Abstract—Nowadays, butyl acetate, a pineapple flavor has been applied widely in food, beverage, cosmetic and pharmaceutical industries. In this study, Butyl acetate, a flavor ester was successfully synthesized via green synthesis of enzymatic reaction route. Commercial immobilized lipase from Rhizomucor miehei (Lipozyme RMIM) was used as biocatalyst in the esterification reaction between acetic acid and butanol. Various reaction parameters such as reaction time (RT), temperature (T) and amount of enzyme (E) were chosen to optimize the reaction synthesis in solvent-free system. The optimum condition to produce butyl acetate was at reaction time (RT), 18 hours; temperature (T), 37°C and amount of enzyme, 25 % (w/w of total substrate). Analysis of yield showed that at optimum condition, >78 % of butyl acetate was produced. The product was confirmed as butyl acetate from FTIR analysis whereby the presence of an ester group was observed at wavenumber of 1742 cm$^{-1}$.

Keywords—Butyl acetate, immobilized enzyme, esterification, flavor ester, green synthesis

I. INTRODUCTION

GREEN chemistry is the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances. In the last decade, green chemistry has been recognized as a scientifically based new approach for environmental protection. From feedstock to solvents, to synthesis and processing, green chemistry actively seeks ways to produce materials in a way that is more benign to human health and the environment [1]. Biocatalyst is one of the fundamental pillars and satisfies the green chemistry concept. Moreover, biocatalysts are generally more efficient than chemical catalysts. For example, biocatalysts suffer from instability and substrate product inhibition. In addition, the process is also environmentally friendly compared to chemical production method.

Nowadays, there is an increasing demand for natural flavors. The worldwide market for natural “green notes” is estimated to be 5 – 10 metric tons per year [2]. These flavors are used in foods, cosmetics and pharmaceutical industries. Traditionally, the flavors had been isolated from natural sources or produced by chemical synthesis. However, several problems were encountered for natural extract out of natural flavors due to the high production cost and limited supply to support worldwide demand. While flavor produced by chemical synthesis caused the bad side effect due to the use of hazardous chemicals. Moreover, other problems uncounted are the separation process, high toxicity and inflammability [3].

Flavor ester is a short chain ester derived from a reaction between short chain acid and alcohol. The process involved is an esterification reaction whereby alcohol reacted with carboxylic acid with the elimination of water. Ethyl, isobutyl, amyl and isoamyl acetates were frequently used as components in flavoring, and isopropyl, benzyl, octyl, geranyl, linanlyl and methyl acetates were important additives in perfumes. Flavor esters were generally produced by free and immobilized lipases from various sources in organic solvents [4]. Until now, there are several research have been carried out in synthesizing flavor esters such as ethyl valerate, hexyl acetate [2], isoamyl acetate [5] and benzyl acetate [6].

Biotechnology appears to be attractive in the use of various ester preparations under milder conditions and may be given the natural label of the product. This includes three principal techniques of biosynthesis that can be distinguished in the following ways: use of enzymes; use of microorganisms; plant cells and culture of tissues. Among those, application of enzymes is the most frequently used technique of biosynthesis. Flavor esters have been generally produced by lipases from various sources in organic solvents. However solvent toxicity and high production costs are the major problems in most reactions [4]. To facilitate these, solvent free system offers more advantages, where the absence of solvents facilitates downstream processing, thus offering significant cost saving and minimizing environmental impact.

There are a number of publications regarding the enzymatic synthesis of flavor esters in nonconventional media, particularly in the presence of solvents [2, 5-6, 7-8]. However, the number of articles that discussed the enzymatic synthesis in solvent free systems is considerably low due to the low production yield. Therefore a study has to be embarked in order to determine the main effect governing enzymatic synthesis of flavor ester. The presence paper focuses on the synthesis of butyl acetate, a pineapple flavor using immobilized lipase Rhizomucor miehei (Lipozyme RMIM) as biocatalyst using solvent free system. This is a preliminary study of synthesizing flavor ester before scaling up the reaction using an appropriate reactor system. The main objectives are to synthesize a flavor ester and to better understand the relationships between reaction variables (reaction time, temperature and amount of enzyme) and the yield of flavor (butyl acetate).
II. MATERIALS AND METHODS

A. Materials

Lipozyme RMIM (from Rhizomucor miehei) immobilized onto macroporous anion exchange resin manufactured by Novozenzyme A/S (Denmark) is used as received without pretreatment. Substrate as acetic acid was obtained from Merck KgaA (Germany) and butanol from Fisher Scientific UK Limited (UK). All other reagents used were of analytical grade and used as received.

B. Synthesis of Butyl acetate

The reaction system consisted of 50 mmol of acetic acid, 50 mmol of butanol and 5% (w/w of total substrates) of Lipozyme RMIM. All substrates were placed in screw-capped vials and the reaction assayed in triplicates. The reaction mixtures were incubated at 37°C in a horizontal water bath shaker with shaking speed of 150 rpm and continuously reacted for 24 hours. The product of butyl acetate was determined by titration method with 0.1 M of NaOH using modern automatic titrator (Metrohm, Switzerland).

C. Identification of reaction product

Preliminary detection of butyl acetate was tested using Thin layer chromatography, TLC (Merck type DC-plastic folein Keisgel 60 F254). Hexane and diethyl ether were used as developing solvent in ratio of 9:1 (v/v). Then, the synthesized butyl acetate was analyzed using spectroscopy methods of Fourier transform infrared spectroscopy (FTIR) to confirm the product obtained.

D. Analysis of reaction product

The yield of butyl acetate produced was expressed as a percent (%) of converted acetic acid as compared to the total fatty acid in the reaction mixture using the following equation:

\[
\text{Conversion (\%)} = \frac{\text{Vol}_{\text{酯酸}} \ (w/o \text{enzyme}) - \text{Vol}_{\text{酯酸}} \ (with \text{enzyme})}{\text{Vol}_{\text{酯酸}} \ (w/o \text{enzyme})} \times 100\% \ (1)
\]

In this study, the butyl acetate produced was presented in term of relative percentage conversion (%).

E. Effect of reaction time

The reaction mixture was incubated at 37°C continuously using a horizontal water bath shaker with continuous shaking speed of 150 rpm. The reaction was assayed at various reaction time intervals (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours). The percentage conversion was determined as described earlier.

F. Effect of temperature

The reaction mixture was incubated at various temperatures (30, 37, 45, 50, 55, 60, 70 and 80°C) using a horizontal water bath shaker with continuous shaking speed of 150 rpm. The percentage conversion was determined as described earlier.

G. Effect of amount of enzymes

The reaction mixture was catalyzed by varying amount of enzymes (5%, 10%, 15%, 20%, 25% and 30% w/w) of Lipozyme RM at optimum temperature and reaction time. The percentage conversion was determined as described earlier.

III. RESULTS AND DISCUSSIONS

A. Identification of reaction products

Preliminary detection of butyl acetate was carried out using thin layer chromatography (TLC) analysis. Hexane and diethyl ether were chosen as mobile phase in the analysis with a ratio of 9:1 (v/v). The pure synthesized butyl acetate, unreacted acid and alcohol were detected as brown spots on the silica gel plate when visualized by an iodine reagent [9]. RF values for each spots are calculated by comparing the distance of solute and the distance of mobile phase. The particular distance for each substance spot was measured from the starting point of the substance spot. This analysis showed that the RF values for butyl acetate, acetic acid and butanol were 0.82, 0.57 and 0.44, respectively. The synthesized butyl acetate was analyzed using Fourier transform infrared (FTIR) spectroscopy. According to Fig. 1, a strong peak was observed at 1742 cm\(^{-1}\) (%T = 18.13%) which indicated the presence of ester-carbonyl group (C=O). Similar finding was reported by Mat Radzi et al. (2005) [9] in the synthesis of oleyl oleate, a wax ester whereby the ester bond was detected at wavenumber 1742 cm\(^{-1}\).

B. Effect of time on the esterification reaction

Time course studies insight into the performance of an enzyme as the reaction progress. Such progress curves help determine the shortest time necessary for obtaining good yields and so enhanced cost-effectiveness of the process. This is a very important factor especially in large scale processing to obtain high performance of production yield. The profile of production of flavor ester at various time intervals is presented in Fig. 2. Generally, the relative percentage conversion of butyl acetate was increased with increasing reaction time. The graph shows the relative percentage yield was increased gradually from 5 hours to 18 hours. The initial reaction acceleration was due to the production of water needed for active conformation during reaction which increases lipase activity [10]. After 18 hours of reaction time, the relative percentage of ester started to decrease. This may be due to the large amount of water produced during the reaction occurs. Similar finding was reported by Mat Hadzir and co-workers (2001) [11] in the synthesis of oleyl oleate by enzymatic alcoholysis reaction between triolein and oleyl alcohol.

C. Effect of temperature on the esterification reaction

The effects of temperature can be apportioned to its effect on substrate solubility as well as its direct influences on the reaction [12]. Effect of varying reaction temperatures on enzymatic synthesis of butyl acetate is shown in Fig. 3. Initially, the relative percentage conversion of ester was increased with increasing temperature from 30-40°C. However, at higher temperatures, the relative percentage of yield was gradually decreased from 40-80°C. This is probably due to beyond a critical temperature; the lipase may have been deactivated [13]. As shown in the Fig. 3, the highest relative percentage of ester was obtained at 37°C. The yield started to
DECREASE at 45°C to 81%, followed by 60% at 50°C and 30% at 80°C. Thermal inactivation of the enzyme might have occurred at a high temperature, reducing the conversion percentage [14]. Romero et al. (2005) [15] have observed that an increase in temperature above 50°C causes the conversion yield of isoamyl acetate synthesis to decrease even through the commercial provider recommended Novozym 435 is optimally to be used at 70°C. According to Abdul Rahim et al. (2005) [16] the decrease in yield may have been due to the denaturation of enzymes as a result of thermal disruption of bonds required for maintaining the active enzyme conformation.

**D. Effect of amount of enzyme on the esterification reaction**

From an applied point of reaction, the substrate concentration should be as high as possible to obtain a higher degree of esterification. Simultaneously, from economic point of view, the amount of enzyme used should be as low as necessary to obtain the desired product [17]. The influence of varying amount of enzyme on the esterification reaction of acetic acid and butanol is shown in Fig. 4. The result shows a slightly increase in the relative percentage conversion of ester when 10% (w/w) to 25% (w/w) of enzyme was used and drastically decreased at 30% (w/w) of enzyme was used. Torres and Otero (2001) [18] had also indicated that the use of large amount of enzyme could significantly increase the fraction of acyl donor molecules to form acyl-enzyme complexes. Moreover, their active sites would not be exposed to the substrate and remain inside the bulk of enzyme particles without contributing significantly to the reaction. According to Karra-Chââbouni et al. (2006) [2], in the presence of high amount of lipase, not all active sites are exposed to the substrates and resulted molecules of the enzyme tend to aggregate together. However, too small amounts of enzyme may have been insufficient for complete substrates conversion within the specified reaction period [19]. They also suggested that another factors also contribute to the lost of enzyme activity such as pH, reaction-medium and temperature. Foresti and Ferreira (2005) [20] also reported that agglomeration using immobilized lipases in a solvent-free system leads to aggregate formation and inhomogeneous enzyme distribution.
Three parameters were chosen to optimize the synthesis of lauryl palmitate, namely reaction time ($T$), reaction temperature ($E$) and amount of enzyme ($E$). The optimum yield of butyl acetate ($>78\%$) can be obtain under optimal condition at 18 hours of reaction time, temperature of 37°C and amount of enzyme of 25 % (w/w of total substrate). This work suggests that lauryl palmitate, a wax ester can be produced at a very high yield via green enzymatic reaction route.

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Fig. 4 Effect of amount of enzyme on the esterification reaction of acetic acid and butanol by Lipoyze RMIM. Reaction condition: reaction time; 18 hours, temperature; 37°C and agitation speed of 150 rpm.

IV. CONCLUSION

The production of isoamyl acetate using immobilized lipase from Rhizopus oryzae, a food grade fungus, was optimized by studying the effect of three parameters, namely reaction time, temperature and amount of enzyme. At optimal conditions, a high yield of butyl acetate ($>78\%$) was obtained under optimal conditions.

The findings of this study suggest that lauryl palmitate, a wax ester, can be produced at a very high yield via green enzymatic reaction route. Further investigations are needed to optimize the process for industrial application.