Improvement of *Aspergillus flavus* saponin hydrolase thermal stability and productivity *via* immobilization on a novel carrier based on sugarcane bagasse

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ABSTRACT

Soyasapogenol B (SB) is known to have many biological activities such as hepatoprotective, anti-inflammatory, anti-mutagenic, antiviral and anticancer activities. Enzymatic conversion of soyasaponins to SB was carried out using saponin hydrolase (SH) extracted from *Aspergillus flavus*. The partially purified enzyme was immobilized on different carriers by physical adsorption, covalent binding or entrapment. Among the investigated carriers, Eupergit C and sugarcane bagasse (SCB) activated by DIC and NHS were the most suitable two carriers for immobilization (the immobilized forms recovered 46.5 and 37.1% of the loaded enzyme activity, respectively). Under optimized immobilization conditions, immobilized SH on Eupergit C and on activated SBC recovered 87.7 and 83.3% of its original activity, respectively. Compared to free SH, immobilized SH on Eupergit C and on activated SCB showed higher optimum pH, activation energy, half-lives and lower deactivation constant rate. Also, their SB productivities were improved by 2.3- and 2.2-folds compared to free SH (87.7 and 83.3 vs. 37.5%, respectively). Hence, being SCB more sustainable and an inexpensive material, it can be considered a good alternative to Eupergit C as a support for SH immobilization. SH immobilization on industrially applicable and inexpensive carrier is necessary to improve SB yield and reduce its production cost. The chemical structure of SCB and the resulting cellulose derivatives were studied by ATR-IR spectroscopy. The thermal analysis technique was used to study the chemical treatment of SCB and coupling with the enzyme. This technique confirmed the removal of lignin and hemicellulose by chemical treatment of SCB.

1. Introduction

Saponin hydrolase (SH) is an enzyme that catalyzes the hydrolysis of natural saponins to more biologically active compounds; mainly their aglycones. For example, conversion of glycyrrhizin, Licorice saponin, by *Aspergillus niger* hydrolase into glycyrrhetinic acid, which is widely used as antiulcer, antiallergic, antiviral, antibacterial and hepatoprotective agent [1]; conversion of major ginsenosides, ginseng saponins, to more active minor ginsenosides by intestinal bacteria [2]; and conversion of yellow ginger saponins by *Trichoderma harzianum* CGMCC 2979 to diosgenin, which acts as precursor for human steroidal drugs [3]. Also, hydrolysis of soyasaponins by *Aspergillus flavus* SH resulted in a production of soyasapogenol B (SB) [4], which is a candidate therapeutic agent for chronic hepatitis [5]. SB (3 β, 22 β, 24-

trihydroxyolean-12(13) ene) has been reported to have other biological activities, such as platelet aggregation suppressing effect, therapeutic activities for immune diseases, anti-inflammatory activity, and growth suppression effect on human breast cancer, colon cancer, ovarian cancer and hep G2 cells [6–8].

Enzymatic production of SB from soybean saponin is often advantageous as compared to acid hydrolysis due to its high selectivity, specificity, efficiency, eco-friendly status and minimum production of byproducts. Consequently, microbial enzymes are considered as a competitive tool for clean manufacturing of valuable pharmaceutical compounds on an industrial scale. However, industrial applications required a selection of an efficient technique that permits to improve the enzyme features to provide extended active lifetime [9]. Enzyme immobilization has been reported to improve many enzyme properties

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including; enzyme stability, specificity, selectivity, activity, and reduction of inhibition by products or medium [10]. Moreover, immobilization permits easy biocatalyst–product separation and facilitates recovery and reusability of enzymes, hence, providing cost-effectiveness use of enzymes in continuous biocatalytic processes [11]. Methods of immobilization could be classified into four main methods; namely: physical adsorption, ionic bonding, covalent bonding and entrapment [12]. Only one research on SH immobilization has been reported [13], but there are no reports on SB production from soybean saponin by immobilized SH.

In the context of our research on the microbial production of SB from soyasaponins by SH enzyme as a biocatalyst, the present work investigates *Aspergillus flavus* SH immobilization on different carriers using different methods (physical adsorption, covalent binding, and entrapment). In addition, optimization of the immobilization conditions was carried out targeting to develop an improved biocatalyst for SB production. Comparison between the immobilized and free SH regarding their catalytic properties and their thermal stabilities has been also studied.

2. Materials and methods

2.1. Materials and carriers for enzyme immobilization

Rice straw and corn cob were supplied by local farmers. SCB was obtained from Egyptian Sugar and Integrated Industries Company (ESII), El-Hawamdia, Giza, Egypt. Sawdust was supplied by local wood processing factories. Cotton and loafa were obtained from local markets. Eupergit C was supplied by Röhm Pharma Polymers (Darmstadt, Germany). Sodium alginate was supplied by BDH chemicals Ltd., Poole, England. Polyethyleneimine (branched, 10000 MW) (PEI), 50% Glutaraldehyde (GA), *N-N'*-diisopropylcarbodiimide (DIC) and N-hydroxysuccinimide (NHS) were bought from Alfa-Aesar (Karlsruhe, Germany). Chitosan was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of SH enzyme

SH preparation was obtained from *A. flavus* by a method described in a previous paper [14]. The concentrated culture filtrate was fractionated by 75% acetone. This partially purified enzyme (specific activity 12.7 mU/mg protein) was lyophilized and used for the preparation of the immobilized enzyme.

2.3. Immobilization methods

Cellulosic agricultural leftovers (SCB, saw dust, rice straw, corn cob and loafa) were treated with 1.25% (w/v) sulphuric acid and 1.25% (w/v) sodium hydroxide at 100 °C for 1 h each time, followed by washing with distilled water. Then it was dried in hot air oven at 100 °C for 2 h.

2.3.1. Physical adsorption

A. flavus SH immobilization by physical adsorption was carried out by loading 1 ml of enzyme solution (20 mg of lyophilized partially purified enzyme was dissolved in 1 ml of 20 mM phosphate buffer (pH 7), which equivalent to 2 mg protein/ml) on 0.5 g of non-activated cellulosic carriers.

2.3.2. Entrapment in calcium alginate

1 ml of 4% sodium alginate solution was mixed with 1 ml of an enzyme solution (20 mg/ml). In another set of experiments, 4% gel solution was treated with GA (0.2%, w/v) before the addition of the enzyme sample. The whole mixture was made into beads by dropping the alginate solution in 0.1 M CaCl$_2$ solution. The resulting beads (1.0–1.5 mm in diameter) were collected and washed with phosphate

buffer (20 mM, pH 7) to remove the unbound enzymes [15].

2.3.3. Covalent binding

SH immobilization on Eupergit C (acrylic epoxy-activated resins). Unless stated otherwise, 0.5 g of Eupergit C was covered with 1 ml of an enzyme solution (20 mg/ml) in 20 mM phosphate buffer (pH 7) and stirred (150 rpm) for 2 h at room temperature.

SH immobilization on different activated carriers: SH covalent linkage to different cellulosic carriers or chitosan was performed using four different coupling agents' procedures as follows.

a) Activation of the carrier by glutaraldehyde (GA). 0.5 g of each support was suspended in 5 ml of 1% (w/v) GA in 50 mM phosphate buffer pH 8 and gently stirred for 2 h, at room temperature. The activated carriers were then filtered and washed 3 times with distilled water before drying. GA is a bi-functional reagent that can react with different enzyme moieties, mainly involving primary amino groups of proteins. Nevertheless, it is known that it can also react with other groups (thiols, phenols, and imidazole) [16].

b) Activation of the carrier by sodium periodate (PI). 0.5 g of different carriers was soaked in 20 ml of PI solution (30 mg/ml) in 0.1 M acetate buffer (pH 4.0). The mixture was placed in the dark and stirred at ambient temperature for 96 h. The oxidized carriers were then washed with distilled water several times to remove the oxidant and used for the immobilization of SH without drying.

c) Activation of the carrier by DIC and NHS. This was carried out using two different procedures [17]. In the first case, carriers (0.5 g each) were immersed in 5 ml of 0.1 M, pH 3.5 MES buffer and dried at 40 °C under vacuum. Afterwards, carriers were immersed in dimethylformamide (DMF) containing 2 (or 4) mM DIC and 5 (or 10) mM NHS and kept shaken for 2 h at 150 rpm. Then, the activated carriers were centrifuged to remove excess solvent (DMF) and washed with buffer 20 mM phosphate buffer, pH 7.0 to ensure that there no remaining DMF. In another procedure carriers were dipped directly in 5 ml of 0.1 M, pH 3.5 MES buffer containing 2 (or 4) mM DIC and 5 (or 10) mM NHS and kept shaken for 2 h at 150 rpm. Then washed with 20 mM phosphate buffer, pH 7.0 and dried overnight under vacuum.

A specified amount of activated carriers (0.5 g) was stirred separately with 1 ml of an enzyme solution (20 mg/ml) in 20 mM phosphate buffer (pH 7) for 2 h at 150 rpm and 4 °C. The carriers with the immobilized enzyme were separated by filtration and washed thoroughly with bi-deionized water. The carriers with immobilized enzyme designated as immobilized SH were dried in a desiccator at room temperature and finally, activities were assayed from hydrolysis of soyasaponins. Supernatants and washings were assayed for SH activity and protein.

2.4. Enzyme activity and protein determination

1% soyasaponins suspended in 0.2 M acetate (pH 5) was added to the carriers with the immobilized enzyme or the control supports or the free lyophilized enzyme, and the mixtures were allowed to react at 40 °C for 1 h. The reaction product (SB) was found to increase linearly up to 4 h. Reaction products were extracted with double its volume ethyl acetate. The quantity of SB in the sample was analyzed by high-pressure liquid chromatography (HPLC). One unit of enzyme activity is defined as the amount of free or immobilized enzyme that produces one μmole of aglycone (SB) *per* min from the substrate. Recovered activity was used for evaluation of immobilization efficiency and it was calculated as follows:

$$RA\ (\%) = A_I/A_F * 100 \qquad (1)$$

Where A_F and A_I refer to the activities of free and immobilized SH,

respectively.

Protein was determined by Bradford method [18]. Protein bound on carriers was calculated as the difference between that loaded and free in supernatants and washings. Immobilized protein yield (Yp) was calculated using the following equation:

$$Yp\ (\%) = P_1/P_0*100 \qquad (2)$$

Where P_1 represented the amount of the immobilized protein and P_0 was the initial amount of protein in the loading enzyme solution.

2.5. SB analytical methods

Thin layer chromatography (TLC) was carried out on pre-coated silica gel plate (Merck, silica gel 60F-254). The plate was chromatographed for SB with a solvent system of benzene/ethyl acetate/acetic acid (12:4:0.5, v/v/v). SB having a Rf value of 0.35 was detected on TLC plates by acid charring (10% H_2SO_4, 120 °C, 10 min).

High-performance liquid chromatography (HPLC) was performed with Waters Alliance HPLC System (Model NO.E2695 XE Separations Module, Austria) under the following conditions: column, Sun Fire Prep C18 (5 μm, 10 × 150 mm); column temperature, 40 °C; mobile phase, acetonitrile-methanol-water (50/15/35); flow rate, 1 ml/min; and UV detector operating 200 nm. An aliquot of the ethyl acetate containing reaction products was diluted 10-fold with the mobile phase. A 10 μl sample of this diluted solution was analyzed by HPLC, and the quantity of SB in the sample was determined by comparison with authentic SB [19].

$$SB\ conversion(\%) = \frac{[SB\ weight/SB\ MW] \times 100}{[soyasaponin\ I\ weight/soyasaponin\ I\ MW]} \qquad (3)$$

Where MW is the molecular weight; soyasaponin I represents soyasaponins.

2.6. Determination of optimum temperature and thermal stability of free and immobilized SH

To investigate the effect of temperature on the activity of free and immobilized SH, samples were incubated in 0.2 M acetate buffer (pH 5.0) and assayed for SH activity at temperatures ranging from 30 to 50 °C.

Thermal stability assays of the free and immobilized enzymes were studied at 40 and 50 °C. In a predetermined time interval, a sample was removed and assayed for enzymatic activity as described above. The half-life of an enzyme is the time it takes for the activity to reduce to a half of the original activity. It was determined by plotting the log of residual activity against time, at temperatures causing inactivation (50, 55, and 60 °C), according to the following equations [13]:

$$Half\text{-}life = 0.693/slope \qquad (5)$$

$$Deactivation\ energy = slope\ of\ the\ straight\ line. \qquad (6)$$

2.7. Attenuated total reflection infrared spectroscopy (ATR-IR)

ATR-IR measurements of the samples were performed with a PerkinElmer FTIR spectrometer Frontier equipped with single bounce attenuated total reflectance (ATR) accessory. Solid samples were placed directly on the ATR crystal applying maximum pressure. All spectra were averaged from 32 scans from 650 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Spectra were baseline corrected with the PerkinElmer Spectrum software

2.8. Thermogravimetric analysis (TGA)

Thermogravimetric measurements of the samples (10–15 mg) were performed on a Netzsch TG209 F1 analyzer. Samples were heated in a

pure nitrogen atmosphere from room temperature to 900 °C at a rate of 20 K/min with a flow rate of 30 ml/min. The weight loss (TG) and first derivative (DTG) were recorded as a function of time and temperature. The Proteus software (version 6.1.0) was utilized to acquire and analyze the TG and DTG data.

3. Results and discussion

3.1. Immobilization of SH on various carriers

The final application of the biocatalyst is taken into consideration while the supporting materials are chosen. Considering the importance of SB as a candidate therapeutic agent for chronic hepatitis and various cancer diseases [6–8], development of new techniques for SH immobilization on an industrially applicable and inexpensive carrier is necessary to improve its yield and reduce its production cost. Table S. 1 (Supplementary data) shows results of SH immobilization on different carriers by different immobilization procedures. In particular, three different methods were applied, namely, physical adsorption, covalent binding, and entrapment. Physical immobilization of SH on loafa and corn cob were accompanied by relatively low percentage of immobilized protein (15 and 19.2%, respectively). This might be attributed to the existence of weak interactions between carrier and enzyme in case of physical immobilization [9]. It is worthy to note that, they retained relatively high SH activities (22.4 and 32.7%) and had the highest SH specific activities of 10.5 and 11.9%, respectively. However, SH immobilized by entrapment in Ca alginate beads had the highest percentage of immobilized protein (95.8%), among all carriers, although it was accompanied by the lowest recovered activity. This could be due to mass transfer limitations that limit the accessibility of the substrate to the internally trapped enzyme molecules [15].

On the other hand, SH immobilization by covalent binding to different carriers resulted in higher immobilized protein percentages compared to those obtained by physical immobilization method. SH covalently bound to Eupergit C showed the highest recovered activity of 46.5%. Eupergit C is epoxy-activated acrylic beads that bind protein via their epoxide groups. Epoxide groups could bind to different nucleophiles (e.g., amino, thiol, and hydroxyl groups) present on the enzyme molecules surface [20] and, to a lesser extent, with carboxylic groups [21]. Thus, epoxy-activated carriers are very convenient for immobilization and stabilization of enzymes via multipoint covalent attachment [22]. A relatively high percentage of immobilized enzyme was also obtained using SBC activated via DIC/NHS method (37.1%). The chemical modification of SBC by addition of DIC to buffered (MES, pH 3.5) SBC and in the presence of NHS generates succynimidyl esters with carboxyl group of residual lignin and hemicellulose supposed to be present in the SCB after acid-base treatment, which covalently linked with amino groups of enzyme through amide linkage [23,17]. In general, the values of the covalently immobilized enzyme on carriers activated by other methods (GA or PI) were lower. In fact, the activity of enzyme could be reduced after immobilization as a result of protein denaturation or conformational changes [10]. Therefore, both of Eupergit C and SBC activated by DIC and NHS were selected to be applied for SH immobilization in the further experiments by considering the obtained activity of immobilized SH.

3.2. Optimization of covalent binding protocols using Eupergit C and SBC functionalized by DIC and NHS

Immobilization conditions of an SH preparation (containing 50 mU and 0.2 mg protein/g carrier) on Eupergit C and on SBC functionalized by DIC and NHS were optimized taking into accounts different parameters such as time (2–24 h), temperature (4–40 °C), enzyme loading (2–6 mg protein/ml), support weight (0.5-1.5 g/ml), and buffer pH (5–8). Optimization of the immobilization conditions using functionalized SBC was started by a preliminary experiment investigating the

Table 1
Effect of SBC activation procedure on *A. flavus* SH immobilized activity and protein.

SBC activation procedure	[a] Recovered activity (%)	[b] Immobilized protein (%)	Specific activity (mU/mg protein)
2 mM DIC & 5 mM NHS in DMF	24.20	88.64	4.1
4 mM DIC &10 mM NHS in DMF	24.19	89.63	4.0
2 mM DIC & 5 mM NHS in MES buffer	37.09	78.28	6.0
4 mM DIC & 10 mM NHS in MES buffer	64.32	91.12	11.9

Values are the average of three independent experiments and the maximal mean deviation is ± 6%.

[a] Recovered activities were based on the ratio of immobilized and free enzyme activities obtained at a certain condition and expressed as a percentage, see Section 2.4. 100% activity corresponds to 50 mU/g carrier.

[b] The immobilization was performed with the enzyme dose of 4 mg protein/g support in 50 mM phosphate, pH 7, at 4 °C for different times 24 h.

effect of the concentration of DIC (2 or 4 mM) and NHS (5 or 10 mM) and the solvent type in which they were dissolved MES buffer or DMF. Results in Table 1 reveal that using higher concentrations of DIC and NHS in MES buffer allowed 1.7- and 1.2-folds increase of SH recovered activity and immobilization yield, respectively. Contrary to MES buffer, the use of DMF as the solvent resulted in an inhibition of the enzyme activity.

It was found that the percentage of immobilized protein did not depend on the immobilization time as no significant change in immobilized protein values was noticed by time (data not shown). However, by increasing the immobilization time from 2 to 24 h, the recovered activities of both SH immobilized on Eupergit C and on functionalized SBC decreased from 46.5 to 32.9 and from 64.3 to 32.9, respectively. This result suggests a negative effect of the carrier on the stability of SH or the increased coupling of the enzyme perhaps involving some important amino acids that would result in lowered activity of the immobilized enzyme. Therefore, by considering the recovered activities, 2 h was selected as the optimum immobilization time.

To establish the optimum temperature for immobilization, SH immobilization was performed at 4, 25 and 40 °C. As seen in Table 2, the percentage of the immobilized protein on Eupergit C and SBC increased from 69.7 to 85.3% and from 78.3 to 95.8%, respectively, by increasing the immobilization temperature from 4 to 40 °C. However, immobilized SH on Eupergit C and on functionalized SBC showed their highest recovered activities of 46.5 and 64.3%, respectively, when the immobilization temperature was 4 °C. This behavior could be explained by the denaturation effect of high temperature range (25–40 °C) on SH.

A parameter that plays an important role in the immobilization step is the pH value. In the case of Eupergit C, the epoxide groups can react with different nucleophiles of the protein according to the pH value at which the reaction is carried out. Thus, immobilization could be conducted in a pH range from pH 4 to 10, which could also include the attachment of epoxy groups to carboxyl or amino groups on the enzyme molecule [10]. As shown in Fig. 1, both immobilized SH activity and percentage of immobilized protein were found to be depended on the

pH at which immobilization was conducted. The highest recovered activity (69.7%) and immobilized protein (93.0%) were achieved at pH 5. The lowest percentage of immobilized protein was determined at pH 7. This finding agrees with the results reported by Mateo et al. [10] who observed that the epoxy groups show low reactivity at neutral pH. At pH above 7, the amount of immobilized protein was slightly increased but SH recovered activity was sharply decreased. This may be due to the instability of SH at high pH values. Moreover, it is worth pointing out that, although the use of supports having many amino groups can accelerate the adsorption, higher pH values will hinder the multipoint covalent attachment [10].

In order to avoid excessive use of enzyme and enzyme multilayers on the support that can cause a reduction of the enzyme specific activity because of the hindered access to the deeper layers of the enzyme molecules, it is important to determine the optimal enzyme/support ratio. The highest activities of immobilized enzyme on both of functionalized SBC and Eupergit C were obtained when the concentration of the enzyme solution loaded on 0.5, 1 or 1.5 g of support was 40 mg/ml. Above this concentration, a decrease of immobilized SH activity was noticed (Fig. 2A and B). This result suggests that above this enzyme concentration the enzyme might form aggregates that do not allow a homogeneous distribution of the enzyme molecules on the surface of the support. At 40 mg/ml SH concentration, the percentage of immobilized protein was directly proportional to the weight of the support (Fig. 3), or, in other words, to the enzyme/support ratio. In particular, by increasing the amount of both Eupergit C and functionalized SBC from 0.5 to 1.5 g, the percentage of immobilized protein was increased from 69.7 to 100% and from 76.7 to 100, respectively. However, the maximum recovered activities of 88 and 82% were achieved using 1 and 0.5 g/ml of Eupergit C and functionalized SBC, respectively. Therefore, considering the activity of immobilized SH, the optimal ratio of enzyme to Eupergit C or functionalized SBC was chosen as 40 mg of protein per 1or 0.5 g, respectively. Consequently, the optimum ratio of the enzyme to Eupergit C was 1/25 (w/w), while that of the enzyme to SBC was 1/12.5 (w/w). This difference in the optimum ratio could be due to the different nature of the two supports, and to the fact that immobilization might depend not only on the covalent linkage but also on the electrostatic or ionic interaction of the enzyme molecule with the charged surface support. In fact, SBC is more polar because it is rich in cellulose, hemicellulose, and lignin [24] and they have different functional groups capable of chemical modification providing specific functionalities [25]. Instead, compared to SBC, Eupergit C is a more hydrophobic copolymer, which is formed of methacrylamide/N, N′-methylen-bis (acrylamide)/monomer carrying oxirane groups [22]. Moreover, multiple covalent linkages between the enzyme and the support may disturb the globular structure of the protein and hence the activity [26].

3.3. Effect of reaction temperature and thermal stability on the activity of free and immobilized SH

As shown in S. 2, the optimum temperature was found at 40 and 45 °C for the free and immobilized SH on Eupergit C, respectively. In
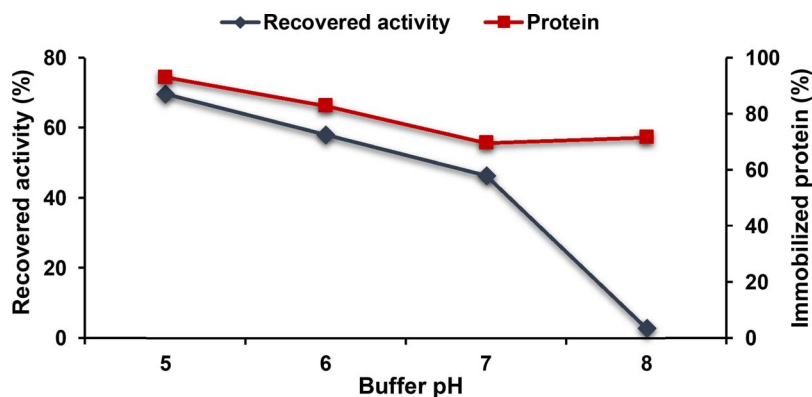
Table 2
Effect of immobilization temperature on *A. flavus* SH recovered activity and immobilized protein on Eupergit C and on functionalized SBC.

Immobilization temperature (°C)	[a] Recovered activity (%)		[b] Immobilized protein (%)		Specific activity (mU/mg protein)	
	Eupergit C	SBC	Eupergit C	SBC	Eupergit C	SBC
4	46.5	37.1	69.7	78.3	7.1	11.9
25	36.5	29.2	75.4	84.7	5.1	7.4
40	36.4	29.1	85.3	95.8	4.4	6.4

Values are the average of three independent experiments and the maximal mean deviation is ± 7%.

[a] Recovered activities are expressed as in Table 1. 100% activity corresponds to 50 mU/g carrier.

[b] Immobilization was performed with the enzyme dose of 4 mg protein/g support in 50 mM phosphate, pH 7 for 2 h at different temperatures.

Fig. 1. Recovered activity (♦) and protein (■) as a function of pH of the buffer used for SH immobilized on Eupergit C. Values are the average of three independent experiments and the maximal mean deviation is ± 8%.

case of immobilized SH on functionalized SBC by NHS and NIC, the optimum temperature range is broad from 40 to 45 °C. Moreover, immobilization resulted in a slower decline of the activity as the assay temperature was increased over the optimum value as noticed in S. 2. However, at temperatures lower than 40 °C, the relative activity of the free form was higher than those of the immobilized ones. These could be due to internal diffusion restrictions of the substrate into the support [13] and so on a lower effective concentration of substrate. This explains why the calculated Ea of the free SH (3.4 Kcal/mol) was lower than those of the immobilized SH on Eupergit C or on functionalized SBC (9.8 and 8.2 cal/mol, respectively).

Thermal deactivation kinetics of free and immobilized enzyme at 40 and 50 °C are shown in Fig. 5. Results revealed that immobilization of SH on Eupergit C and on functionalized SBC by NHS and NIC slowed down SH deactivation rate in comparison to that of the free form,

changing its thermal deactivation profile. At 40 °C, the immobilized SH on Eupergit C and on functionalized SBC retained 90 and 95% of their activities, respectively, after 30 min, whereas the free form retained only 58% of its original activity under identical conditions (Fig. 4). By comparison of the calculated half-life values of the free and immobilized enzymes at 40 °C (Table 3), it can be concluded that immobilized SH on Eupergit C and on functionalized SBC were 8 and 11-fold more stable than the free enzyme, respectively (expressed as the ratio of half-lives of immobilized and free enzymes). Moreover, at 50 °C, immobilized SH on Eupergit C and on functionalized SBC retained 45 and 54% of their original activity after 15 min incubation, respectively, while the free form retained only 16% of its activity at the same temperature. Also, the calculated deactivation rate constant values of free SH at 40 and 50 °C (0.038 and 12.97 min^{-1}, respectively) were higher than those of immobilized SH on Eupergit C (0.005 and 0.015 min^{-1},
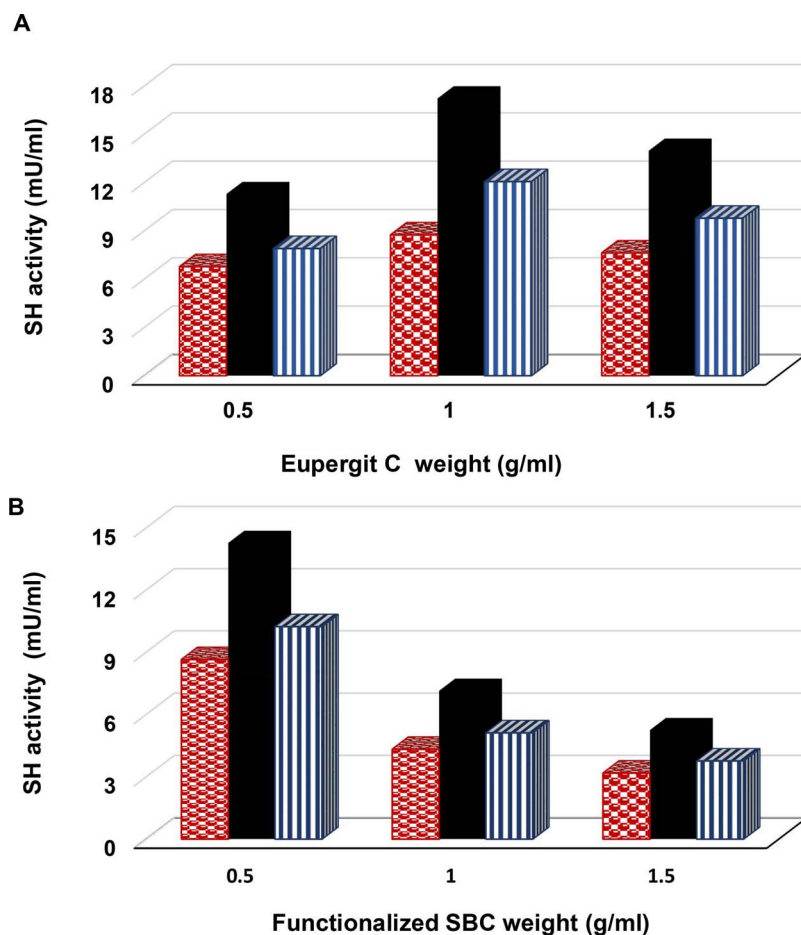
A

B

Fig. 2. Effect of carrier weight and initial enzyme loading of 20 (■ red), 40 (■ black), 60 (■ blue) mg/SH on immobilized SH activity. Values are the average of three independent experiments and the maximal mean deviation is ± 9%.
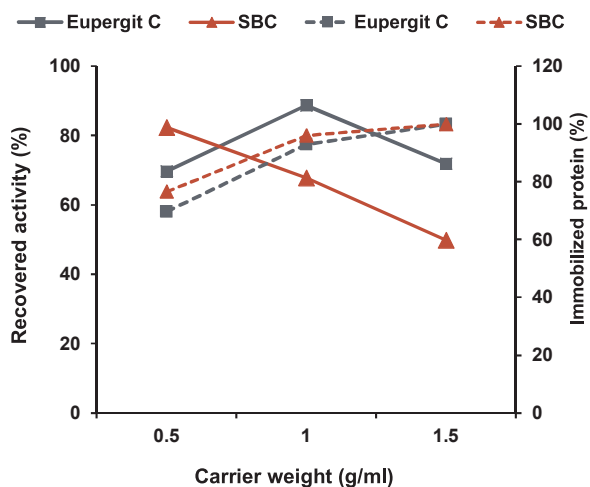
Fig. 3. Recovered activity (solid lines) and immobilized protein (dotted lines) using different weights of Eupergit C and functionalized SBC. Values are the average of three independent experiments and the maximal mean deviation is ± 7%.
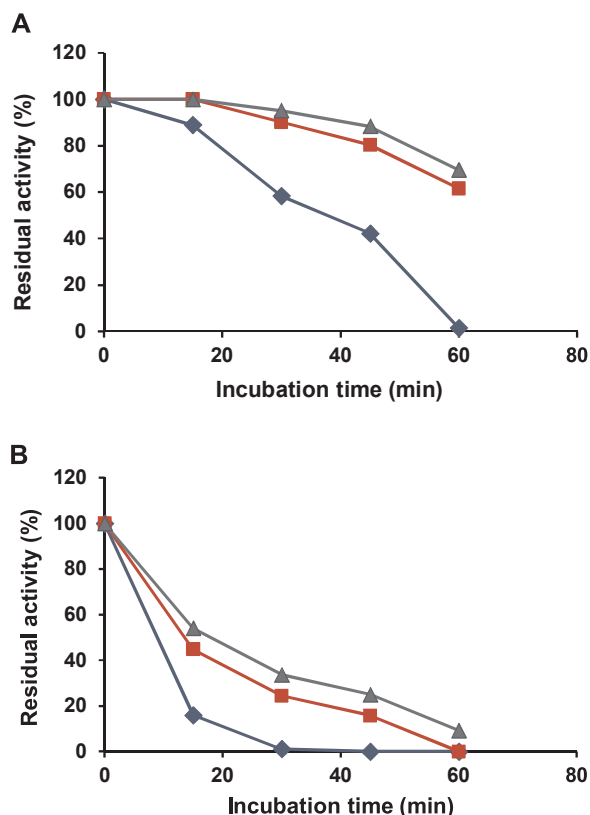

Fig. 4. Thermostability of free (♦) and immobilized SH on Eupergit C (■) and functionalized SBC (▲) at 40 °C (A) and 50 °C (B). Values are the average of three independent experiments and the maximal mean deviation is ± 8%.

Fig. 5. Course of SH activities (A) and SB yields (B) of free (♦) and immobilized A. *flavus* SH on Eupergit C (■) and functionalized SCB (▲). Values are the average of three independent experiments and the maximal mean deviation is ± 7%.

Table 3
Deactivation rate constants and half-lives time at different temperatures of free and immobilized A. *flavus* SH on Eupergit C and on functionalized SBC.

Enzyme property	Free SH	Immobilized SH	
		Eupergit C	SBC
Deactivation rate constant k (min^{-1})			
40 °C	0.038	0.005	0.003
50 °C	12.97	0.015	0.011
Half life time (min)			
40 °C	18.1	152.1	205.3
50 °C	0.1	45.9	62.3

3.4. Comparison of SB production by free and immobilized SH on Eupergit C or functionalized SBC

The course of SH activities and SB yields of free and immobilized SH are shown in Fig. 5. Results revealed that SB yields of free and immobilized SH were directly proportional to the reaction period. The obtained SB yield by free SH was higher than those obtained by immobilized SH on both Eupergit C and functionalized SBC after 1 h incubation. On contrary to the latter findings, SB yields of immobilized SH on both Eupergit C and functionalized SBC (87.7 and 83.3%, respectively) were comparably higher by 2.4 and 2.2-folds than free SH (37.5%) after 24 h incubation, respectively (Table 4). This could be due to a removal of SB from the bulk reaction medium by its adsorption on the immobilization matrix which consequently favors the conversion rate of soyasaponins to SB. This behavior was proved by extraction of SB, at the end of the incubation period, by ethyl acetate from the reaction mixture before or after the separation of the immobilization support. Results (data not shown) indicated that SB was detected only before the separation of the immobilization support and almost no SB

respectively) and on functionalized SBC (0.003 and 0.011 min^{-1}, respectively). Immobilized SH showed higher thermal stability due to the multipoint bindings of the enzyme on Eupergit C and SBC through the epoxy groups [27] and functional groups located on the surface of SBC activated by DIC/NHS [23], respectively. Results indicated that SH immobilized on SBC was more stable than SH immobilized on Eupergit C.
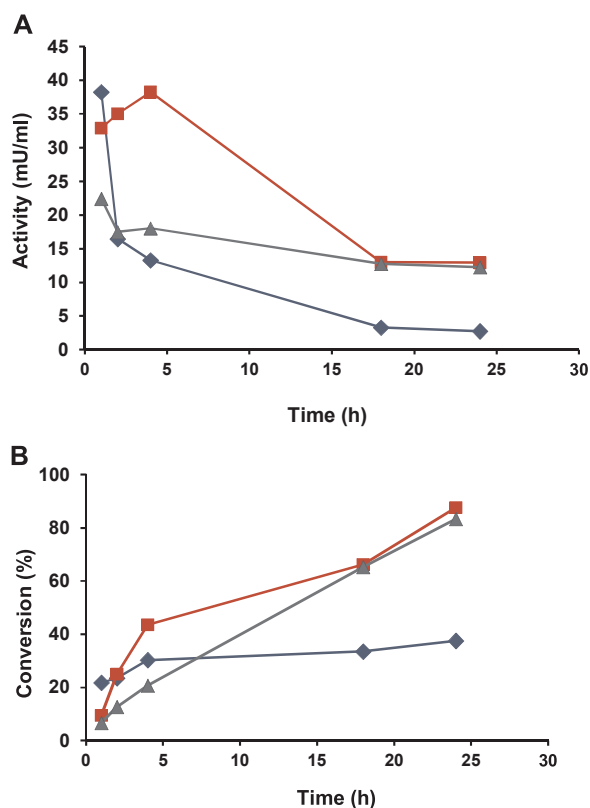
Table 4
Comparison of the properties of *A. flavus* free and immobilized SH on Eupergit C and on functionalized SBC.

| Enzyme property	Free SH	Immobilized SH	
		Eupergit C	SBC
SB conversion (%)[a]	37.5	87.7	83.3
Recovered activity (%)	[b]100	88.7	82.4
Specific activity (U/mg protein)	12.7	12.9	11.9
Optimum temperature (◦C)	40	45	45
Activation energy Kcal/mol	3.4	9.8	8.2

[a] After 24 h reaction.
[b] The initial activity of free enzyme was considered as 100%.

was detected in the ethyl acetate extract if the immobilization support was previously removed from the reaction medium. On contrary, in case of free SH, produced SB was accumulated in the reaction mixture obstacle further conversion of soyasaponins by a so called product feedback inhibition [28]. Additionally, the higher stability of the immobilized enzyme over the free one may play a role in SB enhanced production, as also suggested inform of the different SH activity profiles as a function of time of free and immobilized SH (Fig. 5).

3.5. Characterization and thermal properties of lignocellulosic matrix

To evaluate the full suitability of the lignocellulosic matrix as an enzyme support, it was carried out more in depth the characterization of its composition and stability. Native SCB (A) and corresponding derivatives (SCB treated with 1.25% of H_2SO_4 followed by 1.25% NaOH under reflux, activated with DIC/NHS and coupled with enzyme were signed as B, C, and D, respectively) were characterized by infrared spectroscopy. As shown in S. 3, the most visible differences that can be noticed in the spectrum for A in relation to B, C and D are found in the region from 1800 to 700 cm^{-1}. The absorption bands at 1730, 1640, 1600, 1510, 1245 and 825 cm^{-1} were observed in the spectrum of native SCB (A) compared with other B, C, and D spectra. These absorption bands are corresponding to functional groups in hemicellulose and lignin associated with cellulose. The absence of absorption bands at 1730, 1510 and 1245 cm^{-1} in the spectra B, C and D indicated that the most of lignin and hemicellulose were removed. Similar band assignments were reported by Pereira et al. [29]. Furthermore, the removal of lignin and hemicellulose were confirmed by TGA (S. 4 and Fig. 6). Unfortunately, because of the low sensitivity of infrared spectroscopy and the low enzyme/carrier ratio, it was not possible to monitor the amide functional group peak.
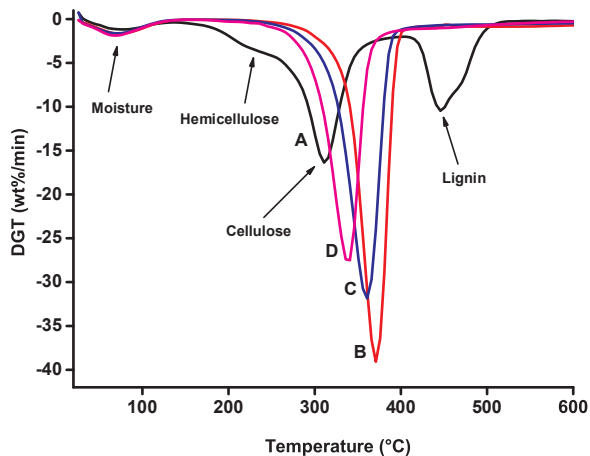
The thermal behavior of SCB and modified derivatives was studied by TGA in the range of room temperature to 900 °C at a rate of 20 °C min^{-1} under a nitrogen atmosphere. S. 4 and Fig. 6 show TGA and differential thermogravimetry (DTG) curves, respectively for native SCB, acid-base-treated SCB, DIC/NHS-activated SCB and enzyme-coupled SCB. TGA curve of native SCB (A) exhibited four degradation stages. The first stage of the weight loss (30–115 °C) corresponds to the moisture content in the sample. The second stage of the weight of native SCB (A) presented the thermal degradation peak around 230 °C which attributed to hemicellulose. The third stage represented the major degradation peak at 310 °C, which corresponds to the cellulose. As for the fourth stage of weight loss occurred at 445 °C represented the degradation of lignin. As can be seen in Fig. 6, cellulose fiber of samples B, C and D showed higher thermal stability. DTG curves for B, C and D samples showed only one main degradation peak at 370, 360 and 340 °C, respectively, whereas cellulose in sample A showed DTG peaks at 310 °C. These changes in the decomposition temperature of cellulosic materials can be attributed to the changes in the crystallinity of cellulosic polymer due to the chemical treatments and coupling of cellulose with DIC/NHS followed by enzyme immobilization.

4. Conclusion

The study herein presented to identify an immobilization support that added improved properties to SH enzyme from *A. flavus* in a perspective of its exploitation for an industrial scale-up of soyasaponins hydrolysis to SB. Immobilization of the enzyme on SBC functionalized by DIC and NHS or Eupergit C seemed to have much better performances compared to other carriers. Optimization of SH various immobilization parameters on Eupergit C and functionalized SBC led to active immobilized biocatalysts, which recovered 87.7 and 83.3% of the originally added enzyme activity, respectively. Moreover, the immobilized biocatalysts showed higher thermal stability and SB productivity compared to free SH. The chemical structure and thermal property of SCB and its corresponding functionalized derivatives were studied by ATR-FTIR and TGA, respectively. These two techniques confirmed the removal of lignin and hemicellulose by chemical treatment but unfortunately, were not able to prove the enzyme covalent coupling. Concerning the different origin of the two immobilization supports, it worth pointing out that the use of SBC, which is an agricultural by-product, seemed to be more sustainable than Eupergit C, which is a synthetic polymer matrix. This cheap eco-friendly support, SBC, was biocompatible with SH, rendering an immobilized derivative with characteristics similar to or even better than that obtained using Eupergit C. However, more studies are planned to evaluate the economic feasibility of this enzyme/support preparation for an industrial application. This investigation can be regarded as the first report describing the possibility of using immobilized SH in bioconversion of soyasaponins to SB.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.btre.2017.12.007.

Fig. 6. Derivative thermogravimetric analysis of: A) native SBC, B) treated SBC with 1.25% H_2SO_4 followed by 1.25% NaOH, C) activated SBC with DIC/NHS and D) immobilized enzyme on SBC.

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