Provisional chapter

Solvent-Free Isoamyl Acetate Production via Enzymatic Esterification

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Additional information is available at the end of the chapter

Abstract

Isoamyl acetate is an organic compound which is mainly used as flavor additive in food industries. Traditionally, the food flavor has been produced by extraction from plants, followed by chemical synthesis route which then shifted to biocatalytic route due to consumer's awareness and inclination toward natural products. This study was carried out to examine the reaction synthesis between acetic anhydride and isoamyl alcohol in the presence of *Candida antarctica* Lipase-B (CALB) as a catalyst in solvent-free system (SFS). Results show that two reactions took place between acetic anhydride and isoamyl alcohol. The effect of different reaction parameters on the final yield of isoamyl acetate and the optimization of process parameters using a statistical tool were also investigated with response surface methodology (RSM). It was found that the optimum isoamyl acetate yield is at reaction temperature 30°C, acid/alcohol molar ratio 0.10, and enzyme loading 4.14%. The regression coefficient for optimization based on RSM was 0.9961. Errors resulted from model validation is less than 1% and is acceptable for real-life application. RSM model and first principle model were selected to determine the reaction kinetics and yield of reaction for isoamyl acetate. The results showed that RSM model provides a good predication of the esterification system with R^2 value of 0.90.

Keywords: enzymatic, esterification, isoamyl acetate, solvent-free system, lipase

1. Introduction

Esters are one of the most common of all naturally occurring organic compounds which contain –COOR as functional group. Many simple esters are pleasant-smelling liquids and mainly used as fragrant odors of fruits and flowers. For example, methyl butanoate is an

element found in pineapple oil, whereas isoamyl acetate is an element of banana oil [1]. These esters are also naturally present in animal fats and oil [2] and in many biologically important molecules. Esters are ubiquitous and contain "nature-identical" substance that can be used to substitute natural flavor and fragrances. The demand for flavor and fragrance products is fairly high for most applications in developed countries. In 2009, flavor and fragrance industry faced a decline due to global economic crisis, but rapidly recovered a year after. The market was forecasted to continue expanding at a CAGR of 5.6% during 2011–2013 [3].

As the demand on flavored food increased tremendously throughout the years, consumers were also concerned about the natural ingredients of it by considering food with natural flavored in their list. The term "natural" has been clearly defined by the U.S. Code of Federal Regulations 101.22(a)(3) as "…the essential oil, oleoresin, essence or extractive, protein hydrolysate (product of hydrolysis), distillate of any product of roasting, heating or enzymolysis, which contains the flavoring constituents derived from a spice, fruit juice, vegetable or vegetable juice, edible yeast, herb, bud, bark, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products or fermentation products thereof, whose significant function in food is imparting flavoring rather than nutritional" [4].

Esters naturally available in plants and flowers were extracted for traditional flavor production. However, the traditional extraction of flavor from plants is too expensive for commercial exploitation, limitation of raw materials, and only small amount of esters produced. On the other hand, the demand of esters kept increasing; therefore, researchers overcome the problems with alternative production route via chemical synthesis. Esterification via chemical synthesis is based on Fischer esterification method. Its drawbacks attributed to the chemicals used and consumers' awareness toward chemicals added to their food makes the synthesis is not favored in the food industry. Hence, a new method of ester synthesis is required to produce large number of esters for industrial application with high economic benefit and a purer end product.

Synthesis of isoamyl acetate in organic solvent has been introduced. Due to region- and stereospecificity expressed by most lipases in mild operation conditions and high degree of purity produced, lipase-catalyzed esterification in organic solvent has recently received greater consideration relative to the traditional chemical synthetic methods, particularly in the production of natural flavor and fragrance. Despite the higher conversion yields of esters, organic solvents undoubtedly bring about negative impact on solvent toxicity, inflammable, and need extra action on separation process. In addition, some organic solvents used are too expensive to allow profitable commercial scale-up [5]. Hence, a solvent-free system was introduced in the esterification process.

The absence of solvents in the solvent-free synthesis gives advantages on the downstream processing as there would be fewer components present in the reaction mixture at the end of the esterification process. Moreover, the production cost can be minimized. In addition, Yahya et al. [6] has stated that it is possible to use high substrates' concentrations in a solvent-free system. Hence, it is scientifically and environmentally wise to produce ester via solvent-free biotechnological route that would eliminate all the disadvantages of traditional and chemical synthesis route of producing esters.

1.1. Enzymes

Enzymes react by accelerating both the rate and specificity of metabolic reactions of the substrates without changing its original shape and amount [5]. In the food industry, flavor production from enzyme-catalyzed process has been reviewed by Christen and Munguia [7]. Common types of enzymes used in food flavor are hydrolases and oxidoreductases [7, 8]. One of the hydrolases enzymes is lipase. Lipase is also capable to form ester bounds under reverse hydrolytic conditions which allow catalysis of various other types of esters [9]. The demand for natural and environmental friendly product is the main key point of the usage of lipase in ester synthesis. Therefore, lipases are considered as enzymes of high commercial potential due to their flexibility in application. There are several lipases which have been used in isoamyl acetate production. Summary on the isoamyl acetate production based on different enzymes is shown in **Table 1**.

From **Table 1**, it can be observed that a lot of works have been done by using *Candida antarctica* as compared to other lipases. Based on **Table 1**, *T. lanuginosus* has the minimum amount of ester conversion.

Recyclability of enzymes is also an important factor to be considered for industrial-scale applications as it can reduce the cost of raw materials. For esterification in the organic solvent, *S. simulans* can be reused up to 4 cycles [20] and *Rhizomucor miehei* up to 10 cycles [16], while *Candida antarctica* can be recycled more than 10 cycles [10, 14]. Previous studies also had compared the performance of enzymes toward the esterification reaction. Guvenc et al. [11] have done a study on solvent-free system and have found that Novozym 435 from *Candida antarctica* was more efficient than Lipozyme from *Rhizomucor miehei*. This is agreed by Romero et al. [13] who extended the study on kinetics of enzymatic esterification of isoamyl acetate using Novozym 435 in an organic solvent. The advantages and the benefits of using *Candida*

Lipase origins	Maximum conversion, %	References
Candida antarctica	95.5	Gubicza et al. [10]
	80	Guvenc et al. [11]
	100	Romero et al. [12]
	96	Romero et al. [13]
	100	Feher et al. [14]
	∼99	Wolfson et al. [15]
Rhizomucor miehei	>90	Krishna et al. [16]
	∼40	Romero et al. [13]
Mucor miehei	>80	Krishna et al. [17]
	96.4	Mittelbach and Trathnigg [18]
S. simulans	64	Ghamgui et al. [19]
T. lanuginosus	∼42	Romero et al. [13]

Table 1. Enzymes and its corresponding maximum conversion of substrates based on previous studies.

antarctica in isoamyl acetate production in solvent-free system, as listed in previous research works, provide a sound basis of choosing this lipase in this present study.

2. Materials and methods

2.1. Materials and chemicals

In this study, isoamyl acetate was produced experimentally by reacting acetic anhydride and isoamyl alcohol with the presence of enzyme, *Candida antarctica* Lipase-B (CALB) in a solvent-free system. The chemicals used in this study were analytical grades and are summarized in **Table 2** together with the respected purity, usage, and supplier. The chemicals were used as received without further purification.

2.2. Equipment

The production of isoamyl acetate enzymatic synthesis from acetic anhydride was done in lab scale. All of the experimental works were carried out using 100-ml Erlenmeyer flasks with stopped rubber, which then were placed in an incubator shaker (Benchmark Incu-shaker mini, New Jersey). Incubator shaker was used to maintain the mixing rate and to control the temperature. Then, flame ionization detector gas chromatography (GC-FID) (Agilent Technologies, 7820A GC system, USA) was used to analyze the concentrations of compounds in the sample taken.

2.3. Isoamyl acetate syntheses

Isoamyl acetate syntheses were carried out without any organic solvent in 100-ml stopped-rubber Erlenmeyer flask with working volume of 15 ml. Enzyme was added into the reaction media containing a mixture of isoamyl alcohol and acetic anhydride at various temperatures. The reaction mixture was then incubated in an incubator shaker (Benchmark) at 150 rpm for 6 h. The basis of this experimental method was taken from [16].

2.4. Analysis of esterification

About 0.5 ml of the reaction mixture was withdrawn periodically starting from t = 0 h, until t = 6 h for analysis. The withdrawal was done using micropipette and transferred into

Materials/chemicals	Purity	Usage	Supplier
Isoamyl alcohol	99.8%	Production medium	Merck Co., Malaysia
Acetic anhydride	98%	Production medium	ACROS Organics, Malaysia
Isoamyl acetate	100%	GC standard	Merck Co., Malaysia
CALB (≥5000 U/g)	N/A	Production medium	Sigma-Aldrich, Malaysia

Table 2. List of materials and chemicals used.

microcentrifuge tube. Samples were analyzed using gas chromatograph (Agilent Technologies 7820A) equipped with a hydrogen flame ionization detector and a SGE BP21 (FFAP) column (60 m × 0.32 mm × 0.25 μm). Helium was used as a carrier gas at a flow rate of 5 ml/min. After injection of samples, the oven temperature was kept at 100°C and linearly increased to 140°C. The rate of temperature increase was set at 70°C/min, and was kept at 140°C for the remaining time of analysis. Injector and detector temperatures were set at 200 and 250°C, respectively.

Quantification of data was done by calibration with standards samples. Each sample required 4.08 min to be analyzed by GC-FID. The retention times of peaks were as follows: isoamyl acetate, 2.26 min; isoamyl alcohol, 2.38 min; acetic anhydride, 2.48 min; and acetic acid, 3.2 min.

2.5. Effect of reaction temperature

The effects of reaction temperature on the enzymatic esterification were studied at various temperatures: 30, 40, and 50°C. About 15 ml working volume of the medium in a 100-ml Erlenmeyer flask was incubated in an incubator shaker with agitation speed of 150 rpm for 6 h reaction time. Samples were taken periodically until 6 h of reaction time and analyzed by GC-FID for isoamyl acetate production.

2.6. Effect of acid/alcohol molar ratio

The effect of acid/alcohol molar ratio was studied at various acid/alcohol molar ratios: 0.1 (excess alcohol), 1 (equimolar), and 2 (excess acid). The medium was incubated in an incubator shaker at 40°C reaction temperature, and with agitation speed of 150 rpm for 6 h reaction time. Samples were taken periodically until 6 h of reaction time and analyzed with GC-FID for isoamyl acetate content.

2.7. Effect of enzyme loading

The enzyme loading effects were studied at various percentages of enzymes in medium: 4, 8, and 12%. The calculation was based on the overall mass of substrates used in the reaction. The medium together with the enzyme was incubated in an incubator shaker at a temperature of 40°C. The agitation speed was set to 150 rpm; samples were taken at different time intervals until 6 h of reaction time and were analyzed using GC-FID for isoamyl acetate content.

2.8. Optimization process using response surface methodology (RSM)

Optimization studies are carried out using response surface methodology (RSM). RSM is a collection of statistical and mathematical analysis for developing, improving, and optimizing processes in which the response developed is influenced by several variables. It has an important application in the process development, design, and formulation of new products, as well as in the improvement of existing product design.

			Levels		
Variables	Coding	Unit	−1	0	+1
Temperature	A	°C	30.00	40.00	50.00
Ac/Al ratio	B	—	0.1	1	2
Enzyme loading	C	%	4	8	12
Reaction time	D	h	2	4	6

Table 3. List of variables and its value.

2.9. Model fitting and statistical analysis

Optimum conditions for isoamyl acetate enzymatic esterification can be obtained by using optimization software, Design Expert 6.0.6. The method used was Central Composite Design (CCD), under RSM. CCD is the best design for response optimization [21]. In this study, three levels and four factor variables were chosen. The three levels represent the three points between the lower and upper limit of the parameters, whereas the four factors represent the four parameters that are studied in this section, which are reaction temperature, ac/al molar ratio, enzyme loading, and reaction time. The details of the parameters and levels studied were shown in **Table 3**.

2.10. Sensitivity analysis

Sensitivity analysis is useful for testing the robustness of the result of a model developed, to show the relationships between input and output variables in a system, and for model simplification by removing the insensitive or insignificant variables.

Based on the optimization step before, a sensitivity analysis for each individual parameter and the interaction between parameters in this study were done using application tools provided by RSM. It was done to decide the interaction between parameters, and the most sensitive parameters in esterification process.

3. Enzyme kinetic modeling

3.1. Response surface methodology (RSM) model

RSM model was developed using DoE software by designing new experiments for enzyme kinetic model. Three-level and four-factor designs which consist of 27 sets of experiment were constructed using parameters listed in **Table 4**.

3.2. First principle model

First principle model is an application of conservation of mass to the analysis of a physical system by taking account of material entering, leaving, generating, consuming, and accumulating in the system.

Variables	Coding	Unit	Levels		
			−1	**0**	**+1**
Temperature	β_1	°C	30	40	50
Mass enzyme	β_2	wt%	4	8	12
Reaction time	β_3	h	2	4	6
Reciprocal of anhydride concentration	β_4	l/mol	0.12	0.20	1.18

Table 4. Experimental range and levels of variables.

The mathematical general equation of a balanced mass conservation quantity by using conservation law in a system is:

$$Input + Generation = Output + Accumulation \tag{1}$$

In term of general mole balance, the above equation became

$$Q_0 C_{j0} + \int_V r_j dV = \frac{d}{dt}\int_V C_j dV + Q_1 C_{j1} \tag{2}$$

By assuming component j enters and leaves the element only by convection with the inflow and outflow streams by neglecting diffusional flux through the boundary of the volume element, Q_0 and Q_1 are the mass of component j at the inflow and outflow, respectively, C_{j0} and C_{j1} are the concentration of component j at the inflow and outflow, respectively, $\int_V r_j dV$ is the rate of generation of component j, and $\frac{d}{dt}\int_V C_j dV$ is the rate of accumulation of component j.

In a close system (batch process), assuming with well stirred substrate, the above equation reduces to:

$$Generation = Accumulation \tag{3}$$

Therefore,

$$\int_V r_j dV = \frac{d}{dt}\int_V C_j dV \tag{4}$$

where r is the rate of reaction, V is the volume, and C_j is the concentration of product produced by time in the reaction system. Since the volume of the reactor is constant in batch system, thus Eq. (4) reduces to:

$$r_j = \frac{dC_j}{dt} \tag{5}$$

The reaction rates can be derived in detail by enzyme kinetic equation.

3.3. Validation of kinetic model

The entire model developed will then need to be validated to assure the models are reliable and can be used in industrial application. Validation of the kinetic model was done by

comparing the output from the model developed with the experimental data collected. The results were compared and plotted in a graph, and error value based on the regression analysis was done.

4. Results and discussions

4.1. Isoamyl acetate syntheses

Theoretically, acetic anhydride possesses two acyl groups. In a reaction with isoamyl alcohol, one of the acyl from acetic anhydride will bind with isoamyl alcohol and discharge one H^+ to form isoamyl acetate and acetic acid. Then, excess acyl (from acetic acid) will react with excess alcohol to form another isoamyl acetate and water. The details of reaction scheme are shown below:

a. First reaction

$$Isoamyl\ alcohol + Acetic\ anhydride \rightarrow Isoamyl\ acetate + Acetic\ acid$$

b. Second reaction

$$isoamyl\ alcohol + acetic\ acid \rightarrow isoamyl\ acetate + water$$

c. Overall reaction

$$isoamyl\ alcohol + acetic\ anhydride \rightarrow isoamyl\ alcohol + water$$

Based on the chemical reaction scheme shown previously, esterification of isoamyl acetate undergo two reactions, first is between the acetic anhydride and isoamyl alcohol, producing acetic acid and isoamyl acetate; and the second reaction is between the acetic acid and excess isoamyl alcohol, producing another isoamyl acetate and water as by-product. An overall analysis on the effect of substrates concentration was done at 8% enzyme loading, 6 h of reaction time at acid/alcohol ratio of 0.1, 1, and 2, and the results were plotted in **Figures 1–3** respectively.

From **Figure 1**, the concentration of isoamyl acetate increased rapidly at the initial of the reaction until 15 min of reaction time. During that time, acetic anhydride was consumed until 98% of its initial concentration, whereas the concentration of acetic acid and isoamyl acetate was increasing. This is in line with the reaction mechanism involved, where the reaction between acetic anhydride and isoamyl alcohol will produce acetic acid and isoamyl acetate initially. As clearly shown in **Figure 1**, as acetic anhydride was completely consumed, acetic acid produced will then react with the excess isoamyl alcohol, producing isoamyl acetate and water. This is evident by the reducing of acetic acid concentration after 15 min of reaction time and consequently the rapid increase of isoamyl acetate concentration. Throughout that time,

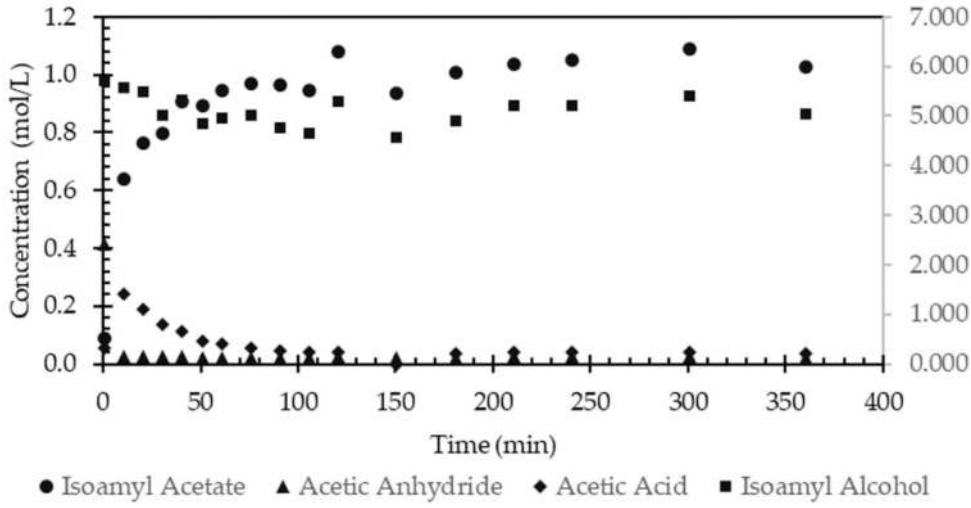

Figure 1. Overall concentration of substrates and product during the reaction for acid/alcohol ratio of 0.1.

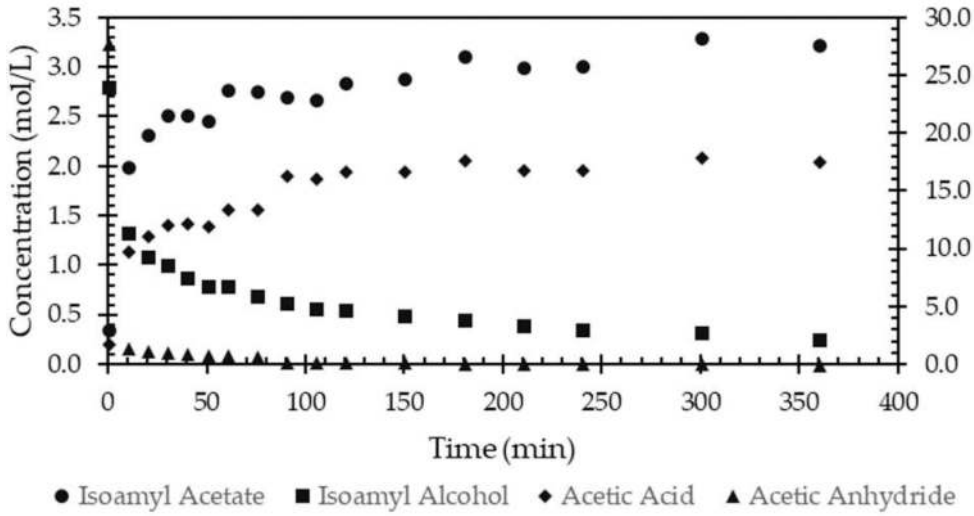

Figure 2. Overall concentration of substrates and product during the reaction for acid/alcohol ratio of 1.0.

concentration of acetic acid starts decreasing until 80% of acetic acid's initial production. At the end of the 6-h reaction time, isoamyl alcohol was in excess, acetic anhydride and acetic acid were 98 and 80% consumed, respectively.

From **Figure 2** initially, concentration of isoamyl acetate increased rapidly at the first 30 min of reaction time. During that time, acetic anhydride was consumed until 95% of its initial concentration, whereas concentration of acetic acid and isoamyl acetate was increasing, following the reaction mechanism described by the above reaction equation, which is reaction between acetic anhydride and isoamyl alcohol will initially produce acetic acid and isoamyl acetate. Acetic anhydride was fully consumed during 180 min of reaction time, and acetic acid produced at the beginning of reaction was in excess until the end of 6 h reaction duration as well as isoamyl

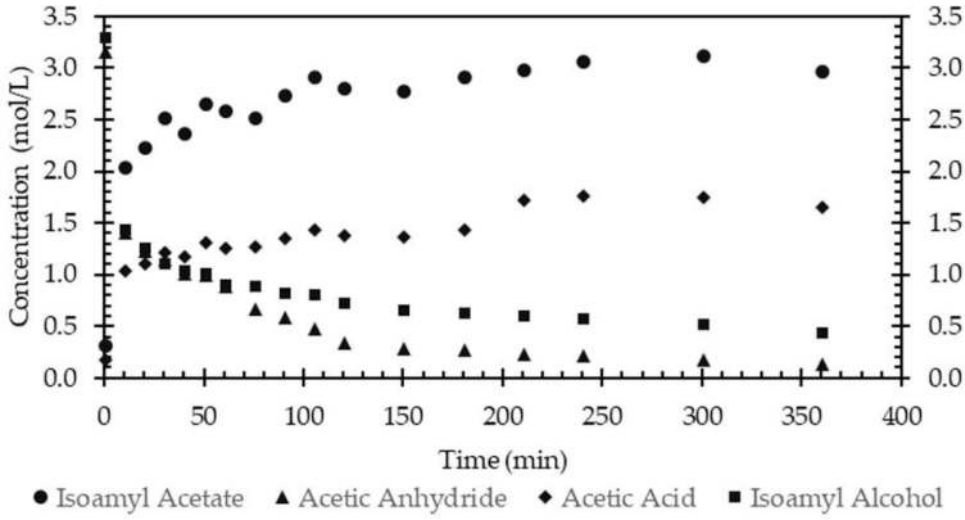

Figure 3. Overall concentration of substrates and product during the reaction for acid/alcohol ratio of 2.0.

alcohol. This is because the production of isoamyl acetate has achieved steady state, and the excess amount of alcohol is lower than the minimum amount which required to be reacted.

Figure 3 clearly shows that in excess acetic anhydride condition, isoamyl alcohol was 98% consumed during the 240 min of reaction time. After that, there is no decreasing trend in either acetic anhydride or acetic acid concentration. Acetic acid produced by the primary reaction at the beginning of the test was in excess since isoamyl alcohol is in limited condition. This shows that both reactions were stopped by limited amount of isoamyl alcohol in the mixture.

Based on **Figures 1–3**, isoamyl alcohol is the critical substrate in this esterification reaction. The final amount of isoamyl acetate produced is highly depending on the amount of isoamyl alcohol at the initial of the reaction. Limited amount of isoamyl alcohol will automatically stop the reaction mechanism, since there is no receiver of acyl in the mixtures. This also shows that acetic anhydride amount in the mixtures need to be restricted to some amount so that there is no excess acyl in the reaction, and hence making this process cost-effective [22].

4.2. Effect of synthesis parameters

4.2.1. Effect of reaction temperature

Reaction temperature of esterification process has an effect on the final yield of isoamyl acetate produced based on the Arrhenius equation (Eq. (6)).

$$k = k_0 \, exp\left\{-\frac{E_a}{RT}\right\} \tag{6}$$

where k is the rate constant, k_0 is the pre-exponential constant, E_a is the activation energy, R is the gas constant, and T is the absolute temperature.

Eq. (6) clearly shows that reaction temperature has parallel effect to the reaction rate constant and hence influences the final yield of isoamyl acetate produced by affecting the esterification reaction rate.

Effect of temperature on the yield of ester produced has been investigated at temperatures 30, 40, and 50°C for 6 h of reaction time. From literatures, the range of temperature studied was between 30 and 65°C. However, the optimum reaction temperature was found to be between 30 and 50°C. Hence, to elucidate the impact of reaction temperature on yield of ester, the synthesis has been studied at a temperature range from 30 to 50°C for 6 h of reaction time, at 8% enzyme concentration and acid/alcohol molar ratio of 1. The results are illustrated in **Figure 4**.

Based on **Figure 4**, initial reaction rate of ester production increased with increasing reaction temperature from 30 to 50°C. This would be explained by Eq. (6), where increasing reaction temperature would increase the kinetics of the reaction, hence encourage the collision rate between molecules in the medium, and thus favor higher production of ester. This result is in agreement with a research done by [23, 24], where increasing reaction temperature will increase the reaction rate and hence produce higher concentration of ester. Similar result on the positive effect of reaction kinetics toward the increasing of reaction temperature was also found by the studies.

As the reaction time increased, the production rate of ester appears to decrease for reaction temperatures of 40 and 50°C compared to the production at 30°C. The final yield of ester produced at 30, 40, and 50°C of reaction temperatures at time 6 h of reaction time were 67.2, 61.8, and 59.1%, respectively. This could be due to the enzyme tertiary structure that starts to disrupt at higher reaction temperature and at longer reaction time, hence losing its catalytic activity, thus lowering the enzyme production rate [25].

4.2.2. Effect of acid/alcohol molar ratio

The effect of acid/alcohol molar ratio has been studied at low anhydride concentration, equimolar, and excess in anhydride, whose ratios were 0.1, 1, and 2, respectively. The reaction

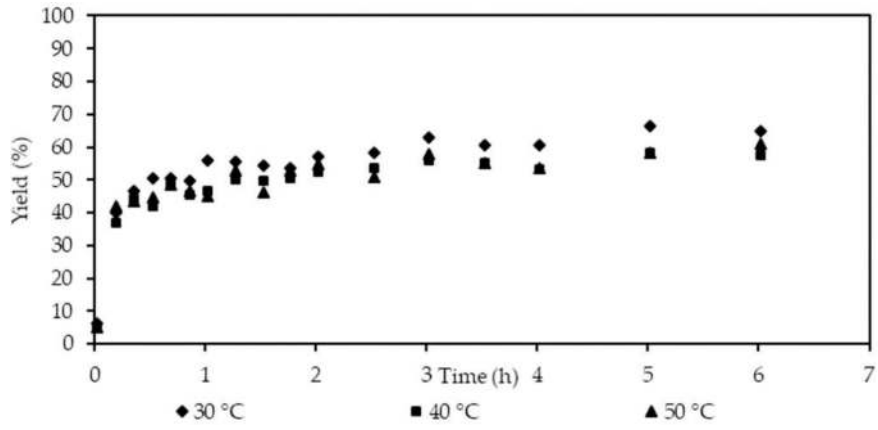

Figure 4. Effect of temperature on the ester production.

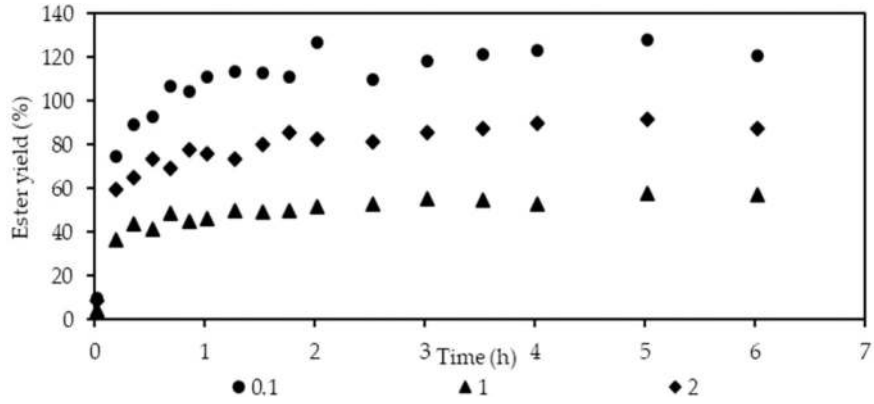

Figure 5. Effect of acid-alcohol ratio on the yield of ester.

temperature was set at 40°C and enzyme loading at 8%. The result for this experimental condition is shown in **Figure 5**.

Based on **Figure 5**, a maximum yield of isoamyl acetate was achieved when isoamyl alcohol is in excess over the acetic anhydride ratio. This is due to the availability of excess nucleophile in the reaction mixture. Based on the reaction scheme shown in Section 4.1, an acyl from acetic anhydride is reacted with a nucleophile available from isoamyl alcohol and producing isoamyl acetate and acetic acid. Then, an acyl from acetic acid produced reacted for the second time with the available nucleophile in the reaction mixture and produced another isoamyl acetate and water. The maximum yield of isoamyl acetate produced at acid/alcohol molar ratios of 0.1, 1, and 2 was 128, 53, and 88%, respectively.

The current study is in agreement with [14] and [26], where higher substrate concentration (high acid/alcohol molar ratio) leads to lower yields of ester. This could also be due to acid concentration which has met the critical concentration needed in the reaction [27]. However, by increasing the molar ratio to excess acid, the yield of ester starts to increase again. This condition happened because the concentration of acid in the reaction mixture has past the critical concentration of acid needed in the reaction medium.

4.2.3. Effect of enzyme loading

The effect of enzyme loading concentration was studied at 4, 8, and 12% of enzyme loading, acid/alcohol molar ratio of 1 and 30°C of reaction temperature. Enzyme loading is economically important to the esterification process as enzyme is costly compared to other materials used in the synthesis. Producing high yield of isoamyl acetate at low quantity of enzyme synthesis was highly preferred in an esterification synthesis. Therefore, in this study, the yield of isoamyl acetate produced versus reaction time was plotted and shown in **Figure 6** for three different enzyme loadings.

Based on the figure above, increasing amount of enzyme loading increases the yield of ester produced. The maximum yield of isoamyl acetate produced by the synthesis for 4, 8, and 12%

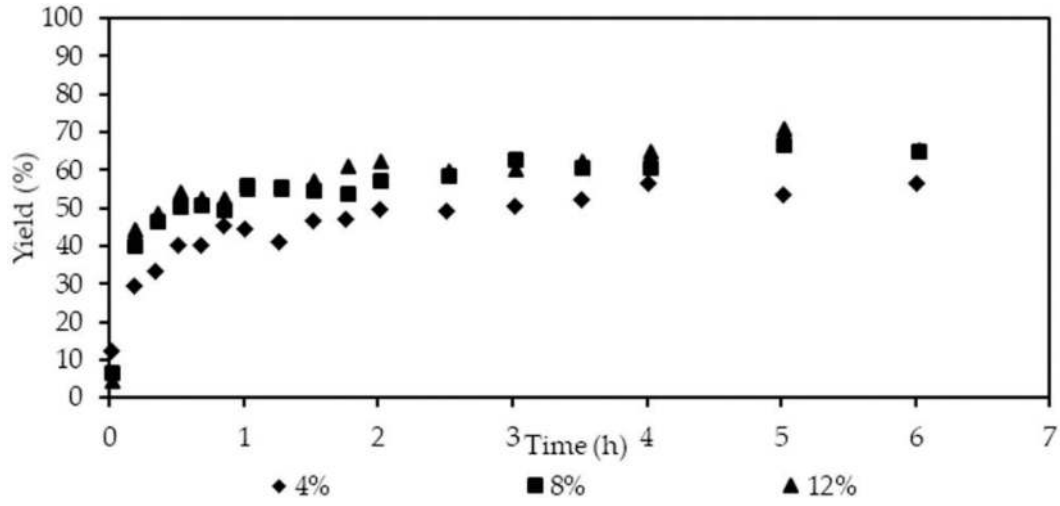

Figure 6. Effect of enzyme loading on the ester production.

are 57, 65.8, and 71.5%, respectively. The graph also shows the clearer trend of enzyme loading effect along the 6 h of reaction time. For lower amount of enzyme loading, longer reaction needed to achieve steady state of the esterification reaction. Increasing the enzyme loading from 4 to 12% shortened the time required for the reaction to achieve steady state which is favorable. It also shows that esterification reaction increases with the increasing amount of enzyme loading and the trends resembles the results of esterification studied by [26]. This concludes that the rate of esterification is dependent on the enzyme concentration used in the mixture.

4.3. Optimization using response surface methodology (RSM)

Design Expert software contained many types of optimization methods that can be used in this study. Based on the optimization methods described, a central composite design (CCD) method was chosen to design all the experiments conducted for esterification synthesis. CCD is generally the best design for response optimization, as stated by [21, 28]. In this study, three-level and four-factor designs were used to determine the optimum condition with four experimental parameters (temperature, acid-alcohol molar ratio, enzyme ratio, and reaction time) and three-level indicated the level of each range (−1, 0, +1). Six replicates which run at the center point (0, 0) of the design were performed to allow the estimation of pure error. All the experiments were carried out in the randomized order to minimize the unexplained variability in the observed responses due to irrelevant factor.

Fitting of the data to models in RSM and their subsequent ANOVA showed by **Figure 7** confirmed that the enzymatic esterification reaction of acetic anhydride and isoamyl alcohol was most suitably described by quadratic model. The equation of the model based on the actual values is shown by Eq. (7).

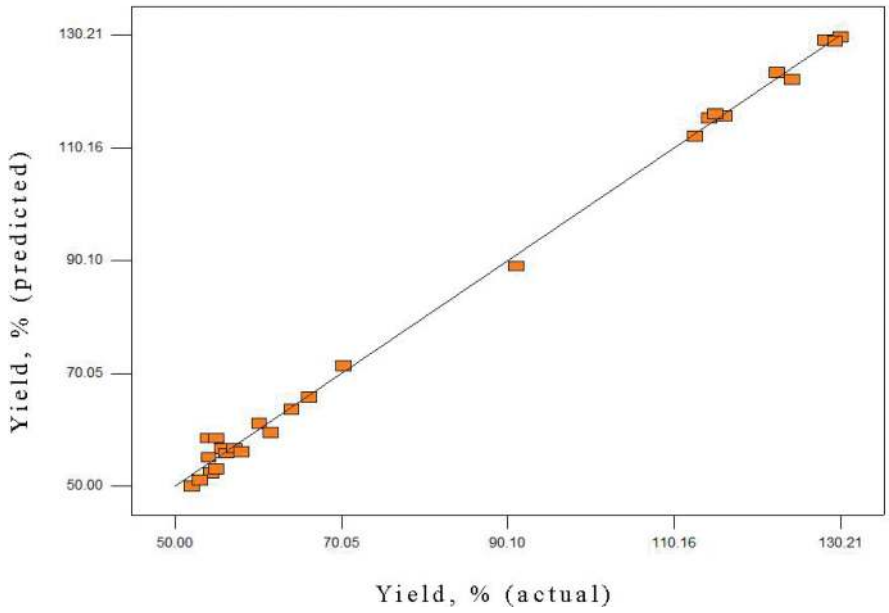

Figure 7. Parity plot for relation between observed and predicted isoamyl acetate synthesis.

$$\begin{aligned} yield = {} & 271.8778 - 11.0681\boldsymbol{A} - 303.3621\boldsymbol{B} + 9.9557\boldsymbol{C} \\ & - 11.5634\boldsymbol{D} + 0.1459\boldsymbol{A}^2 + 36.25\boldsymbol{B^2} - 0.4381\boldsymbol{C}^2 - 1.2452\boldsymbol{D}^2 \\ & + 10.2656\boldsymbol{AB} - 0.0588\boldsymbol{AC} + 0.3626\boldsymbol{AD} + 0.4859\boldsymbol{BC} \\ & - 8.678\boldsymbol{BD} - 0.2537\boldsymbol{CD} - 0.1443\boldsymbol{A}^2\boldsymbol{B} + 0.0011\boldsymbol{A}^2\boldsymbol{C} \\ & - 0.0072\boldsymbol{A}^2\boldsymbol{D} + 0.3997\boldsymbol{AB}^2 - 0.0222\boldsymbol{ABC} + 0.2123\boldsymbol{ABD} \\ & - 0.0037\boldsymbol{ACD} + 0.0151\boldsymbol{BCD} \end{aligned} \tag{7}$$

The quadratic response function represents the yield of ester produced, where $\boldsymbol{A}$ is reaction temperature, $\boldsymbol{B}$ is the acid/alcohol molar ratio, $\boldsymbol{C}$ is percentage of enzyme used, and $\boldsymbol{D}$ is the reaction time.

Closer the value of R^2 to unity, the better the empirical models fit the experimental data. Parity plot between actual data and the predicted data is done and shown in **Figure 7**. As can be seen, the predicted values match the actual values reasonably well within the ranges of the experimental parameter conditions, with R^2 value of 0.9961. At this stage, this result suggests the applicability and reliability of the equation in representing the esterification reaction between acetic anhydride and isoamyl alcohol by using CALB in a solvent-free system with sufficient degree of accuracy.

Statistical analysis from the analysis of variance (ANOVA) was done using RSM software (result not shown). The F value of the model (47.022) with a P value of 0.001 implied that the model is significant. Generally, P value lower than 0.01 indicates that the model is considered to be statistically significant at 99% confidence level and values greater than 0.10 indicate that

the terms are not significant [29]. Based on the results, the most significant model terms (F value = 23.014) that give high impact on the yield of ester produced is the acid/alcohol molar ratio, with P value of 0.0087. The small P value (<0.001) and a high regression coefficient (R^2 = 0.9961) showed the suitability of the model for representing the real relationship between all the reaction parameters and yield of the ester produced. Adequate precision value measured the signal-to-noise ratio for the model. Ratios greater than 4 indicated adequate model discrimination [28]. In this study, the adequate precision for developed model was found to be 17.6666; this indicates that the model could be used to navigate the design space for this enzymatic esterification reaction.

Model developed by RSM needs to be validated to make sure it is reliable and acceptable. **Figure 8** shows the comparison of ester yield from the actual experiment and calculated by model developed. The comparison was done for 27 runs of experiments.

Based on **Figure 8**, the yield of ester's model developed by RSM appear to fit well with the actual results of ester yield from the experimental data. This clearly shows that the model developed is reliable to use as prediction of the real process of enzymatic esterification synthesis between acetic anhydride and isoamyl alcohol catalyzed by CALB in solvent-free system.

4.3.1. Numerical optimization

Numerical optimization was done by setting the desired optimum condition and product, and then DoE software will generate few solutions based on the model developed before. Based on the desired optimized condition setting, four sets of solutions for optimized experimental condition for enzymatic esterification in SFS were developed as shown in **Table 5**.

Based on the numerical optimization, the most desirable reaction conditions for optimum isoamyl acetate yield was set at minimum reaction temperature, enzyme loading, and reaction time. Minimum reaction temperature is required to reduce the energy usage, hence lower the

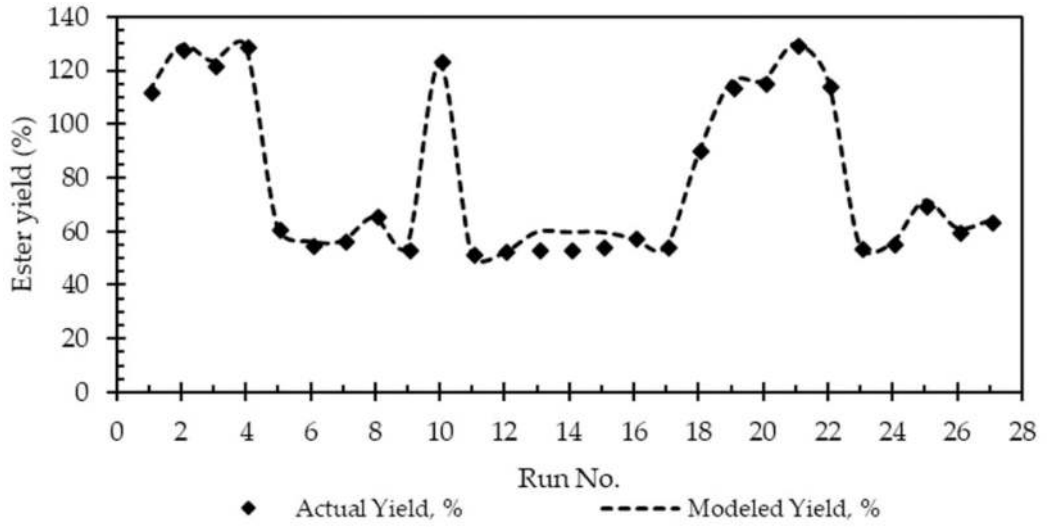

Figure 8. Comparison between the RSM model developed and the actual experimental data.

Parameters	Constrains			Solution
	Goal	Lower limit	Upper limit	
T (°C)	Minimize	30	50	30
Ac/Al	Is in range	0.1	2	0.10
Enzyme (%)	Minimize	4	12	4.14
Time (h)	Minimize	2	6	2.00
Yield (%)	Maximize	52.00	130.21	112.83

Table 5. Optimized condition suggested by DoE software.

operating cost of esterification process. Enzyme loading was set to minimum in order to reduce the usage of enzyme in esterification synthesis as enzyme is the most expensive material in esterification synthesis. Lowering reaction time to the minimum can help to accelerate the production rate of isoamyl acetate and give benefit to the industry. The results for optimum condition are shown in **Table 6**.

Experimental result showed that there was no significant difference for percentage yield of ester produces between the predicted and actual values. The error value for the optimization experiment is less than 1%. Therefore, the model obtained is reliable to predict the yield of isoamyl acetate in enzymatic esterification of isoamyl acetate by solvent-free system with high accuracy.

4.4. Enzyme kinetic

4.4.1. Response surface methodology (RSM) model

Design of experiment (DoE) software is used again to design the experiment. Three-level and four-factor designs which consist of 27 sets of experiments were done. The effect of temperature, T, mass of enzyme, M, reaction time, t, and reciprocal of initial anhydride concentration, $1/[A]_0$, on the enzymatic reaction rate were investigated using CCD analysis in RSM.

All coefficients obtained from the full quadratic polynomial model were evaluated by regression analysis and tested for their significance. The insignificant coefficients were eliminated based on p values. It was found that coefficients for β_4^2 and β_{23} were highly insignificant, hence the predicted polynomial model was rearranged by eliminating the terms which consist of β_4^2 and β_{23}, resulting in a modified quadratic model. The coefficient of determination ($R^2 = 0.99$) implies that the model was satisfactory.

The final model for reciprocal of enzymatic reaction rate obtained from the CCD analysis is:

Number	Temperature (°C)	Ac/Al ratio	Enzyme loading (%)	Reaction time (h)	Yield (%)		Error (%)
					Predicted	Actual	
1	30.0	0.10	4.14	2.0	112.83	112.63	0.18

Table 6. Result and error analysis of model validation.

$$\frac{1}{r} = 7.69399 - 0.2021\ (T) - 0.3193(M) - 1.0334(t) + 10.0771\left(\frac{1}{[A]_0}\right) + 0.028\left(T^2\right) + 0.0239\left(M^2\right) + 0.1060\left(t^2\right) - 0.024(TM) + 0.0385\ (Tt) - 0.1342\left(T\left(\frac{1}{[A]_0}\right)\right) - 0.0732\left(M\left(\frac{1}{[A]_0}\right)\right) - 0.1623\left(t\left(\frac{1}{[A]_0}\right)\right) \quad (8)$$

4.4.2. First principle model

By using Eq. (8), the general equation for reaction rate developed throughout the experiment is given by:

$$\frac{dC_j}{dt} = r_j \quad (9)$$

Given that general reaction rate equation, r is;

$$r_j = kC_j^n \quad (10)$$

where r_j is the reaction rate for product j, k is the reaction rate constant, C_j is the concentration of product j, and n is the order of reaction.

Since the overall reaction is reversible, hence Eq. (10) becomes

$$r_j = k_1 C_j^n - k_2 C_i^n \quad (11)$$

where k_1 and k_2 are rate constants for forward and backward reaction, respectively, C_i and C_j are concentration for substrates i and product j, respectively, and n is the order number of the reaction.

Overall reaction for isoamyl acetate esterification from acetic anhydride and isoamyl alcohol is:

$$2CH_3CH(CH_3)CH_2CH_2OH + (CH_3CO)_2O \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 2CH_3COOCH_2(CH_3)CHCH_3 + H_2O$$

Based on Eq. (11), there were few reaction rate equations that are possible to be applied for this isoamyl acetate enzymatic esterification reaction; therefore, all of the possible reaction rate equations were listed in **Table 7** and reaction rate constants for all equation developed were solved out using nonlinear equation solver in POLYMATH and the regression of each equation was compared. **Table 8** shows that the accurate reaction rate equation was for the third reaction rate with $R^2 = 0.9385$ and $adj.R^2 = 0.9360$. The kinetic constant for k_1 and k_2 equals to -0.0135 and 0.2530, respectively, with order number of 1. Therefore, the final reaction rate equation for enzymatic esterification reaction from acetic anhydride and isoamyl acetate becomes

$$r = -0.0135C_b + 0.2530C_p \quad (12)$$

where C_b is the concentration of isoamyl alcohol and C_p is the concentration of ester.

No.	Reaction rate equation	k_1	k_2	R^2	$Adj.R^2$
1	$r = (k_1 C_a) + (k_2 C_c)$	0.0461	0.3984	0.5705	0.5533
2	$r = (k_1 C_a) + (k_2 C_p)$	0.0078	0.2426	0.9121	0.9086
3	$r = (k_1 C_b) + (k_2 C_p)$	−0.0135	0.2530	0.9385	0.9360
4	$r = (k_1 C_a^2) + (k_2 C_p)$	0.0013	0.2447	0.9109	0.9073
5	$r = (k_1 C_a^2) + (k_2 C_p^2)$	0.0187	0.0912	0.8111	0.8035
6	$r = (k_1 C_b^2) + (k_2 C_p^2)$	0.0032	0.0961	0.7864	0.7778
7	$r = (k_1 C_b) + (k_2 C_p^2)$	0.0172	0.0951	0.7890	0.7806
8	$r = (k_1 C_a C_b) + (k_2 C_p^2)$	0.1912	0.0909	0.7936	0.7853
9	$r = (k_1 C_a C_b) + (k_2 C_p)$	−0.0147	0.2474	0.9109	0.9073

Table 7. Possible reaction rate equation, constant developed, and regression analysis.

Model	R^2
RSM model	0.90
First principle model	0.89

Table 8. Regression analysis between the model developed and the actual data from experimental results.

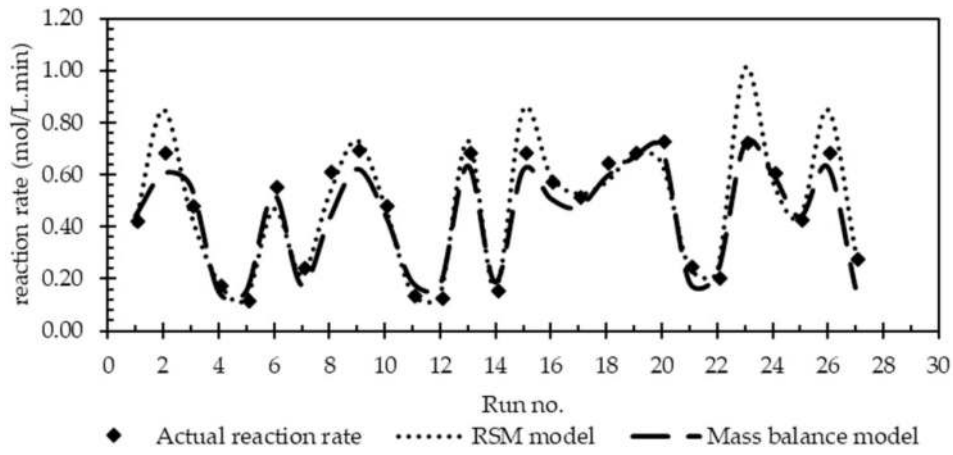

Figure 9. Comparison between the actual reaction rate and the modeled reaction rate.

4.4.3. Validation of kinetic equation

Validation of kinetic equation was done by comparing the experimental data for reaction rate of each run with the reaction rate calculated by Eqs. (8) and (12). Then, the results were plotted in **Figure 9** and regression analysis was done to evaluate the accuracy of models developed.

Regression analysis was done between the models and the actual data by using excel and the result of regression analysis is shown in **Table 8**. The regression value (R^2) calculated for RSM

model is 0.90, and for first principle model is 0.89. Based on **Figure 9** and **Table 8**, first principle model and RSM model was found to have good agreement with the actual data from the experiment. Hence, kinetics of enzymatic esterification of isoamyl acetate in this study can be represented by RSM and mass balance kinetic model.

5. Conclusions

In this study, it was concluded that there are two chemical reactions involved in the esterification of isoamyl acetate from acetic anhydride and isoamyl alcohol. The main reaction is between acetic anhydride and isoamyl alcohol, and the combination of the two reactions results in an overall reaction as follow:

$$2CH_3CH(CH_3)CH_2CH_2OH + (CH_3CO)_2O \rightleftharpoons 2CH_3COOCH_2(CH_3)CHCH_3 + H_2O$$

Between all of the parameters studied, the most critical parameter in isoamyl acetate synthesis is the acid/alcohol molar ratio. This is because the molar ratio will affect the nucleophile and acyl content in the mixture. The least sensitive parameter is the reaction temperature of the ester synthesis. This can be shown by small gap on the yield of ester produced at large different of reaction temperature.

Optimization of enzymatic isoamyl acetate synthesis in solvent-free system was done using RSM. The present process fits well with second order quadratic equation with determination of coefficient (R^2) equals to 0.9961. The numerical optimization suggested that the optimum condition for enzymatic esterification of isoamyl acetate from acetic anhydride and isoamyl alcohol by enzyme CALB in solvent-free system is at 30°C reaction temperature, 0.10 acid/alcohol molar ratio, 2 h of reaction time, and 4.14% of enzyme loading. Two types of mathematical models were selected and validated to determine the reaction kinetics and yield of reaction for isoamyl acetate. From two models selected, the RSM model is the most accurate model for the esterification with R^2 value of 0.90.

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