

Fructose stearate esterify in packed bed reactor using immobilized lipase.

N A Shahrin, G G Yong and N A Serri

Bioprocess Technology, School of Industrial Technology, Universiti Sains Malaysia,
11800 Penang, Malaysia

Email: aziah_serri@usm.my

Abstract . The enzymatic esterification of sugar-fatty acid ester to produce bio-based surfactants or emulsifiers has been recognized as an alternative way to the chemical synthesis due to its environmentally friendly reaction. Therefore, present study aimed to employ an optimal procedure for the continuous synthesis of fructose stearate by using immobilised *Rhizomucor miehei* lipase (RML) in a packed-bed reactor. Briefly, lipase immobilization on chicken eggshells was conducted and characterized using Transmission Electron Microscopy (TEM) and Brunauer-Emmett-Teller (BET) analysis. Subsequently, the screening of enzyme loading was performed. Response Surface Methodology (RSM) based on central composite design (CCD) was applied to optimize the temperature, flow rate and substrate molar ratio. The immobilisation efficiency on eggshells was 63.64%. After immobilization, the BET surface area, total pore volume and pore diameter of the eggshells were reduced to 1.0714 m²/g, 1.003 x 10⁻³ cm³/g and 3.7449 nm, respectively. Furthermore, both BET analysis before and after immobilization revealed that the pore structures of eggshells were classified as Type II isotherm. From preliminary study, enzyme loading of 1.5 g immobilized lipase was selected as the optimum enzyme loading. The quadratic model in RSM analysis was validated to predict the optimum conditions. A maximum of fructose stearate concentration as high as 7.252 x 10⁻¹ mol/L obtained at a better condition of 37.47°C under a flow rate of 0.074 ml/min and 2.82:1 substrate molar ratio of fructose to stearic acid. This work has pronounced the eggshell is as a potential carrier for RML immobilization with ability to be used in packed bed reactor to synthesis fructose stearate.

1. Introduction

Sugar esters are attractive and biocompatible surfactants which possess excellent stability, detergency, foaming, emulsifying and dispersing effects, turning it into one of the most versatile process chemicals [1]. In synthesis of sugar ester, the lipase-catalyzed reaction theoretically inserts a fatty acid into a specific position of the sugar. Lipases have substrate specificity towards the acyl donors that are varying in carbon chain length. *Rhizomucor miehei* lipase (RML) is sn-1,3 specific lipase in which its sn-1,3 position is usually held by unsaturated fatty acids, such as oleic acid, palmitic acid and stearic acid [2]. Adsorption of lipase on hydrophobic eggshells support occurs surrounding the active center at lower ionic strength if compared to other enzymes. Lipase will recognize the supports as a lipid/water interface [3]. During hydrophobic adsorption, lipases are strongly absorbed on the interface of hydrophobic supports and switch the conformational equilibrium of lipase towards the stabilized open form [4]. Enzyme in its immobilized form has many favorable qualities, including enhanced stability towards extreme environment, convenient handling, ease separation of products, and efficient recovery and reuse [5-6].



In the present study, enzymatic esterification of fructose and stearic acid by immobilized RML was conducted in packed bed reactor (PBR). PBR enables the reaction to run at higher enzyme loading and shorter diffusion pathway than that of the batch conditions, leading to higher conversion yield and higher rate of reaction per unit of enzyme [7]. Furthermore, reaction under continuous mode via PBR can increase the automated operation and surmount a problem of the long-time reaction in batch approach due to large amount of downtime to be scheduled for emptying, cleaning, and filling [8].

Hence, this study was carried out to study the efficiency of immobilization approach using eggshells by means of surface and porosity values from Brunauer-Emmett-Teller (BET) method. The aim was also to obtain optimum conditions of temperature, flow rate and substrate molar ratio of fructose to stearic acid for enzymatic synthesis of fructose stearate using immobilized enzyme in packed bed reactor via response surface methodology (RSM).

2. Experimental

Chicken eggshells were collected from the hawker stall around Universiti Sains Malaysia (Penang, Malaysia). Commercial *Rhizomucor miehei* lipase (RML) was purchased from Sigma-Aldrich and other chemicals used throughout the study were purchased from Merck, QRec, R&M Chemical and Sigma-Aldrich.

2.1. Pre-treatment of chicken eggshells

Prior to lipase immobilization, the chicken eggshells were pre-treated by using the optimized conditions obtained from preliminary studies done by Salleh et al. (2016). All dried eggshell was sieved with 63 μm pore size.

2.2. Lipase immobilization into chicken eggshells

Using adsorption technique, RML was immobilized on pre-treated eggshell. About 67 % (v/v) of lipase in phosphate buffer (50 mM, pH 7) was loaded into the flask along with 10 g of eggshell. The immobilization was carried out at 37 °C and 200 rpm for 1 hour and later being stored at 4°C for 16 hours after incubation. Unbound lipase was removed by washing with phosphate buffer [9]. Immobilized lipase was checked for its enzyme activity using tributyrin method [3]. The size and morphology of eggshell before immobilization was determined by transmission electron microscopy (TEM). Surface characterization for both eggshell powder before and after immobilisation was carried out at liquid N₂ temperature of 77K using Micromeritics ASAP 2020. The specific surface area of samples *SBET*, was determined using multipoint Brunauer-Emmett-Teller (BET) method. The Barrett-Joyner-Halenda (BJH) method was used to measure pores' volume, *V_{pore}* and the pore diameter distribution [10-12].

2.3. Continuous synthesis of fructose stearate using packed bed reactor

The PBR system which comprised of a cylindrical glass column (10 mm i.d. × 90 mm length) was filled with starting mixture of fructose and stearic acid well dissolved in ethanol (0.5 M to 1.0 M) in a feeding tank incorporated with immobilized RML. A re-circulating water bath was employed to maintain the desired temperature and by-pass valve was required to lower the pressure of the reactor system, thus allow the substrate to flow back to the feed tank. Range of conditions (temperature, flow rate and substrates molar ratio of fructose to stearic acid) obtained from preliminary was further optimized using response surface methodology. Design Expert 7.0.0 software was used to design and analyze the experimental data. After factors screening in preliminary study, significant parameters were selected to undergo face centered central composite design (CCD) in response surface methodology to determine the optimum condition for producing the highest concentration of fructose stearate. The experimental sequence for a total of 15 experiment runs suggested by software was randomized to reduce the effects of uncontrolled factors.

2.4. High-performance liquid chromatography

The quantitative analysis of residual fructose concentration was performed by Shimadzu high performance liquid chromatography (HPLC) equipped with Thermo Scientific Hypersil™ APS-2 HPLC Columns with mobile phase mixture of acetonitrile and deionized water, 85:15 by volume at a flow rate of 1.0 ml/min. The sample volume of 20 µL was injected into the HPLC system and eluted at 35 °C. The concentration of fructose stearate was calculated as:

$$\text{Concentration of fructose stearate} = (X_0 - X_t)/X_0 \times 100 \quad \text{Eq. 1}$$

where X_0 is the fructose concentration at the beginning of reaction; X_t is the residual fructose concentration in the mixture at the end of the reaction.

One-way analysis of variance (ANOVA) was employed to evaluate the reliability and validity of the results obtained. The results are considered as statistically significant if the *p-value* < 0.05.

3. Results and discussion

The TEM images of eggshell powder before immobilization shown in Figure 1 illustrated that eggshell powder was made up of mixture of irregular rhombohedral and quasi-spherical shapes. Similar observation had been reported by Rahman *et al.*, (2014) for eggshell nanopowder with the particle size in the range of 400 nm to 900 nm. The eggshell powder also exhibited its highly agglomerated nature that revealed many bright stripes, known as the ordered crystal lattice fringes which can be observed in various directions. In general, TEM analyses reflected high degree of crystallinity of eggshell powder. The results further support that the eggshell has high mechanical strength that makes it a suitable carrier in enzyme immobilization [12-14].

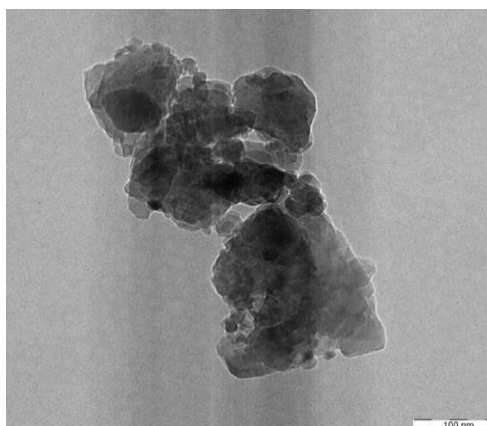


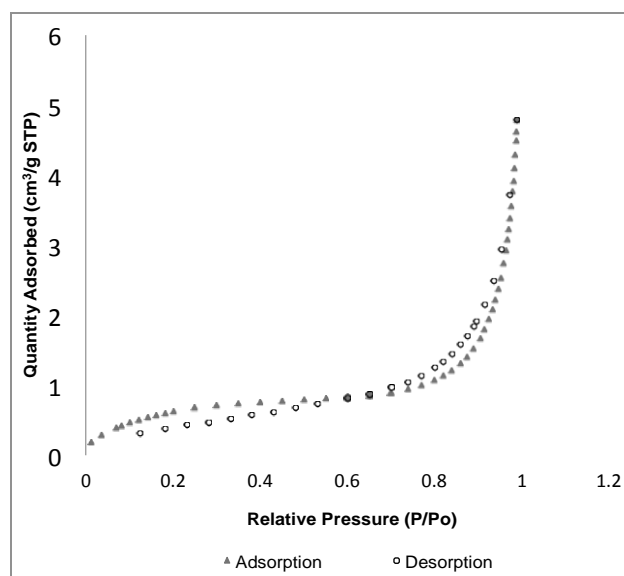
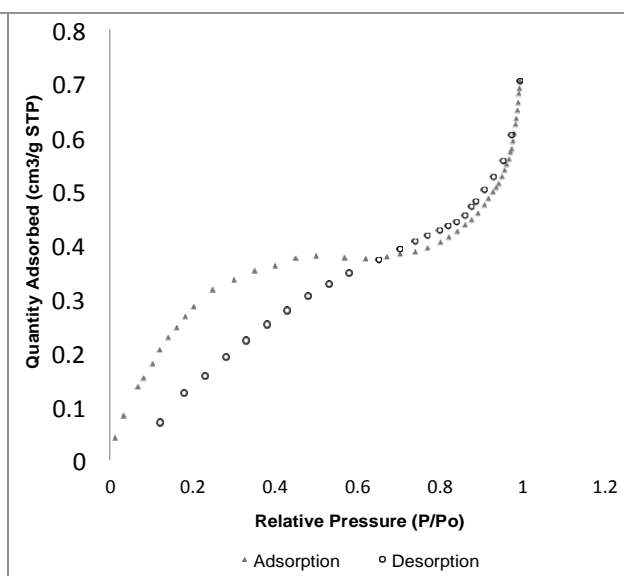
Figure 1 . TEM image of eggshell powder before immobilisation at 50 K magnification

From Table 1, BET analysis showed that surface area decreases from 2.1364 m²/g before lipase immobilization to 1.0714 m²/g after immobilization. There was also a significant decrease in the average pore diameter of the eggshell after immobilization. Reduction of eggshell powder pore volume of eggshell also observed after immobilization. The huge reduction found in surface area, pore diameter and pore volume indicated that the enzyme was successfully adsorbed on eggshell in which was similar as the finding by Rodrigues *et al.*, (2008) in the immobilization of *Candida antarctica* lipase on activated carbon [15]. From tributyrin assay conducted, the enzyme activity before and after immobilization were also determined and a high enzyme immobilization efficiency of 63.64% was obtained (result not shown). Upon immobilization, RML filled up some spaces of the pores and destroyed other pores simultaneously, constructing new pores with various sizes and irregular shapes, resulting in the decrease in surface area, pore diameter and pore volume [16].

Table 1 . The BET surface area, pore diameter and pore volume eggshell powders before and after enzyme immobilisation

	Surface area (m ² /g)	Pore diameter (nm)	Pore volume (cm ³ /g)
Before immobilization	2.1364	11.8392	6.323 x 10 ⁻³
After immobilization	1.0714	3.7449	1.003 x 10 ⁻³

Physical characterization of eggshell powder before and after RML immobilization was carried out to describe the support capacity or affinity for RML. Figure 2 and 3 showed the N₂ adsorption–desorption isotherm test results to determine the surface area, pore volume and pore diameter of the eggshells. According to IUPAC, both the adsorption isotherms for before and after immobilization were classified to Type II isotherms which were frequently obtained when adsorption takes place on nonporous or macroporous materials. The desorption hysteresis loop occurred in Figure 3 was attributed to the co-existence of intrinsic micropores and inter-crystalline mesoporous structure in eggshell. By comparing Figure 2 and Figure 3, the quantity of nitrogen adsorbed also decreased after lipase immobilization which implies that there was fewer number of pores available after immobilization as most of the pores were occupied by the lipase [17].

**Figure 2.** Nitrogen adsorption-desorption isotherms of eggshell powder before**Figure 3.** Nitrogen adsorption-desorption isotherms of eggshell powder after immobilization.

Experimental data obtained was fitted into quadratic model and from the subsequent analysis of variance (ANOVA) test, concentration of fructose stearate was presented in term of actual factors as the following:

$$Y = -1.87 + 0.10X_1 - 10.09X_2 + 0.99X_3 + 0.35X_1X_2 - 6.27E^{-3}X_1X_3 - 0.46X_2X_3 - 1.69E^{-3}X_1^2 - 14.37X_2^2 - 0.11X_3^2 \quad \text{Eq. 2}$$

Where X_1 is the temperature (°C), X_2 is the flow rate (ml/min) and X_3 is the substrate molar ratio of fructose to stearic acid.

Response surface plots using RSM exhibit function of two factors at a time whilst other factors are remained constant. These surface plots are useful in comprehending interaction effects between factors and predicting the yield response for different combination of factor levels. The interaction terms AB (temperature vs flow rate), AC (temperature vs substrate molar ratio of fructose to stearic acid) and

BC (flow rate vs substrate molar ratio of fructose to stearic acid) were observed to have significant effect towards concentration of fructose stearate. For instance, Figure 4 (a) displays the surface plot of an increase in both temperature and flow rate would negatively affect the concentration of fructose stearate. Both the temperature and flow rate have positive effects on the esterification at lowest values in the range of both variables respectively. The temperatures higher than 35°C was found exceeded the optimal threshold of RML, which might lead to unwinding of immobilized RMLs and progressively inactivated the immobilized RML. Previous study also suggested the breaking of intramolecular hydrogen bonds, van der Waals forces, hydrophobic and ionic interactions that gradually destabilizes and disrupt the RML tertiary structure beyond the optimum temperature, thus probably resulted in a lower product concentration [18]. As shown in the Figure 4 (b), a higher concentration of fructose stearate was reached at a moderate temperature (35 °C) along with highest substrate molar ratio of fructose to stearic acid (3:1). An excess of sugar contributes to an enhanced product yield due to the sugar being a lyoprotectant compound was able to reduce the water activity of the medium, favoring the esterification reaction [19-21]. The optimized conditions (temperature = 37.47°C, flow rate = 0.74 ml/min and substrate molar ratio of fructose to stearic acid = 2.82:1) were given by the model by using numerical optimization tool with high product concentration of $7.252 \times 10^{-1} \pm 0.025$ mol/L.

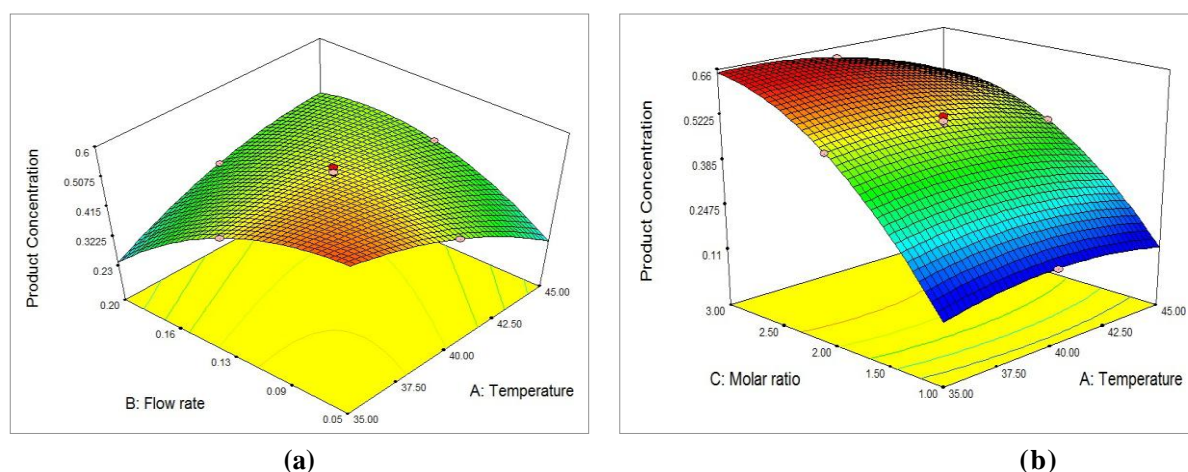


Figure 4 . (a) 3D surface of fructose stearate concentration as a function of temperature (°C) and flow rate (ml/min) at fixed substrate molar ratio of fructose to stearic acid (2:1), **(b)** 3D surface of fructose stearate concentration as a function of temperature (°C) and substrate molar ratio of fructose to stearic acid at fixed flow rate (0.13 ml/min).

4. . Conclusion

A high enzyme immobilization efficiency (63.64%) conducted after the immobilization agreed with the findings from BET, where the adsorption isotherms analysis showed that the Brunauer–Emmett–Teller (BET) surface area, total pore volume and pore diameter of the eggshells decreased significantly after immobilization. Decrement in these surface and porosity values suggested that the lipase was successfully immobilized on the treated eggshells. Subsequently, packed-bed reactor (PBR) was successfully employed in the continuous enzymatic esterification of fructose and stearic acid to produce satisfactory concentration of fructose stearate 7.252×10^{-1} mol/L at optimized conditions of 37.47°C, at flow rate of 0.074 ml/min, using 2.82:1 substrate molar ratio of fructose to stearic acid.

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