World Applied Sciences Journal 11 (4): 401-407, 2010
ISSN 1818-4952
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Green Synthesis of Lauryl Palmitate *via* Lipase-Catalyzed Reaction

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Abstract: Enzyme catalysis is most attractive for the synthesis of fine organic compounds, which are difficult to prepare and to handle by conventional means. In this work, green synthesis of lauryl palmitate, a wax ester was successfully carried out by lipase-catalyzed esterification of palmitic acid and lauryl alcohol. In this study, commercial immobilized lipase from Candida antartica (Novozym 435) was used as biocatalyst. The effect of various reaction parameters were optimized to obtain a high yield of wax esters. The optimum condition to produce lauryl palmitate was at reaction time (RT); 10 min, temperature (T); 40°C, amount of enzyme (E); 0.4 g, molar ratio of substrate (N); 2:1 and organic solvents of log P>3.5. The product was then subjected to characterize using Fourier-transform infrared spectroscopy (FT-IR) and Gas chromatography spectroscopy (GC) to ensure the purity of product obtained. Analysis of yield showed that at optimum condition, lauryl palmitate was produced in short time with high purity, >90%.

Key words: Lauryl palmitate · Immobilized lipase · Esterification · Wax ester · Green synthesis

INTRODUCTION

Green chemistry involves the design of chemical products and processes that reduce or eliminate pollution. Biocatalysts are the one of the fundamental pillars of green chemistry, the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances [1]. Therefore, biocatalysts are going to be one of the important tools for implementing the green chemistry principles [2]. Synthesis of wax ester using enzyme is considered to satisfy the concept of green chemistry due to its environmentally friendly process. Strategic use of enzyme in organic chemistry is the main focus to produce wax ester in high yield and improve the quality and purity of product obtained.

Wax esters consist of long-chain fatty acids esterified to long-chain alcohols are an important class of fine organics compounds. They are produced by a variety of plants, animals, fungi and bacteria and serve a variety of biological function. Wax esters are major constituents of beeswax and plant such as jojoba [3]. Naturally occurring wax esters are chemically diverse. Most of esters of primary alcohols through esters of secondary alcohols can be a major component of the culticular lipids of melanopline grasshoppers [4].

Wax esters are usually harder, less greasy and more brittle than fats. The compounds have many potential applications due to their excellent wetting behavior at interfaces and a non-greasy feeling when applied on skin surfaces. Physical properties of wax esters are very important from the cosmetic formulator point of view [4]. Therefore the physical properties such as melting point, viscosity, specific gravity and reflective index were measured for pure wax ester. For example the melting points of long-chain wax esters (e.g. oleyl palmitate, oleyl oleate) are below 0°C while the boiling points are up to 300°C [5].

Wax esters derived from natural sources such as jojoba oil and sperm whale oil have been widely used in the cosmetics and lubricant industries. For cosmetics, wax ester is highly effective cleanser, conditioner, moisturizer and softener for the skin and hair [6]. Wax ester is also a superior lubricant in high-speed machinery, work tools and metal cutters because it promotes extension of the life of all moving parts. Other examples of the commercial application of waxes are in detergent and polish used for the cleaning and protection of surfaces.

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Since the naturally occurring wax esters are expensive and limited in access, the need to synthesize the compound has grown. Wax ester has been synthesized via chemical [7,8] and enzymatic reactions [9,10]. However, enzymatic synthesis is most preferable due the uses of lower temperature than chemical synthesis and a single product is produced at a higher yield. Moreover, the esters produced through this process can be considered close to 'natural' and can potentially satisfy the recent consumer demand.

Biotechnological production of wax esters with lipases has recently received greater consideration and is undergoing a rapid development. The use if lipases (EC 3.1.1.3) to catalyze reactions in organic solvent are well documented [11,12]. One of the driving forces for this research is the possibility of preparing synthetic wax esters which resemble naturally occurring waxes of commercial interest. The specificity of the lipase to form an ester bond permits control of specific reactions which will also increase yield [13].

A better understanding of various reaction parameters affecting the esterification with long-chain substrates is essential for possible large scale synthesis. This paper shows a high performance green synthesis of lauryl palmitate, a wax ester by enzymatic synthesis route using an immobilized lipase from *Candida antartica* (Novozym 435) in organic solvents.

MATERIALS AND METHODS

Materials: Novozym 435 as 10,000 PLU (from *Candida antarctica* lipase immobilized onto macroporous acrylin resin) was received from Novo Nordisk (Denmark). Lauryl alcohol (purity, 98%) and palmitic acid (purity, 90%) were obtained from Acros Organics (USA). All other reagents were of analytical grade and used as received.

Methods

Lauryl Palmitate Synthesis: The reaction system consisted of 2.0 mmol of palmitic acid, 4.0 mmol of lauryl alcohol, 2.0 mL of hexane and 0.3 g of Novozym 435. The mixture was incubated at 37°C using a horizontal waterbath shaker. The agitation speed was set at 150 rpm and the reaction mixture was continuously reacted for 180 minutes. The reaction was then terminated by dilution with 7.0 mL of ethanol/acetone (1:1 v/v).

Identification of Reaction Product: The product was periodically tested using thin layer chromatography, TLC (Merck type DC-plastic folein Keisel gel 60 F_{254}); Fourier Transform Infrared Spectroscopy, FTIR (Perkin Elmer, model 1650) and gas chromatography, GC (Hitachi, model G 3000). Preliminary detection and identification of reaction product were facilitated by TLC. The developing solvent system used was hexane:ether anhydrous (7:3,v/v). Further identification was carried out by FTIR. Final identification was performed by GC instrument equipped with a medium polar capillary column RTX-65-TG (Restek Corporation, USA). Helium was used as carrier gas at 1.0 mL/min. The initial column temperature was 150°C and the final temperature was set at 300°C. The zone temperature for injector and detector were set at 330°C and 350°C. The temperature was increased at 10°C per minute to 280°C and then it was increased at 5°C per minute to the final temperature.

Analysis of Reaction Product: Determination of the percentage conversion of lauryl palmitate (%): The percentage conversion (%) of lauryl palmitate was measured by determining the remaining unreacted fatty acids in the reaction mixture by titration with 0.1 M NaOH in an automatic titrator (Methrom, Switzerland). All the samples were assayed in triplicate and the experiment was repeated twice.

$$\text{Conversion of wax ester (\%)} = \frac{\text{Volume of NaOH used (without enzyme)-Volume of NaOH used (with enzyme)}}{\text{Volume of NaOH used (without enzyme)}} \times 100$$

Effect of Reaction Time: The effect of time in the wax ester synthesis was investigated by varying reaction periods (5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 minutes) while fixing the other conditions. The percentage conversion was determined as described above.

Effect of Various Organic Solvents: The reactions were studied using various organic solvents (acetone, log P=-0.23; chloroform; log P=2.0, toluene, log P=2.5; hexane, log P=3.5; n-heptane, log P=4.0; nonane, log P=5.1) while fixing the other conditions. Percentage conversion of wax ester was determined as described above.

Effect of Temperature: The reaction mixtures were incubated at various reaction temperatures (30, 37, 40, 50, 60, 70 and 80°C) while fixing the other conditions. Percentage conversion of wax ester was determined as described above.

Effect of Amount of Enzyme: The reactions were studied using various amount of enzyme (0.1, 0.2, 0.3, 0.4 and 0.5 g) while fixing the other conditions. Percentage conversion of wax ester was determined as described above.

Effect of Molar Ratio: The reaction mixtures were reacted with different molar ratio of substrates, mmol lauryl alcohol/mmol palmitic acid (molar ratio = 1:1, 2:1, 3:1, 4:1 and 5:1) while fixing the other conditions. Percentage conversion of wax ester was determined as described above.

RESULTS AND DISCUSSION

Identification of Reaction Product: Products from esterification reaction between palmitic acid and lauryl alcohol catalyzed by Novozym 435 were firstly monitored by TLC. The presence of the lauryl palmitate, palmitic acid and lauryl alcohol were detected as brown spots when visualized by an iodine reagent. Further identification was carried out by FTIR showed a characteristic absorption of ester bond at 1732 cm^{-1}. Final identification of reaction mixture was performed by GC by comparing the ester with a known authentic standard. The profile of GC chromatogram showed major peak of lauryl palmitate presence at retention time of 12.607 minutes and lauryl alcohol at 3.606 minutes.

Effect of Reaction Time: The time course is a good indicator of enzyme performance and reaction progress. It can pinpoint the shortest or adequate time necessary to obtain a good yield and minimize the process cost. Figure 1 shows the reaction time profile for the esterification reaction of palmitic acid and lauryl alcohol catalyzed by Novozym 435. Experiments at nine different times were carried out to analyze its influence on the esterification reaction.

The rate of reaction and overall increased with increasing reaction time. The result suggested that the reaction proceed rapidly within 10 minutes (94.7 %). There after, the percentage conversion remains constant up to 150 minutes (94.9%) and decreased at 180 minutes (72.2%). This was due to the production of water molecule, which had achieved the equilibrium state. As the reaction proceeds, the substrates concentration decreased which led to a fall in the degree of saturation of the enzyme with substrate [14].

According to Trubiano *et al*., [15] the presence of water has only an unfavourable effect on the equilibrium conversion. Since water is one of the products in esterification reactions, it can promote the reverse hydrolysis reaction in the process. It is important to remove water during the course of the reaction to achieve a high product yield. Several methods have been reported in the literature for this purpose including membrane reactors, vaporization, vacuum evaporation, air drying,

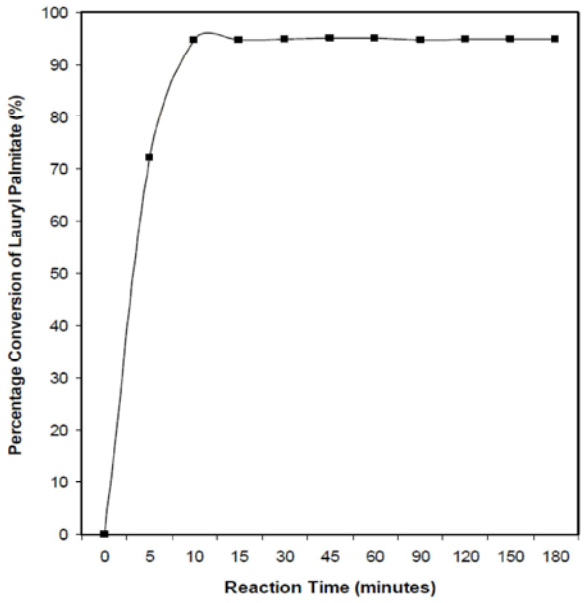

Fig. 1: Effect of reaction time on the synthesis of lauryl palmitate. Reaction condition: temperature; 37 °C, molar ratio of substrate (mmol lauryl alcohol/mmol palmitic acid); 2:1, amount of enzyme; 0.3 g and agitation speed of 150 rpm.

reactive distillation, as well as the use of molecular sieve as media of eliminating water from the reaction mixture. However, this water removal method which is efficient for shifting the equilibrium conversion to completeness was not combined with the benefit of the high temperature stability of the catalyst.

Effect of Various Organic Solvents: During the last decade, the tremendous potential of enzymes as catalyst for chemical processes in non-aqueous environments has been well recognized [16]. The use of biocatalyst in organic solvents offers many advantages over using pure water, such as the increase in solubility of poorly water-soluble organic substrates, avoiding unwanted side reactions and degradation of common organic reagents. It also has the ability to shift the thermodynamic equilibrium of many processes to the synthetic way, thus favouring product recovery [17]. The polarity of the organic solvents employed for the esterification reaction is known to affect the enzyme activity [18]. The log P value of the solvents is widely used parameter to describe solvent polarity and their possible effect on enzyme activity where P is the partition coefficient of a given solvent between water and octanol in a two-phase system. It has been reported that lipase-catalyzed reaction was favored in non-polar solvents with log P>2 [19].

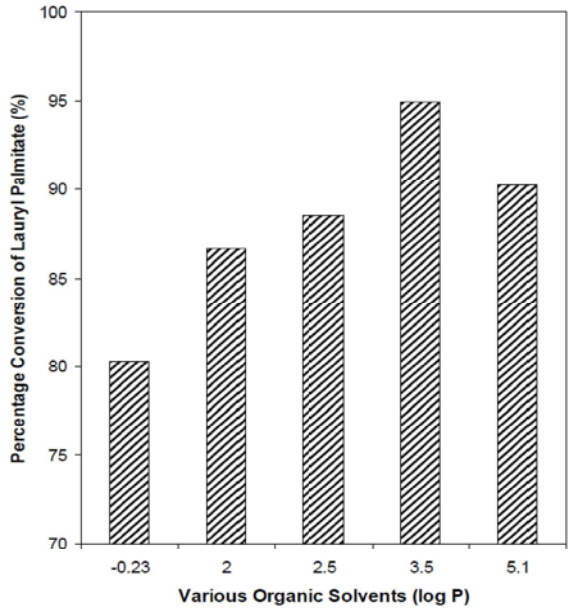

Fig. 2: Effect of various organic solvents on the synthesis of lauryl palmitate. Reaction condition: reaction time; 60 minutes, temperature; 37 °C, molar ratio of substrate (mmol lauryl alcohol/mmol palmitic acid); 2:1, amount of enzyme; 0.3 g and agitation speed of 150 rpm.

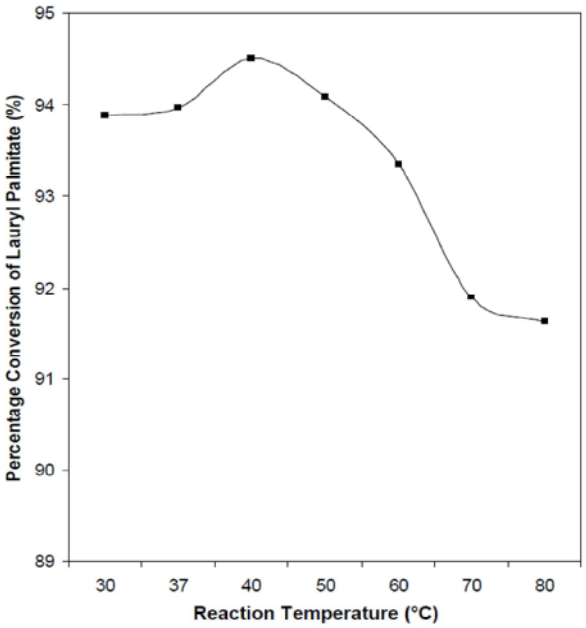

Fig. 3: Effect of temperature on the synthesis of oleyl oleate. Reaction condition: reaction time; 60 minutes, molar ratio of substrate (mmol lauryl alcohol/mmol palmitic acid); 2.1, amount of enzyme; 0.3 g and agitation speed of 150 rpm.

Figure 2 depicts the effect of various organic solvents on the synthesis of lauryl palmitate. Novozym shows lowest activity in polar solvents as occurred in acetone (80.3 %) and chloroform (86.7 %), with log P=-023 and log P= 2.0, respectively. According to Hari Krishna *et al*. [18] this was probably due to the following reasons, (i) they enhanced dissociation of weak organic acid and built up the net proton concentration in the homogeneous phase, which led to the reverse reaction (hydrolysis) and (ii) they might also strip off the essential water around the enzyme present as microaqueous layer thereby, affecting the active conformation of the enzyme and denaturing the biocatalyst. Such solvent having higher log P values have indeed been recommended for optimal lipase activity and stability [20]. More specifically, hexane has been reported to be the optimal solvent for lipase-catalyzed reactions [21]. This was an agreement with our finding, whereby the best solvent for the enzymatic synthesis of lauryl palmitate is hexane (94.9 %) with log P=3.5.

Effect of Temperature: Changes in the reaction temperature can affect the activity and stability of the enzyme and thus the rate of reaction. Effect of temperature also can be apportioned to its effect on substrate solubility as well as its direct influences on the esterification reaction and the enzyme [22]. Figure 3 shows the influence of temperature on the esterification reaction within temperature range between 30°C – 80°C. Initially, the percentage conversion of lauryl palmitate increased with increasing temperature from 30°C (93.9 %) to 40°C (94.5 %). This due to the fact that, energy received from heat of higher temperature was used to increase the frequency of collision between the molecules. The percentage conversion was slightly constant at maximum range of 40°C - 50°C and slightly decreased at 60°C (93.3 %). High percentage yield is also attributed to the enzyme immobilization, which has conferring stability to the lipase. Novozym 435 is a lipase from *Candida antartica* immobilized on a microporous acrylic resin is a tolerance product with maximum activity in the high temperature. The percentage yield was sharply dropped at 70°C (91.6 %). This is probably because beyond a critical temperature, the lipase may have been deactivated.

The results are similar in the findings by most reviewed papers that Novozym 435 was optimally used at between 40°C - 60°C [23]. The conversion decreased slightly after 60°C probably caused by the vibration and movement of the enzyme molecule, which would affect the hydrogen bonds and other bonds in the lipase structure. Hence, the enzyme molecule will unfold and alter its tertiary and quaternary structure or globular structure

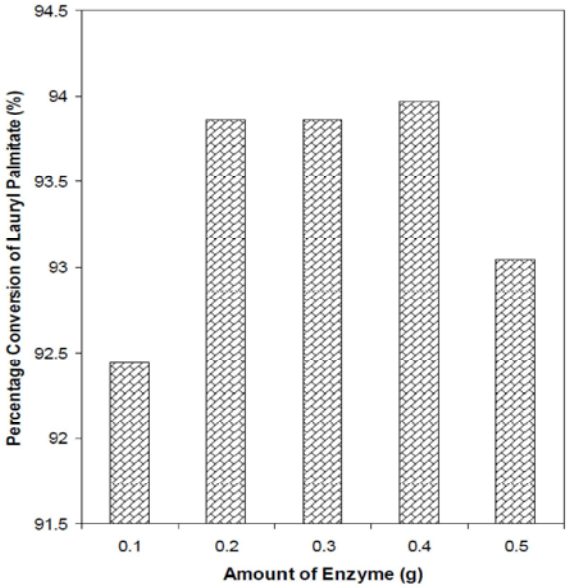

Fig. 4: Effect of amount of enzyme on the synthesis of lauryl palmitate. Reaction condition: reaction time; 60 minutes, temperature; 37 °C, molar ratio of substrate (mmol lauryl alcohol/mmol palmitic acid); 2:1 and agitation speed of 150 rpm.

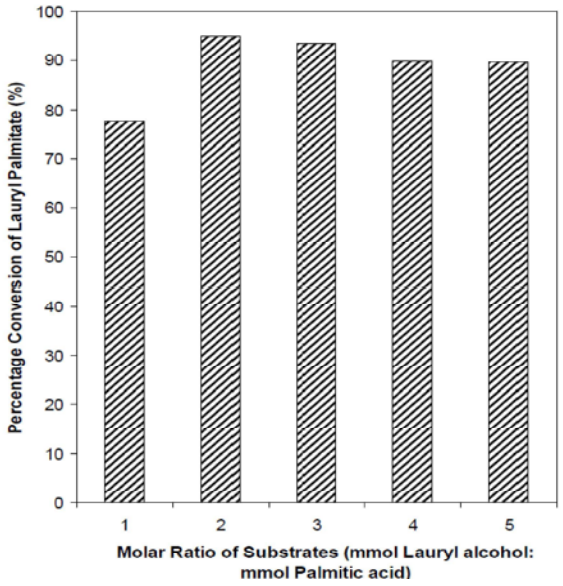

Fig. 5: Effect of molar ratio of substrate on the synthesis of lauryl palmitate. Reaction condition: reaction time; 60 minutes, temperature; 37 °C, amount of enzyme; 0.3 g and agitation speed of 150 rpm.

(three-dimensional conformation). Consequently the catalytic power of lipase will be reduced, because denaturation process has occurred. At high temperature, a higher water evaporation rate may shift the position of the equilibrium to the product side and increases the yield. It also promotes collisions between enzyme and substrate molecules to result in accelerated rates of reaction. According to Trubiano *et al*. [15] immobilization on lipase will alter its sensitivity to temperature. Change in the reaction temperature can affect the activity and stability of the enzymes and thus the rates of reaction. On the other hand, at low temperature the solubility is reduced with the subsequence high viscosity causing mass transfer limitations, retarding reaction rate and lowering final yields.

Effect of Amount of Enzyme: From an applied point of reaction, the substrate concentration should be as high as possible to obtain a higher degree of esterification. Simultaneously, the amount of immobilized enzyme used should be as low as necessary to obtain the desired product [24]. Amount of enzyme plays a crucial role in any biocatalytic process especially in large scale production. Its influence on the reaction was therefore assessed to facilitate determination of the minimal amount necessary for achieving goods yield. The influence of varying amount of enzyme corresponding to 0.1-0.5 g on the esterification reaction of palmitic acid and lauryl alcohol was shown in the Figure 4. The percentage conversion had increased from 0.1 g (93.5 %) to 0.2 g (95.4 %) and kept constant when the amount of enzyme increased to 0.3 g (95.1 %). Amount of enzyme at 0.2 g (95.2 %) to 0.4 g (95.4 %) were sufficient to catalyze this esterification reaction.

The result shows an excess of enzyme amount did not contribute to the increase in the percentage conversion. This is also similar with what was reported by Torres *et al*., [25] and sometime it would decrease the yield of the product. At saturation point, all the substrates are bound to the enzyme and added enzyme molecule could not find any substrate to serve as a reactant. If we increase the enzyme concentration above this point, the reaction will slightly decline due to the s teric hindrance produced by excessive enzyme. In such condition, the reaction can increase by increasing the substrate concentration, because substrate was the limiting factor. In esterification reaction, the amount of enzyme will influence the total reaction times, which are required to achieve desired conversion [26]. According to Aracil *et al*., [27] the most significant main effect in enzymatic esterification reaction is the initial catalyst concentration. This effect has positive influence on ester yield.

Effect of Molar Ratio of Substrates: Relative proportions of the various substrates in a reaction mixture define the physical and chemical properties of a reaction system. High acylation yields can be achieved with high substrate concentrations in the reaction media. The effect of molar ratio of substrates on the esterification reaction is shown in Figure 5. The optimal molar ratio (mmol lauryl alcohol/mmol palmitic acid) was 2:1 (94.91 %). Increasing the mole ratio of lauryl alcohol to palmitic acid beyond this point (molar ratio = 2:1) would decrease the esterification activity. This observation may reflect the ability of the excess lauryl alcohol to distort the essential water layer from enzyme. At the same time, the excess of lauryl alcohol will hinder the interaction frequency between substrate and lipases. This may be due to the presence of high substrates concentration, the viscosity of the reaction mixture surrounding the enzyme molecule may be increased due to the increase in the alcohol leading to ineffective mixing of reactants and subsequent reduction in reaction rate [28].

As reported by Garcia *et al*., [29] for acid concentration larger than 0.2 mol/L, the esterification rate will increase with increasing the fatty acid concentration for any alcohol concentration. However, below 0.2 mol/L, when the acid concentration decrease in reaction rate for large molar ratios (7:1 and 10:1). This behavior indicates the presence of inhibition effects at different molar ratios. Others authors have reported a similar behavior that has been attributed to inhibition effects caused by the alcohol on the catalyst. Firstly, when alcohol is added to the system and due to its high polarity, it undergoes hydrophilic interactions with the aqueous boundary layer on the lipase surface causing modifications of the protein tertiary structure and therefore enzyme inhibition. On the other hand, inhibition phenomenon is given by the formation of binary complexes between the free enzyme and the alcohol (or ester) as well as the formation of inactive ternary complexes between the fatty acid or the ester and the enzyme acid-complex.

CONCLUSION

This work suggests that lauryl palmitate, a wax ester can be produced at a very high yield via green enzymatic reaction route. The elimination of long reaction time can save cost and this approach is becoming more significant to future industrial biotechnology application in specialty and fine chemicals industry.

ACKNOWLEDGEMENTS

This project was financed by a grant from Universiti Sains Islam Malaysia (Project No. PPPP(U)/2006).

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