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A facile enzymatic synthesis of geranyl propionate by physically adsorbed *Candida rugosa* lipase onto multi-walled carbon nanotubes

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ABSTRACT

In view of several disadvantages as well as adverse effects associated with the use of chemical processes for producing esters, alternative techniques such as the utilization of enzymes on multi-walled carbon nanotubes (MWCNTs), have been suggested. In this study, the oxidative MWCNTs prepared using a mixture of HNO_3 and H_2SO_4 (1:3 v/v) were used as a supportive material for the immobilization of *Candida rugosa* lipase (CRL) through physical adsorption process. The resulting CRL-MWCNTs biocatalysts were utilized for synthesizing geranyl propionate, an important ester for flavoring agent as well as in fragrances. Enzymatic esterification of geraniol with propionic acid was carried out using heptane as a solvent and the efficiency of CRL-MWCNTs as a biocatalyst was compared with the free CRL, considering the incubation time, temperature, molar ratio of acid:alcohol, presence of desiccant as well as its reusability. It was found that the CRL-MWCNTs resulted in a 2-fold improvement in the percentage of conversion of geranyl propionate when compared with the free CRL, demonstrating the highest yield of geranyl propionate at 6 h at 55 °C, molar ratio acid: alcohol of 1:5 and with the presence of 1.0 g desiccant. It was evident that the CRL-MWCNTs biocatalyst could be reused for up to 6 times before a 50% reduction in catalytic efficiency was observed. Hence, it appears that the facile physical adsorption of CRL onto F-MWCNTs has improved the activity and stability of CRL as well as served as an alternative method for the synthesis of geranyl propionate.

1. Introduction

Terpene esters of short-chain fatty acids are essential oils that present a great deal of interest in food, cosmetics and pharmaceutical industries as flavors and fragrances; acetate, propionates and butyrates of acyclic terpene alcohols such as geraniol and citronellol, being the main components of essential oil [1]. Traditionally, these esters are obtained by various methods viz. chemical synthesis, extraction from natural products as well as fermentation [2]. Despite the common use of chemical synthesis for producing esters, the process requires harsh reaction conditions such as high temperature and pressure, involvement of strong acid catalyst and hazardous chemicals, considerably long reaction time while providing low conversion rate [1]. Moreover, the chemical synthesis has been associated with tedious separation processes, extreme exposure of toxicants as well as unwanted harmful reaction byproducts [3]. Hence, the enzymatic production of flavors and fragrances using natural raw materials may prove useful for scientific and industrial settings, considering the ever arising demands for such products [4].

Due to the fact that lipases (triacylglycerol ester hydrolysis EC 3.1.1.3) may catalyze esterification reactions without providing high temperature and pressure conditions and since the procedure remains relatively uncomplicated when compared with the chemical synthesis, such enzymes have acquired popularity as biocatalysts for the reactions [5]. In this context, *Candida rugosa* lipase (CRL), a stable mesophilic lipase, has been commonly used due to its high activity and broad specificity in reaction medium [6]. Considering that the CRL is often unstable in its free form, demonstrating low activity in organic solvents, high tendency of deactivation in

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prolonged exposure toward high temperature and extreme pH [6], immobilization of CRL onto carbon nanotubes may be one of the probable solutions. In addition, advances in enzyme immobilization techniques have enabled the use of a wide range of biocatalysts for various reactions under extreme pH, temperature and pressure conditions [7,8].

Owing to its excellent binding capacity due to large surface area to volume ratio, physicochemical properties as well as biological compatibility, carbon nanotubes have been frequently utilized as the support materials for immobilization of enzymes [9,10]. Enzyme immobilization confers a multitude of advantages such as structural stability, improved activity, specificity and selectivity, reduction of inhibition [11], increased flexibility with enzyme/substrate contact using various reactor configurations [12] and longer half-life of the enzyme [13]. Since these enzymes are expensive and are required in large volumes for industrial needs [14], immobilization of such enzymes onto solid support materials would be helpful in maintaining the catalytically active tertiary structure of enzymes [15], which contributes to its reusability. In this context, the physical adsorption method may have higher commercial values than other methods [16] because it is one of the simplest and cheapest immobilization methods available and importantly, in most cases, the enzyme productivity remains unaffected [17].

This present study was aimed at investigating the application of physically adsorbed CRL on the surface of acid functionalized MWCNTs (CRL-MWCNTs) as potential economical biocatalysts. The CRL-MWCNTs were compared with the free CRL for synthesizing geranyl propionate. In addition, its effects on the incubation time, temperature, molar ratio of acid to alcohol, presence of desiccant and reusability in rendering the highest conversion of geranyl propionate were evaluated.

2. Materials and methods

2.1. Chemicals

Multi-walled carbon nanotubes (MWCNTs) prepared by the chemical vapor deposition method were provided by one of the co-authors (Assoc. Prof. Dr. Nor Aziah Buang). Lipase Type VII of *C. rugosa* (EC 3.1.1.3) with measured activity of 1410 units mg^{1}, substrates, geraniol (98%), propionic acid (99%), phosphate buffer, Bradford reagent, phenolftalein and molecular sieves were all purchased from the Sigma Chemical Co. (St. Louis, USA). Other chemicals that include sodium hydroxide and *n*-heptane were of analytical grade and used without further purification. Distilled water was prepared in our laboratory and used in all experiments.

2.2. Purification and functionalization of MWCNTs

The raw MWCNTs (0.5 g) were transferred into a 100 mL flask containing 4 M HCl (20 mL) and refluxed with stirring at 80 °C for 5 h. After cooling to room temperature, the liquid was decanted, the MWCNTs were washed with distilled water until no residual acid was detected and dried in an oven at 60 °C for 24 h. The purified MWCNTs were refluxed at 120 °C by stirring in a mixture of concentrated HNO_3 and H_2SO_4 with ratio 1:3 (v/v) for 24 h. After cooling to room temperature, the mixture was decanted, washed with distilled water until no residual acid was present and dried at 60 °C for 24 h.

2.3. Adsorption immobilization of lipase and its characterization

The free CRL was immobilized onto the acid functionalized MWCNTs (F-MWCNTs). The MWCNTs (10 mg/mL) were first sonicated in aqueous buffer (pH 7) for 30 min to ensure homogeneous dispersion. Then, the MWCNTs were suspended in a 50 mL flask of phosphate buffer (50 mM, solution pH 7) containing the CRL (10 mg/mL) and incubated at 20 °C with constant stirring at 150 rpm. After 3 h of incubation, the flask containing CRL-MWCNTs was stored at 4 °C for 24 h. Upon completion, the unbound protein was removed by washing with phosphate buffer (pH 7) until no hydrolytic activity was detected in the washing. The supernatant was then subjected to protein analysis. Similar CRL immobilization treatments were also performed to unfunctionalized MWCNTs.

2.3.1. Fourier transform infrared (FTIR)

A ratio 1:100 mass of sample was ground thoroughly with potassium bromide and the resulting powder was pressed into a transparent pellet by a hydraulic press. The FTIR spectra obtained using a BOMEM spectrophotometer in transmission mode between 400 and 4000 cm^{-1} at a resolution of 4 cm were analyzed.

2.3.2. Field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM)

A field emission scanning electron microscope (FESEM) (JEOL JEM-6700F) was used to study the surface morphology of MWCNTs, F-MWCNTs and CRL-MWCNTs. For FESEM analysis, the sample was prepared by taking one drop of acetone containing the dispersed MWCNTs on a silicon wafer and allowing it to dry in a vacuum oven for 30 min. For TEM analysis, electron source was used as W-emitter and LaB6, operating at an accelerating voltage of 200 kV. The analysis utilized an objective lens (S-Twin) with a point resolution of 2.0 nm or better with a 25× to 7500, 200× or higher magnification and a single tilt holder with LCD camera. The MWCNTs, F-MWCNTs and CRL-MWCNTs were dispersed in deionized water and a drop was placed on a copper grid and observed after drying in vacuum.

2.4. Determination of protein loading

Protein content of the enzyme solution, before and after immobilization, in the washing buffer was determined by Bradford method using BioRad protein dye reagent concentrate and bovine serum albumin (BSA) as the protein standard [18] as well as in the reaction mixture.

2.5. Enzymatic synthesis of geranyl propionate catalyzed by free CRL and CRL-MWCNTs

The reaction medium that consisted of 0.25 M propionic acid and 1.25 M geraniol was dissolved in *n*-heptane in a flask followed by the addition of free CRL (10 mg/mL) and CRL-MWCNTs (5 mg/mL), respectively. The reaction mixture was refluxed with continuous stirring at 200 rpm in a paraffin oil bath. The geranyl propionate obtained was expressed in terms of percent conversion i.e. percent of propionic acid converted versus the total acid in the reaction mixture by titrating aliquots of liquid sample (1 mL) withdrawn periodically with 0.05 M NaOH using phenolphthalein as an indicator. Each measurement was performed in triplicates and the standard error was calculated. The percent conversion was calculated according to the equation prescribed by previous researchers [1] detailed below:

$$\%\,\text{Conversion} = \left(\frac{(V_o - V_t)}{V_o}\right) \times 100$$

whereby, V_o is the volume of NaOH at initial time ($t = 0$) and V_t is the volume of NaOH at each hour ($t = t_1, t_2, t_3, \ldots$).

2.5.1. Effect of incubation time

Time course study is a good indicator of enzyme performance as well as product yield and a good performance enzyme requires relatively shorter duration to obtain good yields when compared with the poor ones [19]. The effect of incubation was monitored up to 24 h with sampling intervals of 0, 4, 8, 12, 16, 20 and 24 h, respectively. The reactions were carried out in 50 mL round bottom flasks, stirred constantly at 200 rpm. Upon completion of preliminary screening tests, the reaction temperature was maintained at 40 °C.

2.5.2. Effect of temperature

It has been reported that temperature has a significant effect on the equilibrium of the reaction as well as the activity and stability of lipase [20]. The effect of temperature on the enzymatic synthesis of geranyl propionate of both free CRL and CRL-MWCNT was determined at varying temperatures ranging from 40 to 60 °C, at increasing intervals of 5 °C each.

2.5.3. Effect of the presence of desiccant

Water is a byproduct of esterification that needs to be removed for obtaining higher yield of ester [21]. Following the successful employment as desiccant in many enzymatic esterification reactions [22,23], in this present study too, molecular sieves (4 Å) were chosen. They act as absorbents that are replaceable with new ones when saturated with water [24]. By removing water in media, the reaction equilibrium would shift toward the synthesis of the desired ester hence, higher conversion of ester products [24]. The effects of desiccant on the free CRL and CRL-MWCNTs were evaluated in the presence (1.0 g) or absence (0 g) of molecular sieves.

2.5.4. Reusability of CRL-MWCNTs

The reusability of the CRL-MWCNTs was examined by reusing the recovered lipase for repeated reaction cycles. After each cycle, the enzyme was filtered, washed with similar solvent and allowed to dry before reuse. For investigating the reusability, the reaction mixture that consisted of propionic acid and geraniol dissolved in *n*-heptane with the presence of immobilized lipase was used. Each reaction was monitored for a period of 8 h at 30–50 °C with constant stirring (Table 1).