with 500 µl of reaction buffer (10 mM sodium acetate buffer, pH 5.6) and inverting the micro-filter tube 6 times, followed by a filtration step by centrifugation. The washing solutions 1, 2 and 3 were collected and stored at 4°C. The activity and protein concentration of the filtrate and washings was measured as described above. The immobilization yield was determined by subtracting the total protein and enzymatic activity in the filtrate and the washing solutions from the initial values in the enzyme solution.

Apparent activity of the immobilized biocatalyst

The apparent activity of the immobilized biocatalysts was determined by incubating in a micro-centrifuge filter tube 50 mg of biocatalyst with 200 µl of 100 g/l sucrose solution in working buffer (10 mM sodium acetate buffer, pH 5.6). The mixture was incubated at 40ºC for 10 min in an orbital incubator (Vortemp 1550) with vigorous (900 rpm) agitation. The reaction mixture was separated from the biocatalyst by centrifugation at 5000 x *g*. The supernatant was heated at 95ºC to inactivate the possible lixiviated enzyme. Then, 50 µl of the supernatant (conveniently diluted) were transferred in triplicate to the wells of a microplate, and the concentration of reducing sugars was measured by the DNS assay.

Micro-scale study of operational stability

The operational stability of the biocatalysts was assessed in successive batch reaction cycles. Each cycle included the determination, in the micro-centrifuge filter tube, of the apparent activity of the biocatalyst with fresh substrate (100 g/l sucrose), followed by three washing steps of the solid with 500 µl of buffer. The relative enzymatic activity of each cycle was referred to the apparent activity of the first cycle.

Results and Discussion

Micro-scale procedure for screening of immobilization conditions

A general procedure for enzyme immobilization screening is represented in Fig. 1. For each experiment, approximately 10-200 mg of the carrier is typically employed, depending on the availability of the support and its binding capacity. The carrier is weighed

in micro-centrifuge filter tubes, which are widely available from many suppliers. For aqueous solutions, cellulose acetate membranes were normally used. However, if any of the working solutions contains organic solvents and/or acid/base modifiers, compatible membranes (PVDF, PTFE, nylon, polyether sulfone, polycarbonate, etc.) are also commercially available. The housing of the centrifuge filter tubes is made of polypropylene, which is resistant to most organic solvents.

The enzyme solution and the immobilization buffer (100-500 µl total volume) are added to the support. The enzyme usually comes at a pH value quite far from that of the selected immobilization conditions. We suggest using at least 75% of the immobilization buffer (referred to the total volume) to assure that the final pH is close to the desired value. Using this methodology, it is possible to assess different initial conditions of pH, buffer concentration, enzyme/carrier ratio (w/w) and so on, in order to establish the best immobilization parameters. Depending on the method of immobilization, the range of pH and buffer concentration values can be narrowed. In particular, for adsorption strategies, the knowledge of the isoelectric point of the enzyme and the zeta potential of the carrier may help to limit the number of experiments (Izrael-Zivkovic et al. 2015; Wu et al. 2014).

The immobilization mixture is incubated for a certain time typically in a roller mixer to facilitate the contact between the enzyme and the support. The temperature and the incubation time are two parameters that may exert a great influence in the efficiency of the immobilization process (Berrio et al. 2007; Wang et al. 2014). One of the main advantages of the proposed protocol is the ease to separate the solid from the immobilization solution just by simple centrifugation. The activity and protein concentration of the filtrate is further evaluated to determine the immobilization yield. The retained solid can be washed in the same filter tube (we recommend at least three washings with 500 µl of buffer) and the resulting solutions can be separated by centrifugation and analyzed for protein and enzyme activity. As a result, the immobilized biocatalyst is obtained.

Micro-scale procedure for apparent activity and operational stability assays

The theoretical activity of an immobilized biocatalyst is calculated by subtracting the activity in the filtrate and in the washing solutions from that of the starting enzyme preparation. In contrast, the so-called apparent activity refers to the measured activity of the solid biocatalyst, which is influenced by internal mass transfer restrictions within the carrier (Bolivar et al. 2015; Worsfold 1995). We have applied the micro-centrifuge filter devices to assay the apparent activity of immobilized enzymes with a minor expense of biocatalyst (Fig. 2). Thus, 10-50 mg of biocatalyst are incubated with 100-500 µl substrate during 10-20 min in a thermostated orbital incubator with vigorous agitation to eliminate external diffusion limitations. After that time, the reaction mixture is easily separated from the biocatalyst by centrifugation. It is well reported that, depending on the incubation conditions, enzymes may desorb from the support thus moving to the liquid medium (George & Sugunan 2014). The leached enzyme could continue displaying its activity in the supernatant; to avoid this, an inactivation step, typically by heating to 90-100°C or by addition of an acid or a base, was introduced in the protocol (Fig. 2).

The micro-scale procedure is also useful for the determination of the operational stability of a biocatalyst under batch conditions. The apparent activity can be regarded as the activity at the first cycle. Then, at least three washings of the solid biocatalyst are performed to assure the removal of remaining substrate within the pores of the carrier before the next

cycle. After that, the apparent activity of the immobilized biocatalyst is measured again, following the same protocol as described above. This methodology is very appropriate for the preliminary estimation in a fast and reproducible manner of the biocatalyst reuse in successive batch reaction cycles, with a minimum consumption of enzyme.

Immobilization of inulinase onto aminated resins

We applied the proposed methodology to the immobilization −by adsorption− of a commercial inulinase (Fructozyme L) onto various anion-exchange polymethacrylate resins activated with amino groups. Two resins with different spacer arms (EA, ethylamino; HA, hexamethylamino) were screened. Fructozyme L contains a mixture of the endo- and exoinulinases from *Aspergillus niger*. The inulinases catalyze the hydrolysis of inulin, a fructose polymer with $\beta(2\rightarrow1)$ linkages with a polymerization degree that ranges from 2 to 60 –inulin is usually terminated in a glucose unit–. Inulinases are also able to hydrolyse sucrose (Alvaro-Benito et al. 2012); for that reason, we selected sucrose as substrate instead of inulin in order to favour internal diffusion within the biocatalyst.

We screened different immobilization pH values in the range 2.0-12.0. The isoelectric point of inulinase from *A. niger* is 3.85 for the endoinulinase (Skowronek & Fiedurek 2006) and approx. 3.0 for the exo-acting enzyme (Volkov et al. 2012). This implies that at most of the assayed pHs the enzymes will display a negative net charge ready to interact with the positive net charge of the amino-activated carrier (pK_a of alkyl amino groups is usually higher than 9.0). The immobilization yield and the apparent activity of the resulting biocatalysts were determined (Fig. 3). Standard deviations were in all cases lower than 5%. The results showed that the immobilization yield, in terms of total protein retained, did not always correlate with the apparent activity. With both supports there were two different pH intervals (3.0-4.0 and 8.0-9.0) in which the immobilization yield and the activity seemed to be the optimal. It is worth noting that acidic pH values gave satisfactory results despite the neutral or slightly positive net charge of the inulinases. This fact reinforces the need to screen as many experimental conditions as possible to establish the best immobilization protocol, and for such purpose the micro-scale procedure described in this work may be very useful.

Comparing both aminated resins, Sepabeads EC-HA gave a better performance than EC-EA at practically all immobilization pH values. This fact could be due to its longer spacer arm that minimizes diffusional restrictions. The operational stability of the Sepabeads EC-HA biocatalysts (obtained at the different immobilization pH values) was studied by performing sequential batch reactions with 100 g/l sucrose as described in Fig. 2. The residual activity of each biocatalyst in nine subsequent cycles (referred to the apparent activity of the first reaction cycle) is represented in Fig. 4. Interestingly, the immobilization pH −and thus the strength with which the enzyme is bound to the support− exerts an influence on its operational stability. As shown, the biocatalysts prepared at pH 10-12 displayed the best reusability, with a loss of activity lower than 10% after 9 cycles of reaction. The inulinase immobilized at pH 11.0 even showed a slight activation throughout the different reaction cycles. The biocatalysts prepared at pH 2-3 and 8-9 lost nearly 20% of their initial activity, whilst the loss of those immobilized at pH 4-7 was in the range 30-40%. Although the reaction cycles were quite short (10 min), these results provided useful information about the behaviour of the different biocatalysts under reaction conditions.

Conclusion

This methodology is very helpful to discriminate between different immobilization pH values, not only in terms of the apparent activity of the biocatalysts but also in the simulation of its operational stability in a batch reaction. The micro-scale procedure described here will help to overcome the lack of guidelines that usually govern the selection of an immobilization method, thus favouring the development of stable and robust immobilized enzymes that can withstand harsh operating conditions in industry.

ACKNOWLEDGMENTS

This work was supported by a grant from the Spanish Ministry of Economy and Competitiveness (BIO2013-48779-C4-1-R). We thank the support of COST-Action CM1303 on Systems Biocatalysts. P. S-M. thanks the Spanish Ministry of Education for FPU Grant.

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Figure Legends

Fig. 1. Micro-scale assay to screen the best immobilization conditions.

Fig. 2. Micro-scale assay for the determination of the apparent activity and operational stability of immobilized biocatalysts.

Fig. 3. Protein immobilization yield and apparent activity of the biocatalysts obtained in the immobilization of inulinase from *A. niger* (Fructozyme-L) onto Sepabeads EC-EA and EC-HA at pH values between 2 and 12.

Fig. 4. Operational stability of biocatalysts obtained by immobilization of *A. niger* inulinase onto Sepabeads EC-HA at at pH values between 2 and 12.

Fig. 1

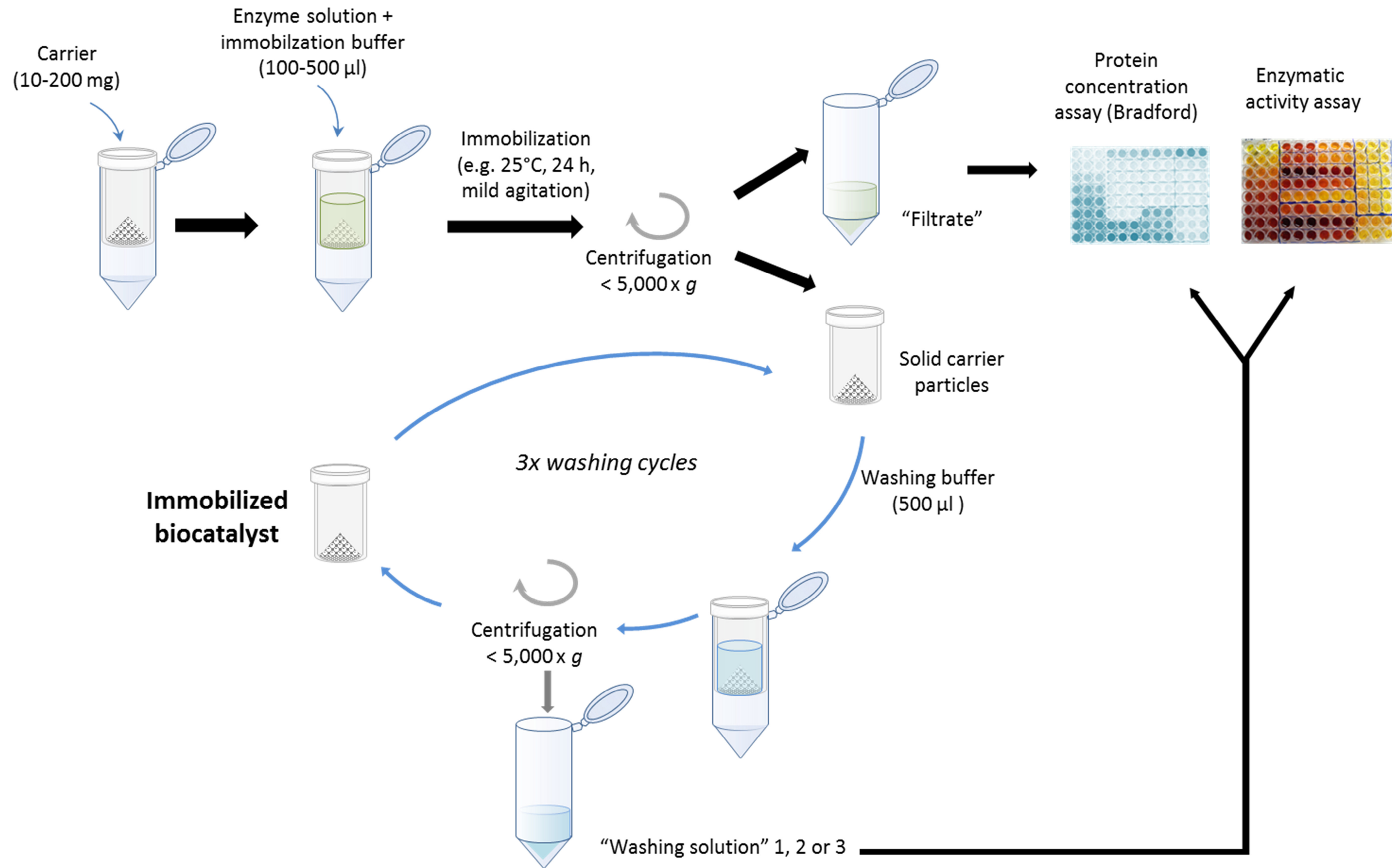

Fig. 2

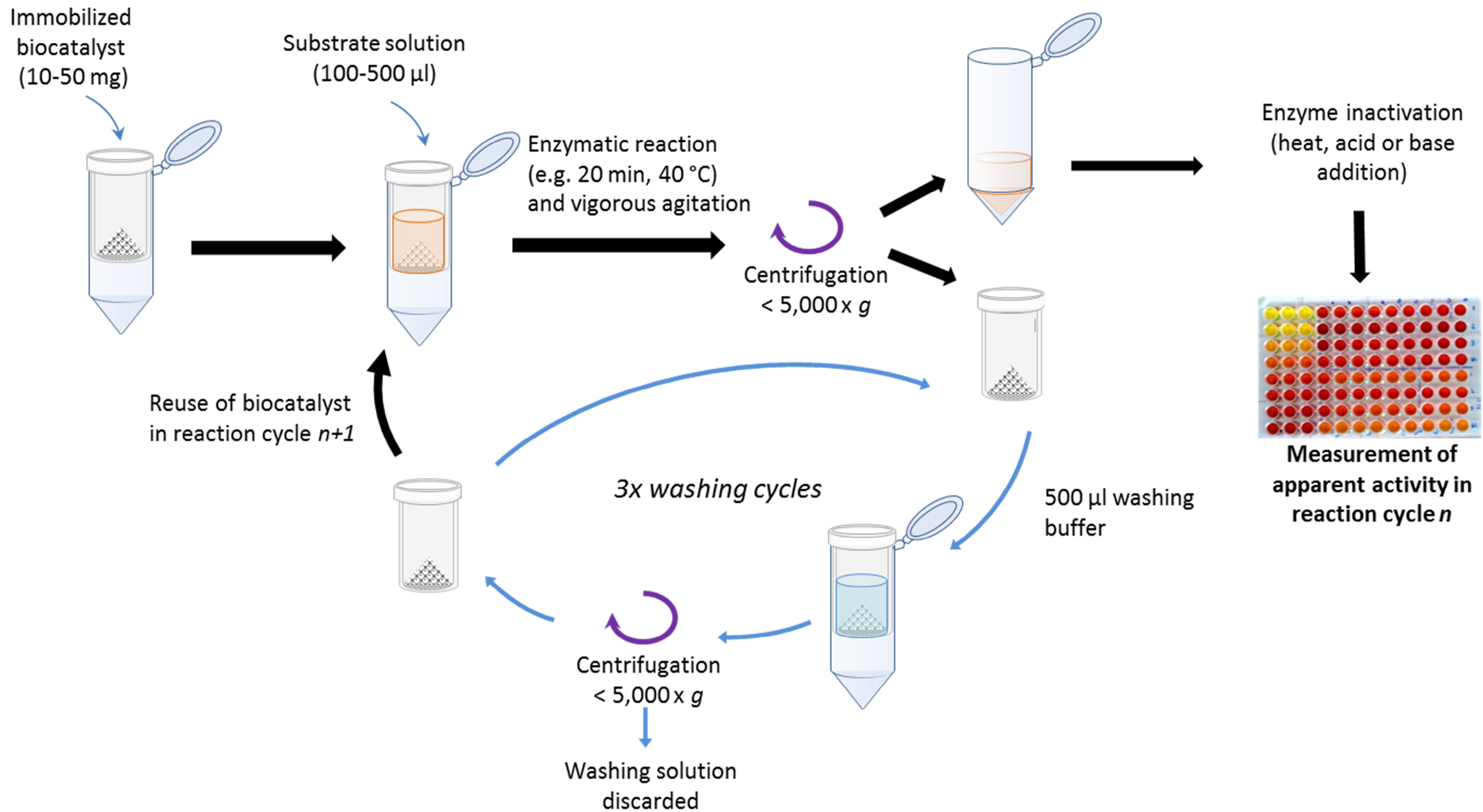

Fig. 3

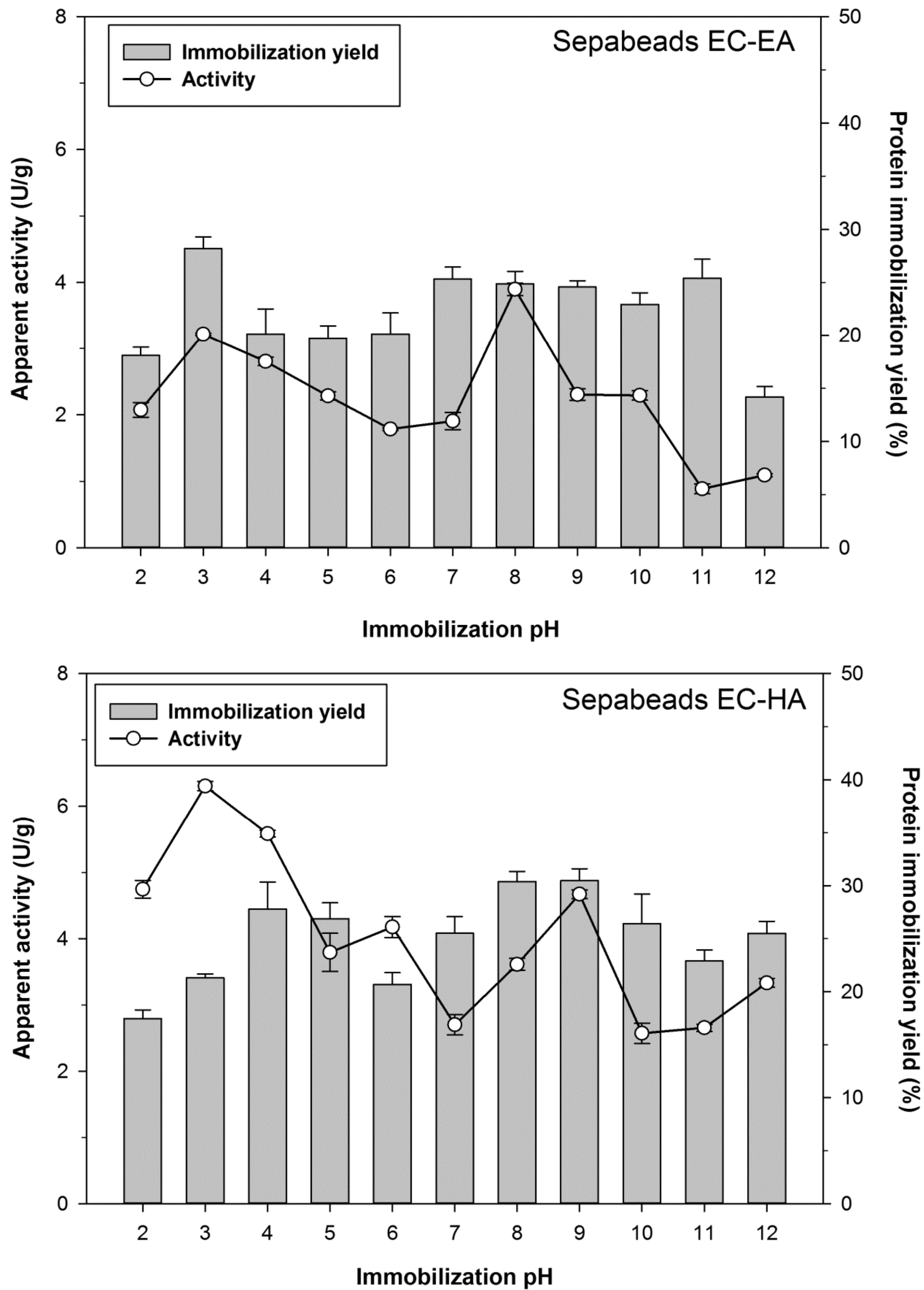

Fig. 4