Comparative studies of free and immobilized *Aspergillus flavus* onto functionalized multiwalled carbon nanotubes for soyasapogenol B production

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Objective
This research focuses on the microbial transformation of soybean saponins to soyasapogenol B (SB) by *Aspergillus flavus* cells producing saponin hydrolase (CSH) as a whole-cell biocatalyst. Multiwalled carbon nanotubes (MWCNTs) have been reported to have several properties that render them ideal support systems with the advantage of being further functionalizable at their surface.
Materials and methods
CSH was covalently immobilized onto carboxy-functionalized MWCNTs by different methods including direct immobilization as well as immobilization by glutaraldehyde or carbodiimide chemistry.
Results and conclusion
Results showed that direct immobilization of CSH onto oxidized MWCNTs was the best method accompanied by about an 80% immobilization yield. The optimum temperature was around 50°C for both the free and the immobilized CSH (MWCNTs–CSH conjugate). The apparent activation energy (*Ea*) was increased from 1.05 to 2.84 kcal/mol by immobilization. The immobilized enzyme also showed significantly improved thermal stability. The calculated half-life values of MWCNTs–CSH conjugate at 70, 80, and 90°C (55, 33.3, and 29.7 h, respectively) were higher than those of free CSH (36.1, 28.0, and 23.2 h, respectively). The MWCNTs–CSH conjugate showed higher K_m (0.45 μmol) compared with the free CSH (0.41 μmol), whereas V_{max} for the MWCNTs–CSH conjugate was smaller than that for free CSH. The MWCNTs–CSH conjugate morphology was examined using transmission electron microscope.

Keywords:
Aspergillus flavus, immobilization, multiwalled carbon nanotubes, saponin hydrolase, soyasapogenol B, soybean saponins

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Introduction

Nanobiocatalysis, which refers to the application of enzymes immobilized on nanostructured materials, has attracted considerable interest in recent years [1]. Immobilization of enzymes onto a high surface area of nanomaterials resulted in higher enzyme loading [1] and improved enzyme properties, including its stability and activity [2]. Among nanostructured materials, carbon nanotubes (CNT) have attracted considerable interest for their extraordinary structural, mechanical, thermal, and electrical properties [3,4]. CNTs are one-dimensional nanomaterials that consist of concentric rolled graphene sheets with a length in the size of micrometers and diameter up to 100 nm [4]. In addition to single-walled carbon nanotubes (SWCNTs), multiwalled carbon nanotubes (MWCNTs) can also be used to immobilize enzymes [5]. Enzyme immobilization onto the surface of CNTs can be achieved using various methods, namely, physical adsorption [6] and covalent bonding [7] of enzymes. Recently, it was found that enzymes can be specifically and precisely bound onto CNTs through specific functional groups that are introduced at their surfaces by functionalization with organic, polymeric, and biological molecules [5,8].

Soybean saponins are triterpenoid glycosides present in soybeans and other legumes [9]. They are composed of an oleanane-type triterpenoid aglycone (soyasapogenols) to which sugar residues are attached [9]. These saponins are reported to have many biological properties, but soyasapogenols are generally more bioactive compared with their glycosides. Saponin hydrolase hydrolyzes soybean saponins group B into their aglycone soyasapogenol B (SB). SB has been reported to have many bioactive properties, such as anti-inflammatory, anticancer, hepatoprotective, antivirus, and anti mutagenic activities [10]. Here, saponin hydrolase was

selected as a model enzyme and immobilized on carbon nanomaterial. *Aspergillus flavus* saponin hydrolase was reported mainly to be a cell membrane enzyme [11]. Thus, we investigated the possibility of covalent immobilization of *A. flavus* whole-cell saponin hydrolase (*A. flavus* CSH) on oxidized MWCNTs directly and by carbodiimide or glutaraldehyde (GA) chemistry. Eventually, a comparative study between the free and immobilized CSH in terms of their catalytic activity, thermal stability, and kinetic properties was carried out.

Materials and methods

Aspergillus flavus culture and media

A. flavus isolated from peanut pods was maintained on potato dextrose agar (PDA-Difco; Laboratories Inc., Livonia, MI, US). Soybean saponin hydrolase production was carried out in production medium consisting of 20 g/l soybean saponin, 40 g/l malt extract, 20 g/l yeast extract, 2 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, 0.3 g/l $MgSO_4 \cdot 7H_2O$, and 0.3 g/l $CaCl_2 \cdot 2H_2O$ with a pH adjusted initially to 9.0 for 48 h at 30°C and 150 rpm.

Oxidation and purification of multiwalled carbon nanotubes

Pure MWCNT (3.0 g) was dispersed in mixed concentrated sulfuric and nitric acids (3 : 1, v/v) at a ratio of 50 ml acid mixture per 10 mg of MWCNT [10]. The resulting mixture was then refluxed at 110°C overnight with continuous stirring to produce oxidized CNTs (MWCNTs–COOH). The samples were washed with ultrapure water until the filtrate became neutral (pH: 7.0). The collected solid was dried under vacuum at 70°C for 12 h. The resulting material was labeled Ox-MWCNTs and set aside for further modification.

Modification of Ox-multiwalled carbon nanotubes

An amount of 10 mg of Ox-MWCNTs was dispersed in 20 ml HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] buffer and 100 mM *N*-hydroxysuccinimide (NHS) was added. The mixture was sonicated for 30 min, followed by the addition of 20 mM 1-ethyl-3-[3-(di-methylamino) propyl] carbodiimide (EDC) to initiate coupling of NHS to the carboxylic groups on the Ox-MWCNTs. The ester complex Ox-MWCNTs/EDC was rinsed thoroughly with buffer to remove excess NHS and EDC. Finally, the material obtained was suspended in 20 mM pH 7.5 HEPES buffer for further investigation [12].

In other experiments, 5 mg of Ox-MWCNTs were sonicated in 9 ml phosphate buffer (PB, 50 mM, pH: 7.5) for 1 h in the presence of 110 µl Tween 20. After the dispersion of nanomaterials, GA was added to prepare a 4% (w/w) solution and the volume was adjusted to 11 ml. The mixture was incubated at 30°C for 30 min under stirring. The modified nanomaterials (MWCNTs/GA) were separated by centrifugation at 6000 rpm for 30 min and washed with buffer solution. Then, 10 ml of PB (50 mM, pH: 7.5) solution was added and the nanomaterials were sonicated for 1 h to form a homogeneous suspension [13].

Immobilization of *Aspergillus flavus* cell saponin hydrolase on multiwalled carbon nanotubes

An amount of 5 mg of the prepared nanomaterials (Ox-MWCNTs, Ox-MWCNTs/EDC, and Ox-MWCNTs/GA) were sonicated in 10 ml PB (50 mM, pH: 7.5) for ~30 min. Then, 5–20 mg of lyophilized *A. flavus* CSH were added and the mixture was incubated under stirring for 1 h at 30°C and then overnight at 4°C. The resulting bioconjugates MWCNTs–CSH, MWCNTs/GA–CSH, and MWCNTs/EDC–CSH were collected separately by centrifugation and then washed several times with buffer. SH activities of the initial CSH, immobilized CSH preparations and in the washing buffer (unbound cells) were determined. The immobilization yield (IY, %) was calculated as follows:

$$\mathrm{IY}(\%) = \frac{\mathrm{I}}{\mathrm{A} - \mathrm{B}}$$

where I is the saponin hydrolase activity units of the immobilized cells, A is the initial activity units offered for immobilization, and B is the activity units of the unbound cells.

Measurement of enzymatic activity

The reaction mixture containing free or immobilized CSH was incubated at pH 5.5 and 45°C using soybean saponin (0.2%, w/v) as a substrate. Reaction products were extracted with ethyl acetate. A volume of 50 µl of ethyl acetate-containing reaction products were diluted with 450 µl of the mobile phase, acetonitrile–methanol–water (50/15/35). A volume of 10 µl of this dilution was analyzed by HPLC and the quantity of SB in the sample was determined by comparison with authentic SB [14]. One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol of aglycone per min from the substrate.

Determination of kinetic parameters and optimum temperature

K_m, V_{max}, and optimum temperature were determined by changing individually the conditions of the saponin hydrolase activity assay (soybean saponin concentration

from 0.5 to 4% and temperature from 20 to 70°C). K_m and V_{max} were calculated from Lineweaver–Burk plots [15].

Thermal stability

Thermal stability of both free and immobilized CSH was evaluated by measuring the saponin hydrolase residual activity of CSH samples exposed to various temperatures (70–90°C) in PB (0.2 M, pH: 6), with different time intervals (1, 2, and 3 h) for each temperature, and the residual activity was then measured.

Activation energy (*Ea*)

Ea is defined as 'the minimum energy required to start a chemical reaction and given in units of kcal/moles. The temperature dependence of the rate constant, for values below the temperature of inactivation, can be described by the Arrhenius equation: $K=A\times e^{-Ea/RT}$, where K is the rate constant, A is the pre-exponential factor, Ea is the activation energy, R is the gas constant (R=1.976 cal/mol/K or 8.314 J/mol/K), and T is the absolute temperature. The apparent activation energy of free and immobilized enzymes was determined from the slope of logarithmic of the activity versus the reciprocal of Kelvin temperature (slope=$-Ea/2.303R$) [16].

Half-life and the deactivation constant rate

The half-life of an enzyme is the time that 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 (60, 70, and 90°C), according to the following equation [17]:

$$\text{Half-life} = \frac{0.693}{\text{slope}}.$$

Deactivation energy=slope of the straight line.

Results and discussion

Immobilization of *Aspergillus flavus* cell saponin hydrolase on Ox-multiwalled carbon nanotubes

A. flavus CSH were immobilized on MWCNTs using three methods, namely, directly on Ox-MWCNT (sample 1 using CSH to nanomaterial weight ratio of 4 : 1), on Ox-MWCNTs/EDC ester complex (samples 2, 3, and 4 using CSH to nanomaterial weight ratios of 1 : 1, 2 : 1, and 4 : 1, respectively), and on Ox-MWCNT in the presence of GA as a crosslinker (sample 5 using CSH to a nanomaterial weight ratio of 4 : 1). The results in Table 1 show that the highest cells' IY of 79.23% was observed in case of direct immobilization on Ox-MWCNT (sample 1). It could also be observed that the formulation using GA as a crosslinker showed a slightly lower IY value of 68.8%, whereas formulations using the Ox-MWCNTs/EDC ester complex were accompanied by much lower IYs ranging from about 22 to 41%; this could be attributed to the inhibitory effect of this complex on cells' enzyme activity or to the high leakage of cells during the immobilization process. Therefore, the direct immobilization of CSH on Ox-MWCNT was chosen as the most suitable method. The fungal cell wall has been shown to be primarily composed of chitin, glucans, mannans, and glycoproteins [18]. This means that it has various free hydroxyl, amine, carbonyl, carboxyl, and imidazole carboxyl groups that offer active sites for covalent binding to Ox-MWCNT in the presence or absence of a crosslinker (GA) [19]. Figure 1a shows unloaded Ox-MWCNT, whereas Figure 1b shows the MWCNTs–CSH conjugate. It was found that CSH were adsorbed on MWCNTs and also appeared in between nanotubes; this yielded more separated and size diameter tubes (Fig. 1b). However, unloaded Ox-MWCNTs were appeared aggregated and sticky to each other.

Comparative study of free *Aspergillus flavus* whole-cell saponin hydrolase and the multiwalled carbon nanotubes–whole-cell saponin hydrolase conjugate

Effect of temperature on free and immobilized Aspergillus flavus whole-cell saponin hydrolase

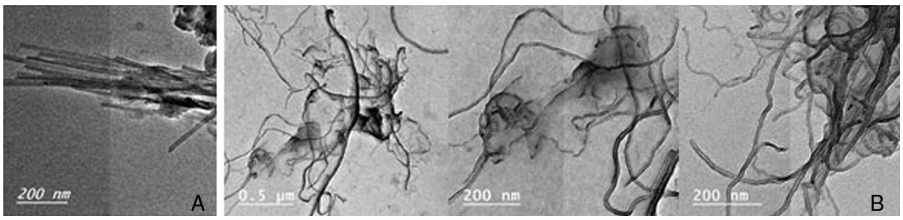
The optimal temperature of free CSH and the MWCNTs–CSH conjugate was 50°C (see Figure 2). Furthermore, the enzyme activity of the free form was higher than that of the immobilized form. This is mainly because of the mass transport resistance to the substrate onto the carrier [20] and thus the lower effective

Table 1 Immobilization of *Aspergillus flavus* cells on different carbon nanotubes preparations

MWCNTs–CSH conjugate	MWCNT : CSH	Immobilization method	Unbound cells (mU) B	Immobilized cells (mU) I	Immobilization yield (IY %)
1	04 : 01	Direct	38.4±1.5	82.5±2.9	79.2±3.8
2	01 : 01	CDE	18.2±0.7	40.7±2.4	32.7±1.6
3	02 : 01	CDE	19.1±0.8	26.9±0.6	21.8±0.6
4	04 : 01	CDE	36.5±1.4	42.9±1.9	40.5±1.9
5	04 : 01	GA	18.2±0.7	85.6±4.3	68.8±2.4

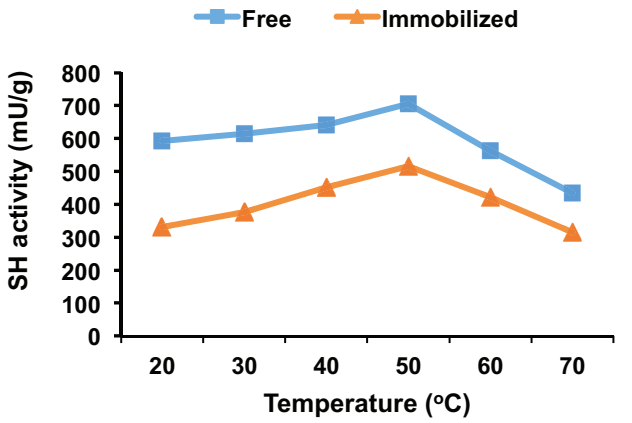
CDE, carbodiimide chemistry; CSH, whole-cell saponin hydrolase (added cells to each carrier, corresponding to 200 mg cells); GA, glutaraldehyde; MWCNTs, multiwalled carbon nanotubes; IY(%)=I/A–B. A=142.6 mU/reaction.

Figure 1



Transmission electron microscope images of unloaded Ox-MWCNTs (a) and MWCNTs–CSH conjugate (b). CSH, whole-cell saponin hydrolase; MWCNTs, multiwalled carbon nanotubes.

Figure 2



Effect of temperature on the enzyme activity of free and immobilized *Aspergillus flavus* whole-cell saponin hydrolase (CSH).

concentration of substrate. This explains why the calculated value of the activation energy (*Ea*) for the free CSH was lower than that of the MWCNTs–CSH conjugate (1.05 and 2.84 kcal/mol, respectively) (Table 2).

Thermal stability of free and immobilized Aspergillus flavus cell saponin hydrolase

It was found that free CSH retained more than 90% of its saponin hydrolase activity at 70°C during all the incubation periods and were generally inactivated at a much slower rate than the MWCNTs–CSH conjugate (data not shown). Also, the free CHS retained 73.3% of the original activity compared with 60.5% retained activity of the MWCNTs–CSH conjugate after 3 h incubation at the same temperature (90°C, data not shown). However, the calculated half-life values of the MWCNTs–CSH conjugate (55, 33.3, and 29.7 h) were higher than those of free CSH (36.1, 29, and 23.2 h) at 70, 80, and 90°C, respectively (Table 3). The high thermal stability of both free and immobilized CSH could be attributed to the high protection of saponin hydrolase enzyme inside cells [21] or the thermal stability of the enzyme itself.

Table 2 Kinetics parameters of free whole-cell saponin hydrolase and the multiwalled carbon nanotubes–whole-cell saponin hydrolase conjugate

Kinetic parameters	Free CSH	MWCNTs–CSH conjugate
K_m (µmol/ml)[a]	0.41±0.019	0.45±0.015
V_{max} (µmol/ml/min)[a]	0.038±0.004	0.031±0.003
V_{max}/K_m	0.092	0.068
Ea (cal/mol)[b]	1051±60.8	2836±90.4

CSH, whole-cell saponin hydrolase; MWCNT, multiwalled carbon nanotube; [a]The apparent *Km* and *Vmax* values were determined by a Lineweaver–Burk plot; [b]The apparent *Ea* values were calculated by an Arrhenius plot.

Table 3 Half-life time and deactivation rate constants at different temperatures of free whole-cell saponin hydrolase and the multiwalled carbon nanotubes–whole-cell saponin hydrolase conjugate

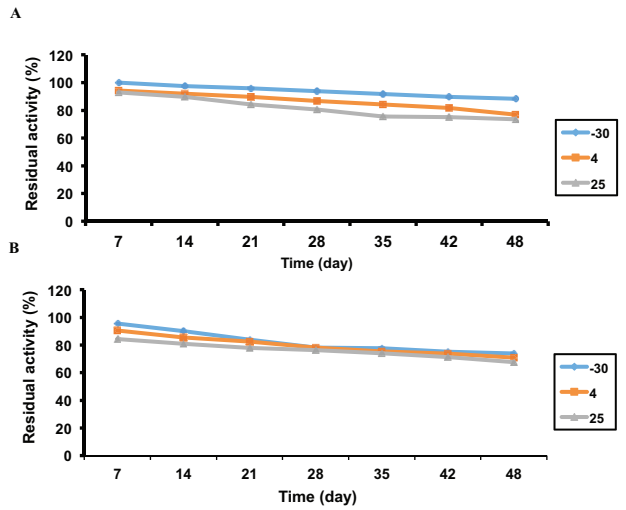
Property	Free CSH	MWCNTs–CSH conjugate
Half-life (hour) at		
70°C	36.1±2.9	55.0±4.7
80°C	28.0±4.1	33.3±2.8
90°C	23.2±3.4	29.7±3.8
Deactivation rate constants ($hour^{-1}$) at		
70°C	1.92×10^{-2}	1.26×10^{-2}
80°C	2.39×10^{-2}	2.08×10^{-2}
90°C	2.99×10^{-2}	2.33×10^{-2}

The half-life and deactivation rate constant values were determined by plotting log residual activity versus time; CSH, whole-cell saponin hydrolase; MWCNT, multiwalled carbon nanotube.

Storage stability of free and immobilized Aspergillus flavus whole-cell saponin hydrolase

In terms of the storage stability, the free and immobilized cells retained high residual activities: about 73.5 and 67.6%, respectively, of their original saponin hydrolase activity at 25°C after 48 days (Figure 3). This extended stability could be attributed to the prevention of structural denaturation as a result of the protection of the enzyme inside the cells in both cases [21]. Furthermore, higher residual activities were retained by the free cells compared with the immobilized ones at 4 and −30°C. This could be attributed to the probability of the inhibition effect of MWCNTs on saponin hydrolase activity over time.

Figure 3



Storage stability of *Aspergillus flavus* free (a) and immobilized (b) whole-cell saponin hydrolase preserved at 25°C (room temperature), 4°C, and –30°C for 48 days.

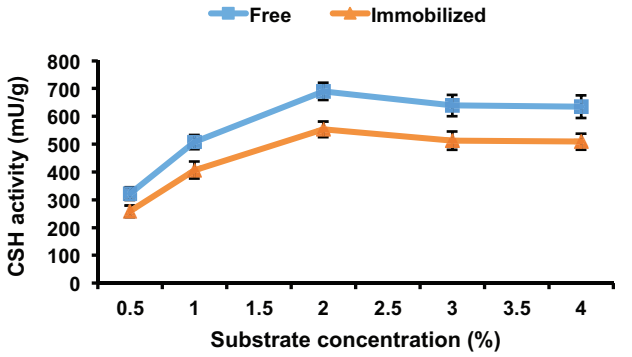
Kinetic properties of free and immobilized Aspergillus flavus whole-cell saponin hydrolase

Both free CSH and the MWCNTs–CSH conjugate generally followed conventional Michaelis–Menten enzyme kinetics with soyasaponin as the substrate (Fig. 4). The results summarized in Table 2 show that the MWCNTs–CSH conjugate retained a maximum reaction velocity (V_{max}) of 0.031±0.003 µmol/ml/min with a corresponding substrate concentration at a half-maximal reaction velocity (K_m) of 10.45 ±0.015 µmol. However, free CSH yielded a higher V_{max} value of 0.038±0.004 µmol/ml/min and a slightly lower K_m value of 0.41±0.019 µmol. With respect to their catalytic efficiency (V_{max}/K_m), free CSH had a catalytic efficiency of 0.092 min compared with 0.068 for the MWCNTs–CSH conjugate.

Operational stability

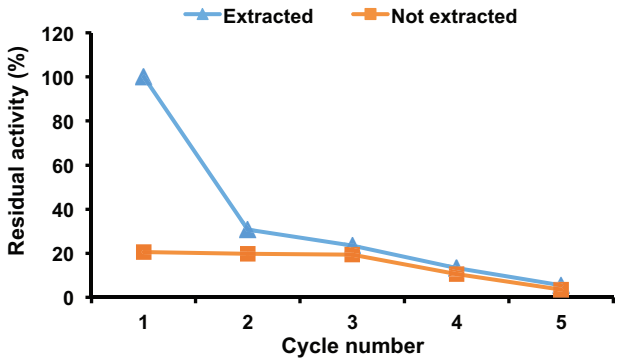
The reuse number of immobilized CSH is one of the most important aspects of industrial application. An increased stability could make the immobilized enzyme more advantageous than the free form [22]. The immobilized *A. flavus* CSH were reused five times; the cells were separated from the reaction mixture before and after extraction with ethyl acetate after each cycle, as shown in Figure 5. In both cases (with and without ethyl acetate extraction), the residual activity decreased gradually to 5.4 and 3.4%, respectively. Thus, the immobilized CSH activities decreased while the reused number increased. The activity decay during recycling may have been related to inactivation of the enzyme caused by denaturation of the protein, or the adsorption and accumulation of reaction products on the biocatalyst [15]. The latter reason is obvious on

Figure 4



Effect of substrate concentration on *Aspergillus flavus* free and immobilized whole-cell saponin hydrolase. CSH, whole-cell saponin hydrolase.

Figure 5



Operational stability of the immobilized *Aspergillus flavus* whole-cell saponin hydrolase. The 100% residual activity corresponds to 515.5 mU/g.

comparing the produced activity at the first cycle when the cells were separated from the reaction mixture before and after extraction with ethyl acetate (30.9 and 6.2 U/g, respectively).

Conclusion

In this study, an innovative nanobiocatalytic system was developed through the immobilization of *A. flavus* CSH on carbon-based nanomaterials for biotransformation of soybean saponins into SB. It was observed that *A. flavus* CSH interacts differently with Ox-MWCNT depending on the method implemented. Although CSH maintained much of its activity (80%) when directly immobilized onto Ox-MWNTs, this was not the case when carbodiimide or GA chemistry was used. Compared with free CSH, the MWCNT–CSH conjugate showed higher K_m value, activation energy, and half-lives, and lower V_{max} value and deactivation constant rate.

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Conflicts of interest

There are no conflicts of interest.

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