PRODUCTION OF GERANYL PROPIONATE BY ADSORPTION OF *CANDIDA RUGOSA* LIPASE ON ACID FUNCTIONALIZED MULTI-WALLED CARBON NANOTUBES

Lok Yen Yen and Roswanira Abdul Wahab
Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru

Abstract

Geranyl propionate is an ester which is in high demand and usually synthesized chemically as a flavouring agent for use in the food and beverage industries. In view of the numerous undesirable effects of the chemical method can have on the environment and human health, efforts to finding alternative means to produce such ester merits scientific consideration. Hence, the biotechnological route to producing geranyl propionate by the biotechnological routeusing physically adsorbed *Candida rugosa* lipase (CRL) type VII onto acid-functionalized multi-walled carbon nanotubes (CRL-MWCNTs) is suggested. The properties and morphology of the developed CRL-MWCNTs were characterizedusing FTIR, FESEM and TGA showing was successful adsorption of CRL onto the surface of the functionalizedMWCNTs.The approach of one-variable-at-a-time (OVAT) based on four parameters viz. time, substrate molar ratio of geraniol to propionic acid and solvent log P were evaluated for comparative esterification reactions catalysed by both the free CRL and the CRL-MWCNTs. The findings revealed that the CRL-MWCNTs were catalytically more efficient than the free CL andafforded improved product conversion of 76.76% as compared to 40.40% in the free CRL under similar optimized reaction conditions of 8 h, substrate molar ratio 4:1 in hexane (log P 3.50) as solvent. Considering such outcome, it can be inferred that the improved biological activity demonstrated by theCRL-MWCNTs that surpassed that of the free CRL may have been attributed to the enhanced rigidity of the CRLprotein following immobilization on the acid functionalized MWCNTs.The outcomesadvocate that the RML/CS/MWCNTs biocatalyst developed here may be a promising alternative to overcome shortcomings associated with the chemically produced geranyl propionate.

Keywords: *Geranyl propionate, Candida rugosa lipase, Multi-walled Carbon Nanotubes*

INTRODUCTION

Demand for flavor and fragrance market is expected to rise 4.4% a year to achieve $26.5 billion in 2016 [1]. To meet customer demand, it is essential for an industry to synthesize flavor based on economic value by using cheaper and more broadly available material. The growing of consumer's preference for natural products has pushed the flavor and fragrance market towards the biotechnological route [2]. Biocatalysis uses natural catalyst to perform chemical transformations on organic compounds, thus the ester prepared may be labeled as "natural" [3]. Production of flavor by using biocatalyst is a green technology [4], environmental friendly and consumes less energy compared to conventional chemical process.

Geranyl propionate is an organic ester with a fruity-floral scent and has a high commercial demand due to it is used as synthetic flavourings. It is traditionally synthesized by direct esterification and transesterification reactions. However, such methods are not economical since most substrates show low miscibility, requiring addition of more organic solvents into the reaction medium [5]. Enzymatic catalysis is a useful tool to overcome these problems as the method enhances stability of substrates and products and also improved selectivity of the enzyme. However, the poor stability of enzymes in solvent leads to low apparent catalytic activity. In order to improve biocatalyst stability, immobilization of enzyme on support material is a preferred method [6]. Herein, the objective of the present research work is produce of geranyl propionate by *Candida rugosa*lipase type VII as biocatalyst immobilized on multi-walled carbon nanotubes. The process parameters were optimized using one-variable-at-a-time method (OVAT) which focused on the effect of reaction time, substrate molar ratio and solvent log P. Besides that, the efficiency of free and immobilized *Candida rugosa* lipase in the production of geranyl propionate was compared.

EXPERIMENTAL

Candida rugosa lipase type VII was immobilized onto functionalizedmulti-walled carbon nanotubes (MWCNTs) *via* physical adsorption.The immobilized *Candida rugosa* lipase was characterized *via* Fourier Transform Infrared (FTIR), Field Emission Scanning Electron Microscope (FESEM) and Thermogravimetric Analysis (TGA). The immobilized *Candida rugosa* lipase was employed to compare the efficiency with free lipase in the production of geranyl propionate for parameters reaction time, substrates molar ratio and solvent log P.

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Experimental Procedure

Purification and Functionalization of MWCNTs

MWCNTs was purified by dispersed in an aqueous HCl of 4M at 80 °C. The reaction was carried out under reflux system on magnetic stirrer at 100 rpm for 8 h. Next, the MWCNTs suspension was washed with distilled water until pH 7.0 by filtering through a 0.8 µm polycarbonate membrane. MWCNTs were dried at 80 °C in an oven for overnight. The purified MWCNTs were impregnated with a 4M acid mixture of H_2SO_4 and HNO_3 (3:1 V/V) in an oil bath at 120 °C for 24 h. Next, the MWCNTs suspension was washed with distilled water to pH 7.0. Finally, MWCNTs were dried at 80 °C in an oven for overnight.

Immobilization of CRL on Multi-walled Carbon Nanotubes

The MWCNTs was dispersed by sonication at a fixed frequency of 40 kHz and at 110 W power in 50 ml phosphate buffer pH 7.0 for30 min, followed by addition of 10 mg/ml of the lipase solution. The mixture was kept at 20 °C with constant shaking by using orbital shaker at 200 rpmfor 5 h. Next, the mixture was incubated at 4 °C for overnight. After incubation, the mixture was centrifuged at 8,000 rpm for 20 min at 20 °C. The supernatant was removedand MWCNTs was washed with distilled water until no hydrolytic activity was detectedin the washings. Finally, the immobilized enzyme was dried in freeze dryer to remove any aqueous by sublimation. The immobilized enzyme was stored in refrigerator at 4 °C until used.

Synthesis of Geranyl Propionate

Geranyl propionate was synthesized through esterification of propionic acid with geraniol using CRL-MWCNT as biocatalyst. The experiment set up consisted of 50 ml two-necked round bottom flask. The entire assembly was immersed in a thermostatic water bath, which was maintained at the desired temperature. As a preliminary step, controlled experiment was performed with geraniol to propionic acid molar ratio of 1 : 1, temperature 40 °C, 1 g molecular sieve and hexane as solvent. The mixture was agitated at 100 rpm for incubation time of 12 hours. Process parameter such as reaction time, propionic acid to geraniol molar ratio and solvent, were investigated to acquire maximum yield of geranyl propionate.

Effect of Reaction Time

The effect of reaction time on progress of reaction was performed as it is a significant parameter helps to establish optimum time to archive maximum yield of product. Consequently, the effect of reaction time on CRL catalyzed esterification for geranyl propionate was executed in a 50 mL round-bottom flask which was placed in a water bath with temperature of 40 °C, agitation speed at 100 rpm, 10 mg/mL enzyme loading, 1 g molecular sieve and reactant molar ratio 1 : 1 .

Effect of Substrate Molar Ratio of Geraniol to Propionic Acid

Variation in the alcohol concentration will affect reaction rated. In order to evaluate the effect of geraniol to propionic acid molar ratio (1 : 1, 2 : 1, 3 : 1, 4 : 1, 5 : 1) on geranyl propionate production, temperature was kept fixed at 40 °C, enzyme concentration at 10 mg/mL, 1 g molecular sieve and agitation at 100 rpm, making possible to establish conversion versus time curves.

Effect of Solvent

Six organic solvents with log P values ranging from -1.0 to 5.0 were tested for production of geranyl propionate. The effect of solvent was determined at temperature 40 °C, enzyme concentration at 10 mg/mL, 1 g molecular sieve and agitation at 100 rpm. In this research work, the organic solvents used were hexane, heptane, chloroform, acetone, dimethylforamide and 2,2,4-trimethylpentane.

Determination of Reaction Conversion Yield of Geranyl Propionate

The geranyl propionate obtained was expressed in terms of percent conversion by titrating reaction mixture (1 mL) with 0.05M NaOH using phenolphthalein indicator solvent (2 mL) as internal standard. The conversion yield was calculated according to Equation 1:

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$$\% \text{ Conversion yield} = \frac{V_0 - V_t}{V_0} \times 100 \qquad \text{(Equation 1)}$$

Where:
V_0 = Volume of NaOH consumed at initial time (t = 0)
V_t= Volume of NaOH consumed at each hour ($t = t_1, t_2, t_3 \ldots$)

Analysis of Geranyl Propionate by Using Gas Chromatography

Identification of synthesized geranyl propionate in liquid samples was carried out by Gas Chromatography equipped with flame ionization detector using a capillary column of fused silica INOWAX. Helium was used as carrier gas at a constant pressure. The temperature program was as follow: 50 °C for 1 min; 5 °C/min up to 300 °C; then steady temperature for 1 min.

Characterization of CRL-MWCNTs

Characterization of surface groups and functional groups present in each step of preparation of the immobilized *Candida rugosa* lipase was executed using FTIR, FESEM and TGA. FTIR used to identify the significant functional group, TGA use to determine the degree of functionalisation and FESEM was used to visualize presence of appropriate functional groups and lipase molecules on the surface of multi-walled carbon nanotubes.

RESULTS AND DISCUSSION

Analysis of FTIR spectroscopy confirmed that of the lipase was successfully immobilized onto MWCNTs-COOH. The FTIR spectrum of immobilized lipase revealed the appearance of adsorption band at 2929.41 cm^{-1} which implied successful attachment of the lipase to the surface of carbon nanotubes as this band was not observed in the spectrum of MWCNTs-COOH **(Figure 1).** Spectrum of oxidized-MWCNT exhibited the absence of a band at 1634.70 cm^{-1} and the corresponding amide carbonyl band from proteins appeared at lower wavelength of 1620.71 cm^{-1} [7]. In addition, the presence of new band at 1032.96 cm^{-1} was attributed to C-N bond, further confirmed the existence of the amide group. Whereas the NH_2 and CH_2 stretchings appeared at 3420.59cm^{-1} and 2929.41cm^{-1}, respectively. Distinctions in the spectra of free and immobilized lipase are observed in the range of 900-1200 cm^{-1}, the spectral region were carbohydrate functional group band [8]. After immobilization of the lipase, a strong peak at 1125.04 cm^{-1} belonging to –CO of the carbohydrate moiety was reduced, expanded and moved to a lower frequency,1116.03 cm^{-1} [7].

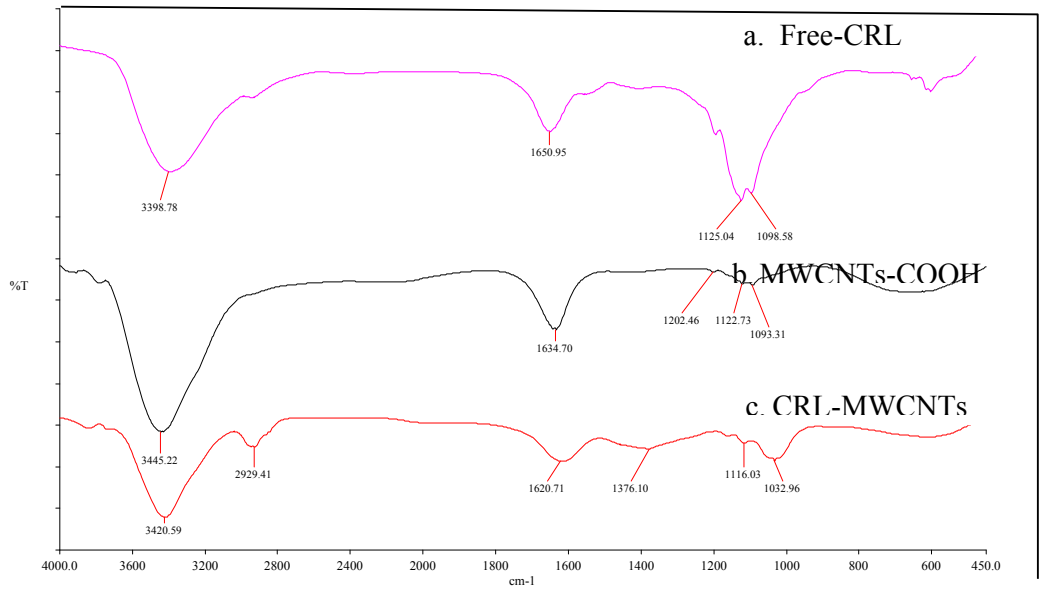

Figure 1: FTIR spectra of (a) Free-CRL, (b) MWCNTs-COOH and (c) CRL-MWCNTs.

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Figure 2 demonstrates the morphology of raw-MWCNTs, MWCNTs-COOH and CRL-MWCNTs obtained by FESEM analyses. The FESEM of raw-MWCNTs revealed open ended tubular structures and are closely attached to each other. The raw-MWCNTs were observed to be of a snake-like shape with very smooth surface. After oxidation using $H_2SO_4$and HNO_3, the diameter of MWCNTS was notably increased and the surface of the MWCNTs turned rough due to the surface erosion [9]. The changes in the diameter of the carbon nanotubes implied successful attachment of the acid functional groups on the surface of MWCNTs [10]. Additionally, the lengths of the MWCNTs were also shorter and the quantity of amorphous carbon had also increased [11].According to **Figure 2(c),** the lipase molecules were clearly physically present on the multi-walled carbon nanotubes.

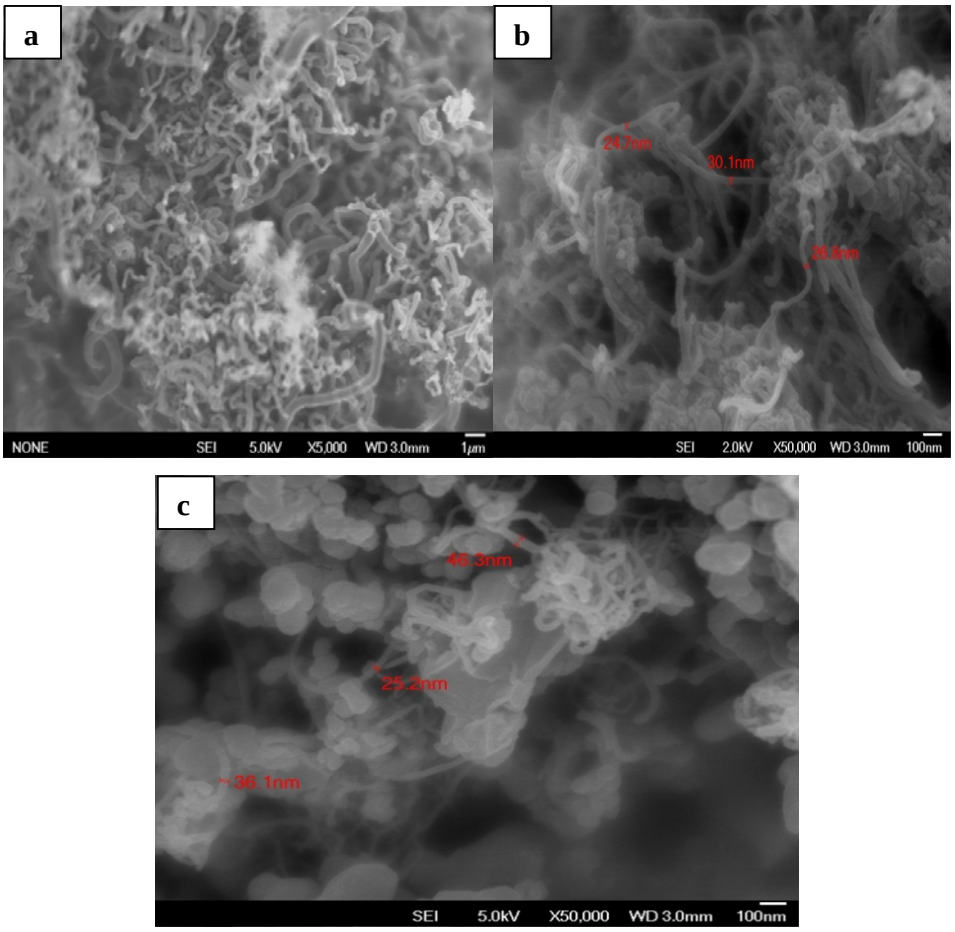

Figure 2: FESEM images of (a) Raw-MWCNTs, (b) MWCNTs-COOH and (c) CRL-MWCNTs.

TGA was employed to examine the weight loss percentage of MWCNTs in range of 30 to 800 °C. **Figure 3** illustrates the TGA results for the MWCNTs-COOH and CRL-MWCNTs. For MWCNTs-COOH, the weight loss was approximately 30%. The MWCNTs-COOH sample exhibited a steady, uniform and multistage weight loss which is due to degradation of various groups present on the surface. As for the CRL-MWCNTs, the sample showed about a 38% weight loss which was attributed to the presence of *Candida rugosa* lipase that was adsorbed on the surface of MWCNTs. In comparison to MWCNTs-COOH, the decomposition curve of the CRL-MWCNTs showed noticeably different weight loss pattern occurring at 250 - 350°C, which can be assigned to the thermal decomposition of lipase [91] which was representative of the thermal stability of CRL. As a result, the weight percentage of attached lipase in CRL-MWCNTs was estimated to be about 13 wt% in this sample. A low weight loss (6.5%) at 30 -150°C is due to the elimination of both surface hydroxyl groups and organic structure of lipase [12].

The effect of reaction time on the progress of reaction was evaluated for the free and immobilized lipase. It was observed that the ester conversion increased with increasing reaction time. In the initial 8 h, the highest conversion of 43% and 60% was achievedfor the free-CRL and CRL-MWCNTs, respectively. It can be seen

that immobilization of CRL onto MWCNTs had improved the esterification process. However, when the incubation time exceeded 8 h, a reduction in percentage yield was observed. A longer reaction time was seen to be unfavorable. This was due to the formation of water in the reaction medium that tend to favor the reverse reaction of hydrolysis of the ester. Therefore, 8 h was selected as optimum reaction time for the subsequent investigations.

(a) MWCNTs-COOH (b) CRL-MWCNTs

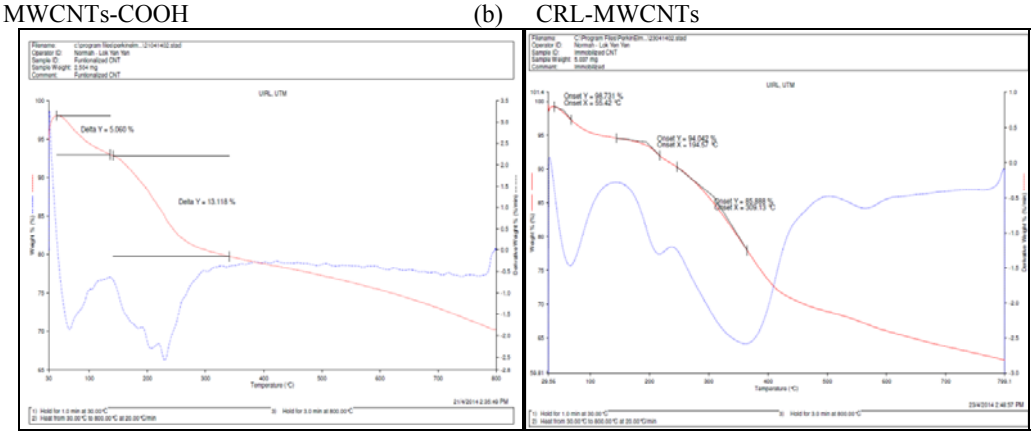

Figure 3: TGA results for (a) oxidized-MWCNTs (MWCNTs-COOH) and (b) immobilized-lipase (CRL-MWCNTs).

The effect of molar ratio of substrates on the esterification reaction was an important parameter to be considered since it defined the chemical and physical properties of a reaction system. The geranyl propionate yield was increased from 25.4 to 50% and 40.4 to 76.76% for the free-CRL and CRL-MWCNTs, respectively, when substrate molar ratio was increased from 1:1 to 4:1. It has been suggested that the increase in the nucleophile concentration would push the equilibrium of the reaction in a forward direction [4]. The investigation found the optimal ratio of geraniol to propionic acid was found to be 4:1. The percentage yield of the geranyl propionate was reduced decreased when the molar ratio of geraniol to propionic acid exceeded 4:1. This observation strongly suggested that the high concentration of geraniol in the reaction mixture had inhibited the reaction and ultimately reduced the reaction rates [13]. The inhibition could be due to the high concentration and density of geraniol that increased the viscosity of the reaction mixture. This subsequently lead to ineffective mixing of the reactants [13]. A higher conversion was achieved for CRL-MWCNTs in compared to free-CRL. The study had successful shown that physical immobilization of CRL onto MWCNTs had enhanced the synthesis of geranyl propionate.

The employment of biocatalyst in organic solvents provides many benefits over solvent-free system, such as increase the miscibility of substrates, avoidance of unnecessary side reactions, enhanced stability of biocatalyst and the shift the thermodynamic equilibrium to the synthetic way, thus leading to high percentage yield of product [13]. In this investigation, six organic solvents with different polarities (log P values ranging -1.0 and 4.51) were employed. In free-CRL and CRL-MWCNTs catalyzed reactions, it was found that hexane (log P 3.5) gives the highest percentage yield of geranyl propionate of 49.89% and 76.76%, respectively. Besides that, heptane (log P 4.0) also gave a high conversion of 63.27%, followed by 2,2,4-trimetylpentane (log P 4.5) at 30.87% for the CRL-MWCNTs catalyzed reactions.These solvents i.e. hexane, heptanes and 2,2,4-trimetylpentane are hydrophobic and they won't strip off water from surface of enzyme. Ultimately, this will result to a flexible protein structure alongside an increased activity [14], ultimately resulted in a high conversion. Other more hydrophilic solvents (log P less than 3.5) such as chloroform, acetone and dimethylfomamide tend to give result in lower conversion of the ester.This could be attributed tothe active conformation of the lipase had been adversely affected. Moreover, hydrophilic solvents tend to strip off the essential water from the enzyme and penetrate into hydrophobic protein core, leading to denaturation of enzyme [14]. Comparison of the free and immobilized CRL-catalyzed reactions, the CRL-MWCNTs offered higher conversion of geranyl propionate. It was noticed that activity of immobilized lipase was better than that of the free lipase. This could be due to hydrophobic nature of MWCNTs surface gave an appropriate microenvironment for the lipase [11].

The synthesized geranyl propionate at 0 h and 8 h were taken and identified by Gas Chromatography equipped with flame ionization detector. The employed mobile phase is helium while the stationary phase consists of a capillary column of fused silica INOWAX. Separation of compounds in the sample is based on the interaction strength of compounds with stationary phase. Chromatogram of the testing samples was shown in

Figure 5. Factors that influenced the separation of the components were based on boiling point and polarity of compounds. The ascending order of boiling point is propionic acid <geraniol<geranyl propionate. Hence, geranyl propionate has longer retention time compared to geraniol and propionic acid. In **Figure 5(b),** the presence of a higher peak at high retention time 13.782 min verified that geranyl propionate was present at 8 h compared to **Figure 5(a).** Previous study done by Thanighai *et al.* illustrated that the retention time of geranyl propionate was 16.11 min.

(a) Sample at 0 hour (b) Sample at 8 hour

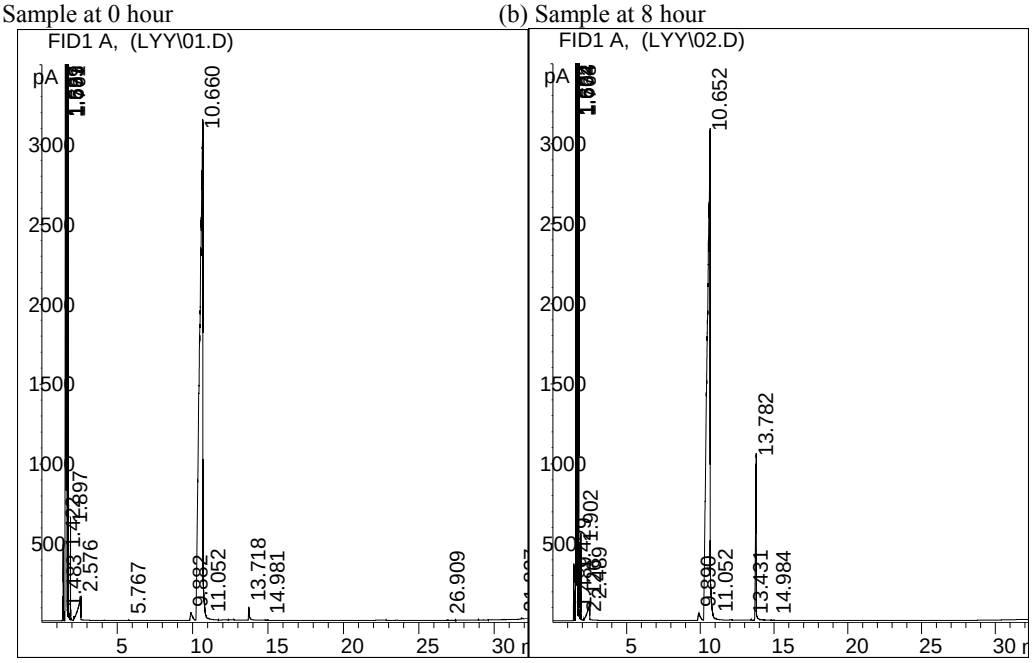

Figure 4.9: Gas Chromatogram of Sample (Synthesized Geranyl Propionate) at 0 hour and 8 hour.

CONCLUSION

This work illustrates that geranyl propionate was successfully synthesized at a reasonably high yield under a short period of time using immobilized *Candida rugosa* lipase type VII by esterification of geraniol and propionic acid. It was confirmed that *Candida rugosa* lipase was immobilized successfully onto MWCNT *via* Field Emission Scanning Electron Microscope (FESEM), Fourier Transform Infrared Spectroscopy (FTIR) and Thermogravimetric Analysis (TGA). Furthermore, it was also noted that physical adsorption of the lipase onto carbon nanotubes had enhanced of enzyme activity when tested against the free lipase for various parameters such as reaction time, geraniol to propionic acid molar ratio and solvent log P. The immobilized lipase afforded a maximum conversion of 76.76% at time 8 h, geraniol to propionic acid (4 :1) molar ratio, in hexane as compared to 49.89% for the free CRL. The study believed that the observably higher percentage yield of the ester in reactions catalysed by the CRL-MWCNTs may have been attributed to the CRL molecule adopting an activated "open lid" conformation after adsorption onto MWCNTs.

REFERENCES

1. Report: Worldwide F&F Market Expected to Reach $26.5B in 2016. (2012). *Perfumer &Flavorist*.
2. Dubal, S.A., Tilkari, Y.P., Momin, S.A., Borkar, I.V. (2008). Biotechnological Routes in Flavour Industries. *Advanced Biotech*. 20 - 29.
3. Gillies, B., Yamazaki, H., Armstrong, D.W. (1987). Production of Flavor Esters by Immobilized Lipase. *Biotechnology Letters.***9 (10),** 709 - 714.
4. Kuperkar, V.V., Lade, V.G., Prakash, A., Rathod, V.K. (2014). Synthesis of Isobutyl Propionate using Immobilized Lipase in A Solvent Free System: Optimization and Kinetic Studies. *Journal of Molecular Catalysis B.* **99**, 143-149.

5. Paroul, N., Grzegozeski, L.P., Chiaradia, V. (2010). Production of Geranyl Propionate by Enzymatic Esterification of Geraniol and Propionic Acid in Solvent-Free System. *Journal of Chemical Technology and Biotechnology.* **85**, 1636-1641.
6. Nevena, Z., Prlainovic, Bezbradica, D. (2013). Adsorption of Lipase from Candida*rugosa* on Multi Walled Carbon Nanotubes. *Journal of Industrial and Engineering Chemistry.* **19**, 279 - 285.
7. Prlainoc, N.Z., Bezbradica, D.I. (2013). Adsorption of lipase from *Candida rugosa* on multi walled carbon nanotubes. *Journal of Industrial and Engineering Chemistry.* **19 (1),** 279-285.
8. Natalello, A., Ami, D., Brocca, S., Lotti, M., Doglia, S. M. (2005). Secondary structure, conformational stability and glycosylation of a recombinant Candida rugosa lipase studied by Fourier-transform infrared spectroscopy. *Biochemical Journal.* **385,** 511-517.
9. Alkhatib, M.F., Mirghani, M.E.S., Qudsieh, I.Y., Husain, I.A.F (2010). Immobilization of Chitosan onto Carbon nanotubes fo Lead Removal drom Water. *Journal of Appplied Sciences.* **10 (21),** 2705-2708.
10. Johan, M.R., Suhaimy, S.H.M., Yusof, Y. (2014). Physico-chemical studies of cuprous oxide (Cu_2O) nanoparticles coated on amorphous carbon nanotubes (α-CNTs). *Applied Surface Science*. 450 -454.
11. Mazova, I., Kuznetsova, V.L., Simonovaa, I.A., Stadnichenkoa, A.I. (2012). Oxidation behavior of multiwall carbon nanotubes with different diameters and morphology. *Applied Surface Science*, 6272– 6280.
12. Castro, H. F., Silva, M.L.C.P., Silva, G.L.J.P. (2000). Evaluation of Inorganic Matrixes as Supports for Immobilization of Microbial Lipase. *Brazilian Journal of Chemical Engineering.* **17.**
13. Radzi, M. S. (2005). High Performance Enzymatic Synthesis of OleylOleate using Immobilised Lipase from Candida *antartica*. *Electronic Journal of Biotechnology.* **8 (3).**
14. Herbst, D., Peper, S., Niemeyer B., (2012). Enzyme Catalysis in Organic Solvents : Influence of Water Content, Solvent Composition and Temperature on *Candida rugosa* lipase CatalyzedTransesterification. *Journal of Biotechonology.* **162 (4),** 398 – 403.
15. Nevena, Z., Prlainovic, Bezbradica, D. (2013). Adsorption of Lipase from Candida*rugosa* on Multi Walled Carbon Nanotubes. *Journal of Industrial and Engineering Chemistry.* **19**, 279 - 285.