REACTION SYNTHESIS AND KINETIC MODELING OF ISOAMYL ACETATE VIA ENZYMATIC ESTERIFICATION IN SOLVENT-FREE SYSTEM

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Abstract

The aim of this study is to determine the reactions involved in esterification reaction of isoamyl acetate, in solvent-free system (SFS), between acetic anhydride and isoamyl alcohol in the presence of enzyme Candida antarctica Lipase B (CALB) as catalyst. This study was done in a batch system using stopped rubber conical shake flask. Results show that there is two main reactions took place, which are: (i) between acetic anhydride and isoamyl alcohol, and (ii) between acetic acid and isoamyl alcohol. Kinetic modeling was also done using First Principle model and found that the kinetic constant for $k_1$ and $k_2$ value equals to 0.0135 and 0.2530 respectively.

Keywords: Esterification, Isoamyl acetate, Solvent-free system, Kinetic modeling.

1. Introduction

Esters are one of the most extensive of all naturally occurring organic compounds which contain functional group, -COOR. Esters are widely used for various purposes in chemical industry. For instance, ethyl acetate is commonly used as solvent in extraction process [1], and dialkyl phthalates are used in plastic industry to keep polymer from becoming brittle [2]. 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 [3]. These esters are also naturally present in animal fats and oil [4] and in many biologically important molecules. Esters are ubiquitous and contain ‘nature-identical’ substance that can be used to substitute natural flavors and fragrances.

Esterification is a process of combining an organic acid (R-COOH) with an
alcohol (R-OH) to form an ester (R-COO-R) and water (H₂O). Esters have great application which primarily used in food and cosmetic industries [5-7] and biodiesel production [8]. Only minimum amount of esters are used as lubricant [7], confectionary [9], and in pharmaceutical product [6]. Traditionally, esters can be directly extracted from plant material or produced by chemical synthesis [10, 11]. However, the high handling cost and low product quantity makes it inappropriate to be implemented for industrial application [12].

Researchers have come out with chemical route in the presence of a strong acid catalyst at high temperature to produce esters at a very low cost. However, there are many by-products and the removal of the catalyst from reaction medium is difficult. In addition, the final product is not natural [10] as has been defined by U.S. Food and Drug Administration in ‘Code of Federal Regulation’ [13]. Then, biotechnological route of ester production was introduced by using free and immobilized lipase from various sources in organic solvent to encounter the problem in the chemical route [14]. Although higher conversion yields, solvent toxicity is a problem for many applications. On top of that, some organic solvents used are too expensive to allow profitable production in industrial scale [15, 16]. Additionally, a solvent-free system increased isoamyl acetate volumetric production thus making the system preferred and attractive for industrial application [17].

Synthesis of isoamyl acetate in this study has been investigated by using acetic anhydride and isoamyl alcohol catalysed by CALB in a solvent-free system.

2. Material and Methods

2.1. Equipment and materials

The experiments were carried out in a 100ml stopped rubber shake flask, which was incubated in Incu-Shaker Mini (Benchmark Scientific, New Jersey) at 150rpm. Temperature was set at 30°C, 40°C and 50°C. Working pressure was at ambient pressure condition.

Isoamyl alcohol used was supplied by Merck Co. (Malaysia), while acetic anhydride (reagent grade, ≥98%) was supplied by Sigma Aldrich (Malaysia). All
substrates were used without any pre-treatment. Immobilized enzyme from *Candida Antarctica*, Novozyme 435 (specific activity ≥ 10000 U/g, recombinant, expressed in *Aspergillus Niger*) supplied by Sigma Aldrich (Malaysia).

2.2. Synthesis procedure

Isoamyl acetate synthesis was carried out without any organic solvent in 100ml stopped rubber shake flask with working volume of 15ml. Acetic anhydride and isoamyl alcohol was added into the flask until alcohol to acid ratio equals to 0.1. Then 1wt% of immobilized enzyme from *Candida antarctica* was inserted into the mixture. The reaction mixture was then incubated in an Incu-shaker mini (Benchmark) at 150 rpm for 360 minutes. Samples were withdrawn every 10 min and analysed with gas chromatograph until 6hr of reaction time. Experimental procedures were repeated for different amount of enzyme and conditions as in Table 1.

2.3. Analysis

0.5ml of the reaction mixture was withdrawn every 10 min. Samples were analysed by Agilent 7820A Gas Chromatograph supplied by Agilent Technologies, equipped with a hydrogen flame ionization detector and a SGE BP21 (FFAP) column (60m x 0.32mm x 0.25 µm). Helium was used as carrier gas at flow rate of 5ml/min. After injection of samples, the temperature of oven 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°C and 250°C, respectively. Quantification of data was done by calibration with standards samples. The peaks of the retention time are as follows: isoamyl acetate, 2.26min; isoamyl alcohol, 2.38 min; acetic anhydride, 2.48min; and acetic acid, 3.21 min [18].

2.4. Experimental design with Response Surface Methodology (RSM)

Experimental design was conducted using Design of Experiment (DoE) software (Design Expert 6.0.6), Start-Ease. A three-level and four-factor design was used to determine the enzyme kinetic constants. Four experimental parameters (operating temperature, T; mass of enzyme, m; reaction time, t; and reciprocal of substrates concentration,1/[A]) were used in this study and 3-level indicated the level of each range (-1, 0, +1).

<table>
<thead>
<tr>
<th>Table 1. Experimental ranges and levels of variables.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
</tr>
<tr>
<td>Temperature</td>
</tr>
<tr>
<td>Mass enzyme</td>
</tr>
<tr>
<td>Reaction time</td>
</tr>
<tr>
<td>Reciprocal of substrates concentration</td>
</tr>
</tbody>
</table>
The list of parameters and levels studied in this experiment are shown in Table 1. Preliminary studies showed that all of the variables are statistically significant and ignoring these variables may affect the design of experiment. Central Composite Design (CCD) was chosen because it provides information on experimental variable effects and overall experimental error in a minimum number of runs. CCD also can be used under different experimental regions of interest and operability. This CCD design consists of 2 parts: factorial points (-1, +1), and center points (0, 0). Six replicate runs at the center point (0, 0) of the design were performed to allow the estimation of pure error. All of the experiments were carried out in the randomized order to minimize the unexplained variability in the observed responses due to irrelevant factor.

A second order polynomial model was predicted by a multiple regression procedure. This resulted in an empirical model related to the response by the equation:

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{j=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_{ij} + \epsilon, \]  

(1)

where \( x_1, x_2, \ldots, x_k \) are the input variables, which influence the response \( Y \); \( \beta_i (i = 1, 2, \ldots, k), \beta_{ij} (i = 1, 2, \ldots, k; j = 1, 2, \ldots, k) \) are unknown parameters; and \( \epsilon \) is random error.

2.5. Validation of RSM model and determination of kinetic constant

RSM model developed needs to be validated in order to evaluate its efficiency. The validation tests were done based on the conditions factor suggested by RSM and the result is compared with the experimental values.

The obtained reaction rate model equation from RSM is then fitted to the modified Michaelis-Menten equation using POLYMATH® software. By using nonlinear equation solver, kinetic constants were determined.

3. Result and Discussion

3.1. Isoamyl acetate reaction synthesis

Theoretically, acetic anhydride possesses two acyl groups. In reaction with isoamyl alcohol, one of the acyl from acetic anhydride will bind with isoamyl alcohol and discharge one \( \text{H}^+ \) 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 as by-product. The existence of water in the mixture by the secondary reaction makes the overall esterification reactions more favorable towards the hydrolysis reaction. This fits with the Le Chatelier’s Principle, where the dynamic equilibrium of the reaction will be shifted towards the left-hand side of the reversible reaction as the by-product increases. This condition was in agreement with the researches done by Ghamgui et al. (2006) and Luhong et al. (2001).
(a) **Main reaction**

\[
\text{(Isoamyl alcohol)} + \text{(Acetic anhydride)} \rightarrow \text{(Isoamyl acetate)} + \text{(Acetic acid)}
\]

(b) **Secondary reaction**

\[
\text{(Isoamyl alcohol)} + \text{(Acetic acid)} \rightleftharpoons \text{( Isoamyl acetate)} + \text{(water)}
\]

(c) **Overall reaction**

\[
\text{(Isoamyl alcohol)} + \text{(Acetic anhydride)} \rightleftharpoons \text{(Isoamyl alcohol)} + \text{(water)}
\]

Fig. 1. The details of reaction scheme.

### 3.2. Response surface analysis

The effect of temperature, mass of enzyme, reaction time and reciprocal of substrates concentration on the enzymatic reaction rate were investigated using response surface methodology. The results of the developed CCD are given in Table 2. Multiple regression coefficients obtained from a least squares analysis used to predict quadratic polynomial model for the reciprocal enzymatic reaction rate are summarized in Table 3.

All coefficients obtained from the full quadratic polynomial model were evaluated by regression analysis and tested for their significance. The insignificant coefficients were eliminated on the basis of \( p \) values. It is well known that smaller \( p \) values (<0.001) indicated significant value of model or factor. From the result, it was found that coefficient for \( \beta_1^2, \beta_{13} \) and \( \beta_{23} \) were highly insignificant, hence the predicted polynomial model was rearranged by eliminate terms which consist of \( \beta_1^2, \beta_{13} \) and \( \beta_{23} \) in full quadratic model. The coefficient of the modified quadratic model can be seen in Table 3. The coefficient of determination for the modified quadratic model \( (R^2 = 0.98) \) is better than the full quadratic model \( (R^2 = 0.97) \) implies that the model was satisfactory.

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

\[
\frac{1}{r} = 7.0784 - 0.1867(T) - 0.3193(M) - 0.8795(t) + 10.0771\left(\frac{1}{[A]_0}\right) + 0.0028(T^2) + 0.0239(M^2) + 0.1060(t^2) - 0.0024(TM) - 0.1341\left(T\left(\frac{1}{[A]_0}\right)\right) - 0.0732\left(M\left(\frac{1}{[A]_0}\right)\right) - 0.1623\left(t\left(\frac{1}{[A]_0}\right)\right)
\]

\[ (2) \]
3.3. Validation of RSM model

Model developed in the above part has been validated by experimental data. The model validation experiment was conducted at 40°C at 8wt% of mass enzyme for 6hrs. Figure 2 shows the comparison of isoamyl acetate reaction rate between model developed by RSM and from experimental data.

Table 2. Experimental results of the CCD.

<table>
<thead>
<tr>
<th>Exp No. M_0 (%)</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>1/[A] (mol/l)</th>
<th>[ester] (mol/l)</th>
<th>[alc] (mol/l)</th>
<th>[anil] (mol/l)</th>
<th>Reaction rate (mol/min)</th>
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<td>1</td>
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<tr>
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<td>0.0600</td>
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<td>1.1040</td>
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<td>0.0310</td>
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<td>0.5800</td>
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<tr>
<td>27</td>
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<td>6</td>
<td>1.1794</td>
<td>0.9760</td>
<td>4.7230</td>
<td>0.0270</td>
</tr>
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</table>

Table 3. Regression coefficients of the quadratic model for CCD.

<table>
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<tr>
<th>Factor</th>
<th>Full quadratic model</th>
<th>Modified quadratic model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prob &gt; F</td>
<td>Std. Error</td>
</tr>
<tr>
<td>Model</td>
<td>&lt; 0.0001</td>
<td>0.581429</td>
</tr>
<tr>
<td>β_1</td>
<td>0.1083</td>
<td>0.120609</td>
</tr>
<tr>
<td>β_2</td>
<td>0.2676</td>
<td>0.120609</td>
</tr>
<tr>
<td>β_3</td>
<td>0.6098</td>
<td>0.120609</td>
</tr>
<tr>
<td>β_4</td>
<td>0.3903</td>
<td>3.431322</td>
</tr>
<tr>
<td>β_5</td>
<td>0.2107</td>
<td>0.209439</td>
</tr>
<tr>
<td>β_6</td>
<td>0.0987</td>
<td>0.209439</td>
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<tr>
<td>β_7</td>
<td>0.0697</td>
<td>0.209439</td>
</tr>
<tr>
<td>β_8</td>
<td>0.9051</td>
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<tr>
<td>β_9</td>
<td>0.2677</td>
<td>0.083963</td>
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<tr>
<td>β_10</td>
<td>0.3775</td>
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<tr>
<td>β_11</td>
<td>&lt; 0.0001</td>
<td>0.152103</td>
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<td>β_14</td>
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<tr>
<td>β_15</td>
<td>0.99</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Adjusted R^2 0.97 0.98
The continuous line indicates the actual result from the experimental data and the dashed line indicates the result from model developed by RSM. The model and experimental result shows a good agreement for reaction rate prediction with $R^2$ value of 0.98. This indicates that the model can be used to find the kinetic constant for this study.

![Reaction rate curve for Isoamyl Acetate production for developed model and experimental data calculation.](image)

### 3.4. Kinetic constant

Based on the general reaction rate equation,

$$r_j = k C_j^n$$  \hspace{1cm} (3)

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.

Isoamyl acetate enzymatic synthesis between acetic anhydride and isoamyl alcohol having forward and backward reaction, hence Equation 3 becomes;

$$r_j = k_1 C_j^n - k_2 C_i^n$$  \hspace{1cm} (4)

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:

$$2\text{CH}_3\text{CH}((\text{CH}_3)\text{CH}_2\text{CH}_2\text{OH} + (\text{CH}_3\text{CO})_2\text{O} \stackrel{k_1}{\rightarrow} 2\text{CH}_3\text{COOCH}_2(\text{CH}_3)\text{CHCH}_3 + \text{H}_2\text{O}$$  \hspace{1cm} (5)

Based on Eq. (4), there were few reaction rate equations that are possible for this isoamyl acetate enzymatic esterification reaction, therefore all of the reaction rate constants for all equation developed were find out using nonlinear equation solver in POLYMATH and the regression of each equation were compared. All of the possible reaction rate equation, constant developed and regression analysis were tabulated in Table 4.

Table 4 shown that the significant reaction rate equation was for the third reaction rate with $R^2 = 0.9385$ and $nadj.R^2 = 0.9360$. The kinetic constant for $k_1$ and $k_2$ equals to -0.0135 and 0.2530 respectively with order number of 1.
### Table 4. Possible reaction rate equation, constant developed and regression analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Reaction rate equation</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$R^2$</th>
<th>Adj. $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$r = (k_1C_0) + (k_2C_1)$</td>
<td>0.0461</td>
<td>0.3984</td>
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<td>0.5333</td>
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<td>2</td>
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<td>0.0078</td>
<td>0.2426</td>
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<td>0.9086</td>
</tr>
<tr>
<td>3</td>
<td>$r = (k_1C_0) + (k_2C_1)$</td>
<td>-0.0125</td>
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<td>0.9360</td>
</tr>
<tr>
<td>4</td>
<td>$r = (k_1C_0^2) + (k_2C_1^2)$</td>
<td>0.0013</td>
<td>0.2447</td>
<td>0.9109</td>
<td>0.9073</td>
</tr>
<tr>
<td>5</td>
<td>$r = (k_1C_0^2) + (k_2C_1^2)$</td>
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<td>6</td>
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<td>0.7864</td>
<td>0.7778</td>
</tr>
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<td>0.7806</td>
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<td>8</td>
<td>$r = (k_1C_0C_3) + (k_2C_1C_5)$</td>
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<td>$r = (k_1C_0C_3) + (k_2C_1C_5)$</td>
<td>-0.0147</td>
<td>0.2474</td>
<td>0.9109</td>
<td>0.9073</td>
</tr>
</tbody>
</table>

### 3.5. Validation of kinetic model

To certify the validity of kinetic constant developed by POLYMATH in above section, comparison of reaction rate from developed kinetic constant and actual reaction rate from experimental data was done. Figure 3 shows the comparison of isoamyl acetate reaction rate calculated by using kinetic constant developed by POLYMATH and reaction rate from experimental data.

The continuous line indicates the actual result from the experimental data and the dashed line indicates the result from model developed by RSM. The model and experimental data shows a good agreement with $R^2$ value of 0.94. This indicates that the developed kinetic constants are valid to be use in the study of enzymatic esterification of isoamyl acetate from acetic anhydride in solvent free system.

Therefore, the final reaction rate equation for enzymatic esterification reaction from acetic anhydride and isoamyl acetate is;

$$ r = -0.0135C_b + 0.2530C_p $$

(6)

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

Fig. 3. Reaction rate curve for Isoamyl Acetate production from developed kinetic constant and experimental data calculation.
4. Conclusion

Synthesis of isoamyl acetate was carried out by reacting acetic anhydride with isoamyl alcohol in a solvent-free system. Candida Antarctica lipase-B (CALB) was used to enhance the production rate of ester. 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 second reaction is between produced acetic acid with the excess isoamyl alcohol. The modelling and optimization process of this enzymatic esterification process were done using RSM based model. A reciprocal of reaction rate model was developed and the ANOVA test implied that the model was satisfactorily representing the real relationship of the main reaction parameters with $R^2$ (0.98). Kinetic constants of the kinetic equation were obtained from POLYMATH software by using the experimental data and reaction rate model developed before by RSM. The final model resulted $k_1$ and $k_2$ value equals to $-0.0135 \text{min}^{-1}$ and $0.2350 \text{min}^{-1}$ respectively. Validation test were done by doing graphical comparison between the kinetic model developed and experimental data, it shows that the kinetic model developed is having a good agreement with the actual experimental data by $R^2$ value of 0.94.

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References


