Titration vs. GC-MS Analysis to Determine the Factors Affecting the Esterification Activity of Candida rugosa Lipase Immobilized onto Celite

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5Research Center for Biomass Valorization, Faculty of Engineering, Universitas Indonesia, Depok 16424, Indonesia

Abstract. The activity of lipases to catalyze the synthesis of esters in a non-aqueous environment can be assessed by performing a simple esterification study. In such tests, titration of the remaining acid has been one of the most used methods to determine the reaction progress due to its simplicity. Nonetheless, the execution of the titration is not always as simple as it sounds. In this study, Candida rugosa lipase is immobilized onto celite matrix, and its esterification activity was evaluated by catalyzing the reaction between butanol and butyric acid. Among the factors affecting the esterification activity of the immobilized enzymes are celite:CRL ratio, buffer pH during immobilization, and post-immobilization drying time. The titration results were analyzed using factorial design, ANOVA test, and Pareto chart. Here, the ambiguity of the titration results is showcased. On the other hand, analyzing the esterification results using GC-MS allowed optimization of the immobilization method to be performed. Finally, factors affecting the activity of the immobilized enzyme can be better assessed when the esterification results were analyzed using GC-MS.

1 Introduction

Many enzyme-catalyzed reactions used lipases as biocatalysts [1,2]. Although their native substrate is acylglycerides, lipases have broad specificity, which allows them to catalyze various kinds of reactions, including hydrolysis, esterification, transesterification, alcoholysis, acidolysis, and aminolysis [3-5]. Among the lipases most widely used in research and industrial applications is Candida rugosa lipase (CRL) [4-6]. This lipases have been

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widely used for waste utilization such as for the production of biodiesel [7,8], wax ester [9], as well as for waste treatment [10].

One of the challenges of using lipase as a catalyst is its higher operating cost due to the limited thermal and operational stability, as well as the difficulty of reusing the enzyme. However, lipases can be immobilized onto a supporting matrix to address these issues [11-14]. Various lipase immobilization methods have been explored. Nonetheless, physical adsorption remained to be one of the simplest methods available [5,15]. This method typically does not alter lipase’s structural configuration because of the mild immobilization process [13,14]. For the supporting matrix, properties that need to be taken into consideration include material inertness, high chemical and thermal stability, compatibility with lipase, and material/production cost [13,16]. One of the materials that meets these criteria is celite. Celite is a porous inorganic material composed mainly of silica (SiO₂). Immobilization of lipase-sol gel into celite has been shown to improve the mechanical stability of the immobilized enzyme [2] and even allowed the obtainment of higher triolein conversion in a packed bed reactor [16].

In this study, CRL is immobilized onto celite through physical adsorption. The variables that significantly affect the esterification activity of immobilized CRL were examined using factorial design. Factorial design is chosen over the One Variable at A Time (OVAT) method because factorial design allows interaction variable(s) to be detected. The esterification activity of the immobilized CRL was initially assessed by titrating the remaining acid. Titration has been one of the most common methods to assess the catalytic activity of enzymes, particularly on the esterification activity of lipases [1,6,17-19]. This method is simple, easily accessible, and inexpensive. However, here, the results of the esterification study using titration were found to be conflicting each time the variables were altered. Finally, the esterification study was assessed using GC-MS, with which the esterification results could be better evaluated.

2 Materials and Methods

2.1 Materials

* Candida rugosa* lipase Type VII (catalogue number L1754) and Celite 545AW – reagent grade were obtained from Sigma-Aldrich. Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate heptahydrate, and NaOH to make the buffer solution were purchased from Merck. Materials for the esterification reaction and the titration were n-butanol, butyric acid, isoctane, ethanol, acetone, and 1% phenolphthalein solution in ethanol were also purchased from Merck.

2.2 Immobilization Procedure

CRL was mixed with a buffer solution, and then 400 mg of celite was added. The initial concentration of the CRL-buffer solution was 40 and 80 mg/mL. The celite:CRL ratio used in this study was 4:1 and 8:1. The suspension was mixed using a stirring rod over a Petri dish, then placed in a desiccator for the CRL adsorption and drying to occur. The immobilized enzyme was dried at room temperature in the desiccator for a determined time, ranging from 1 – 3 days.


2.3 Assessment of the Esterification Activity of the Immobilized Enzyme through Esterification Reaction

The esterification activity of the immobilized CRL was assessed by using it to catalyze the esterification of butyric acid and n-butanol. The method used was modified from previous studies [17,18]. One mmol of each reactant was mixed with isooctane in a conical flask to reach an equimolar initial concentration of 0.2 M. The flask was sealed and placed in a waterbath shaker at 37°C and maximum shaking speed for 3 hours. The reaction was stopped by quenching the reaction mixture with a 1:1 ethanol/acetone mixture.

2.4 Determination of The Esterification Results by Titration Analysis

After the esterification was halted, the esterification mixture was quickly titrated using 0.05 M NaOH solution. A few drops of 1% phenolphthalein were used as the indicator. The titrate is the remaining butyric acid in the esterification mixture. Hence, the esterification product, i.e., butyl butyrate, is calculated from the difference of butyric acid in the blank and the samples. The esterification activity of the enzyme was expressed in μmol butyl butyrate formed/mg immobilized enzyme/minute.

2.5 Determination of The Esterification Results by GC-MS Analysis

The immobilized CRL for this part of the study was obtained from a different method than that described in the subsection ‘Immobilization Procedure’. This is because, with GC-MS analysis, it was possible to optimize the immobilization procedure. The detail of the optimization is described in the subsection “Assessment of Esterification Activity Using GC-MS As The Method of Analysis” in the Results and Discussion. In the optimized method, celite was first prewashed two times with distilled water and once with buffer. In each wash, the suspension was stirred for 30 minutes and then filtered using a vacuum filter. The washed celite was then dried in a desiccator until a constant mass was reached. The immobilization process started by mixing the CRL with buffer to make a CRL solution, and then the prewashed celite was added. The suspension was stirred at room temperature for a determined time. Then, the suspension was poured over a watch glass and dried in a desiccator. The activity of the immobilized CRL was assessed through an esterification reaction (subsection “Assessment of The Esterification Activity of The Immobilized Enzyme through Esterification Reaction”). After 3 hours of esterification, the reaction mixture is directly analyzed using GC-MS.

The GC-MS analysis was done using Clarus SQ 8T GC/MS from Perkin Elmer, with Elite-5MS Column (L: 30 m, ID: 0.25, DF: 0.25) and Helium as the carrier gas. An autosampler was used with a 1 μL injection volume. The injector was set at 200°C. The initial oven temperature was 40°C, held for 3.5 min, ramped 10°C/min to 100°C, held for 3 min, ramped 10°C/min to 250°C and held for 2.5 min.

2.6 Factorial Design

Analysis of the factorial design was performed using Design Expert software V.11 (StatEase, Inc., MN, USA).

3 Results and Discussion
3.1 Assessment of Esterification Activity Using Titration As The Method of Analysis

The independent variables in this study were the operating conditions of the immobilization process, namely the buffer pH and the celite to CRL ratio. Initially (in the first data set), the esterification study was performed using 20 mg of immobilized CRL with a reaction time of 2 hours. A surface plot of the titration results is shown in Fig. 1a. This plot considers all variables (buffer pH, ratio, and their interaction) in the equation. Nonetheless, no significant model or variables were detected during the analysis. Evaluation of the normal probability plot (Fig. 1b) shows that only variable A (buffer pH) lies on the far right side relative to the straight line. Variables appearing on the straight line indicate that their effect on the dependent variable is not significant. On the other hand, the further the variable lies from the straight line, the more significant effect it has on the dependent variable [20]. Hence, variable A (buffer pH) is the most significant variable in the first data set. However, the Pareto chart (Fig. 1c) shows that the effects of the independent variables, including pH, are still lower than the Bonferroni and the t-value limits. This means that these variables are insignificant [20]. A possible reason could be due to the large noise caused by a very small amount of enzyme present in the 20 mg immobilized sample and too short reaction time. Hence, 100 mg immobilized sample and 3 hours of esterification time were used in the following esterification study.

![Surface plot](image1.png)

![Normal probability plot](image2.png)

![Pareto chart](image3.png)

**Fig. 1.** Analysis of the first data set (Independent variables were buffer pH (6 – 7), celite:CRL ratio (8:1 – 4:1), 3 days drying time. The titration was performed using 20 mg of immobilized CRL, with reaction time of 2 hours): a. Surface plot, b. Normal probability plot, c. Pareto chart.
In the second data set, the same immobilized CRL samples as in the first data set were used for the esterification study. The difference is that the esterification reaction was performed for 3 hours using 100 mg of immobilized CRL for each sample. The measurements were done in triplicate. Here, the significant model can be obtained by selecting buffer pH (A) and celite to CRL ratio (B) as the important variables. These are shown from the p-values of the model and the variables in the ANOVA test (Table 1). When the interaction variable (AB) is considered in the equation, the model becomes insignificant.

**Table 1.** ANOVA result of the second data set (Independent variables were buffer pH (6 – 7), celite:CRL ratio (8:1 – 4:1), 3 days drying time. The esterification was performed using 100 mg of immobilized CRL, with a reaction time of 3 hours).

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>6.649E-6</td>
<td>2</td>
<td>3.324E-6</td>
<td>710.98</td>
<td>0.0265</td>
</tr>
<tr>
<td>A – pH</td>
<td>3.815E-6</td>
<td>1</td>
<td>3.815E-6</td>
<td>816.01</td>
<td>0.0223</td>
</tr>
<tr>
<td>B – celite:CRL</td>
<td>2.833E-6</td>
<td>1</td>
<td>2.833E-6</td>
<td>605.96</td>
<td>0.0258</td>
</tr>
</tbody>
</table>

To justify the significant variables affecting the esterification activity of the immobilized CRL, some more immobilization studies were performed by altering the independent variables. However, the results were different in each data set. The independent variables variations and the resulting significant variables in the second and the following data sets are summarized in Table 2, and the Pareto charts are shown in Fig. 2.

**Table 2.** Summary of variations used in data set number 2 – 5 and the resulting significant variables obtained from ANOVA test. The esterification activity was assessed by titration.

<table>
<thead>
<tr>
<th>Data set number</th>
<th>Variations in the immobilization process</th>
<th>Titrant in the titration</th>
<th>Significant variable(s) from the ANOVA test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Celite:CRL ratio</td>
<td>Drying time</td>
</tr>
<tr>
<td>2</td>
<td>6 – 7</td>
<td>4:1 - 8:1</td>
<td>3 days</td>
</tr>
<tr>
<td>3</td>
<td>6 – 7</td>
<td>4:1 - 8:1</td>
<td>1 day with 3x30 minutes vacuum</td>
</tr>
<tr>
<td>4</td>
<td>6 – 7</td>
<td>4:1 - 8:1</td>
<td>1 day with 3x30 minutes vacuum</td>
</tr>
<tr>
<td>5</td>
<td>6 – 8</td>
<td>4:1 - 8:1</td>
<td>2 – 3 days</td>
</tr>
</tbody>
</table>
Fig. 2. Pareto charts for data set number 2 (a), 3 (b), 4 (c), and 5 (d). Variations in each data set are shown in Table 2. The orange bar means the variable gives a positive effect; the blue bar indicates the variable effect is negative.

The Pareto charts (Fig.2) and the ANOVA test results (Table 2, complete data not shown) were in line in deducting the significant variable(s). Here, the significant variable(s) changed as the variation of the independent variable or the titrant was altered. When the drying time was reduced to 1 day (data set 2 vs. 3 in Table 2, Fig.2a vs. 2b), the effect of buffer pH became insignificant. Another problem is that the direction of the effect of the celite:CRL ratio was reversed. This can be seen from the color of the bars in the Pareto chart, where the orange and blue bars indicate positive and negative effects, respectively. In Fig.2a, celite:CRL ratio gave a negative effect on the esterification activity (blue), while in Fig.2b, its effect was positive (orange). The surface plot (Fig. 3) also agrees. In Fig. 3a (2nd data set), a lower celite:CRL ratio gave higher esterification activity of the immobilized CRL, while in the 3rd data set, higher esterification activity was obtained using a higher celite:CRL ratio (Fig. 3b).
Fig. 2. Pareto charts for data set number 2 (a), 3 (b), 4 (c), and 5 (d). Variations in each data set are shown in Table 2. The orange bar means the variable gives a positive effect; the blue bar indicates the variable effect is negative. The Pareto charts (Fig. 2) and the ANOVA test results (Table 2, complete data not shown) were in line in deducting the significant variable(s). Here, the significant variable(s) changed as the variation of the independent variable or the titrant was altered. When the drying time was reduced to 1 day (data set 2 vs. 3 in Table 2, Fig. 2a vs. 2b), the effect of buffer pH became insignificant. Another problem is that the direction of the effect of the celite:CRL ratio was reversed. This can be seen from the color of the bars in the Pareto chart, where the orange and blue bars indicate positive and negative effects, respectively. In Fig. 2a, celite:CRL ratio gave a negative effect on the esterification activity (blue), while in Fig. 2b, its effect was positive (orange). The surface plot (Fig. 3) also agrees. In Fig. 3a (2nd data set), a lower celite:CRL ratio gave higher esterification activity of the immobilized CRL, while in the 3rd data set, higher esterification activity was obtained using a higher celite:CRL ratio (Fig. 3b).

In an attempt to address this issue, for the 4th data set, the esterification reactions of the third data set were redone, but the titrations were performed using ethanolic NaOH. Although many esterification activity tests were done using NaOH solution (in water) as the titrant [6,17,19], alcoholic NaOH solution could also be used [18]. Compared to water, ethanol has better miscibility with the esterification mixture used in this study, i.e., n-butanol, butyl butyrate, and isoctane. Butyric acid, indeed, is the only compound in the reaction system that has good solubility in water. Hence, when the titrant is NaOH solution (in water), there might be some partition of butyric acid between the two phases present. Using ethanolic NaOH, only one (dominant) phase would be formed because butyric acid is also miscible with ethanol. Thus, the partition of butyric acid would be minimized, and the titration is expected to be more accurate.

Apparently, in the 4th data set (Fig. 2c), the direction of the celite:CRL ratio effect is similar to the 3rd data set (Fig. 2b) (orange colored). This means that the celite:CRL ratio has a positive effect on the esterification activity of the immobilized CRL. Comparing data sets 3 (Fig. 3b) and 4 (Fig. 3c), with ethanolic NaOH, the esterification activity of the samples was higher than when the titration was performed using NaOH solution, possibly due to better miscibility of the available compounds. However, in this data set, the significant variable changed again to pH (Fig. 2c).

To do another cross-check, a fifth data set was performed. In the previous data sets, the effect of pH was always positive, and the inflection point has not been observed.
Accordingly, the upper limit of the pH variation in the fifth experiment was increased to 8. The drying time was also varied as 2–3 days to ensure enough drying. 2^3 factorial design with 2 center points per block was used, requiring 12 data points. For each data point, the esterification and titration studies were done in duplicate. The titration was carried out using ethanolic NaOH.

A significant model for the 5th data set was obtained by omitting the drying time variable (variable C) from the equation as it has practically no effect on the esterification activity. The variables that allow the obtainment of a significant model for this data set are shown in Fig 2d. The ANOVA result can be seen in Table 3. The significant variable again changed to be celite:CRL ratio and the interaction variables were not as significant (p-value > 0.05).

### Table 3. ANOVA results of the fifth data set (Independent variables were buffer pH (A), celite:CRL ratio (B), and drying time (C). The titration was performed using ethanolic NaOH).

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>6.423E-5</td>
<td>5</td>
<td>1.285E-5</td>
<td>5.12</td>
<td>0.036</td>
</tr>
<tr>
<td>A-pH</td>
<td>1.266E-5</td>
<td>1</td>
<td>1.266E-5</td>
<td>5.05</td>
<td>0.066</td>
</tr>
<tr>
<td>B-Celite:CRL</td>
<td>1.734E-5</td>
<td>1</td>
<td>1.734E-5</td>
<td>6.92</td>
<td>0.039</td>
</tr>
<tr>
<td>AB</td>
<td>5.126E-6</td>
<td>1</td>
<td>5.126E-6</td>
<td>2.04</td>
<td>0.203</td>
</tr>
<tr>
<td>AC</td>
<td>1.474E-5</td>
<td>1</td>
<td>1.474E-5</td>
<td>5.88</td>
<td>0.052</td>
</tr>
<tr>
<td>ABC</td>
<td>1.435E-5</td>
<td>1</td>
<td>1.435E-5</td>
<td>5.73</td>
<td>0.054</td>
</tr>
</tbody>
</table>

Thus far, the significant variable(s) were always inconsistent between the data sets. In this study, the titrants used, i.e., the NaOH solution and ethanolic NaOH, were set at a concentration of ± 0.05 M. Too concentrated and too dilute titrants need to be avoided in a titration analysis. The former would cause difficult end-point distinctions among samples, especially when only a small amount of titrate is present, such as in this study. The latter is essential, especially when NaOH solution (with water as the solvent) is used due to the non-aqueous nature of the esterification reaction. Too high an amount of water could reverse the reaction into hydrolytic reaction where the formed butyl butyrate is reacted back to produce butyric acid and n-butanol. This would bias the titration result because the esterification activity was assessed indirectly from changes in the amount of butyric acid.

Another problem was observed when performing the titration using ethanolic NaOH. NaOH is less soluble in ethanol than in water. As time went on, the ethanolic NaOH became slightly cloudy, possibly due to the precipitation of the NaOH, even within a few hours. Hence, standardization was performed shortly before the titration. Yet slight changes in NaOH concentration could occur within the duration of the esterification-titration study, again biasing the calculated esterification activity. Hence, another analysis method needed to be used to eliminate these biases.

#### 3.2 Assessment of Esterification Activity Using GC-MS as the Method of Analysis

Using GC-MS, the butyl butyrate product can be directly identified. Here, dodecane was also used as the internal standard to ensure that the butyl butyrate signal was comparable between samples. 50 μL dodecane was added to each reaction mixture and vortexed prior to GC-MS analysis. The ideal internal standard would be to use a more similar compound to butyl
butyrate, such as pentyl pentanoate, for example. However, no other ester was available at the time of analysis. Dodecane was chosen because it is non-reactive towards the reaction mixture, and the chain length is somewhat similar to butyl butyrate.

As a more direct method of analysis, a more confident result can be obtained when the esterification product is analyzed using GC-MS than with titration. This also allowed us to screen for better physical adsorption immobilization procedures among the methods used in previous studies [21-26]. As such, suspicion that the immobilization method used was not good enough could be minimized. The results of the methods screening are shown in Fig. 4.

Two modes of contact between CRL, celite, and buffer were tested, i.e. to mix the CRL, small amount of buffer and celite within a short time on a Petri dish (termed “directly mixed” in Fig.4a) and to pre-mix CRL with buffer to form CRL solution before adding the celite (termed “CRL solution” in Fig.4a). Higher esterification activity was obtained when CRL was dissolved in buffer prior to the immobilization (Fig.4a, left). To recover the immobilized CRL after the immobilization process, simply pouring the suspension over a Petri dish continued by drying in a desiccator was better than filtering the suspension before drying (Fig.4b). The former method would allow more enzyme to be adsorbed onto celite during the drying process. Prewashing the celite with distilled water and buffer solution [23] was better than using unwashed celite (Fig.4c). This was initially unexpected because the celite used was commercial and reagent grade Celite-545 AW, and the pH of the distilled water and the buffer did not change after each wash of the celite. Lastly, using bulk CRL solution (mixing the CRL with buffer and using it directly for immobilization) was better than using the supernatant of the bulk CRL solution (the supernatant is obtained by centrifuging the bulk CRL solution) (Fig.4d).

![Fig. 4. Results of methods screening of the immobilization procedures. The compared procedures were the mode of contact during the immobilization process (Fig.4a), the mode of sample recovery of the immobilized enzyme (Fig.4b), using prewashed versus unwashed celite (Fig.4c) and using bulk CRL solution (CRL + buffer) versus using the supernatant of bulk CRL solution (Fig.4d).](image)

A summary of the optimized immobilization procedure is described in the Experimental Section subheading “GC-MS As The Analysis Method of The Esterification Results”. This method was carried out to immobilize CRL for which the esterification activity was analyzed using GC-MS. With this procedure, several more variables are expected to affect the esterification activity of the immobilized CRL. These variables are summarized in Table 4. The fractional factorial design of $2^{7-3}$ was used to screen the important variables.
Table 4. Variables expected to influence the esterification activity of the immobilized CRL and the range used in this study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Possible variables that affect the esterification activity of the immobilized CRL</th>
<th>Range used</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>pH of the buffer used for washing celite</td>
<td>6 – 8</td>
</tr>
<tr>
<td>B</td>
<td>Buffer volume used for washing celite (ml buffer/g celite)</td>
<td>10 – 30</td>
</tr>
<tr>
<td>C</td>
<td>Celite:CRL ratio</td>
<td>4:1 – 1:1</td>
</tr>
<tr>
<td>D</td>
<td>pH of the buffer used in the immobilization process to dissolve the CRL</td>
<td>6 – 8</td>
</tr>
<tr>
<td>E</td>
<td>Immobilization time (stirring time) (hours)</td>
<td>1 – 2</td>
</tr>
<tr>
<td>F</td>
<td>Stirring speed in the immobilization process (rpm)</td>
<td>200 – 400</td>
</tr>
<tr>
<td>G</td>
<td>Drying time (days)</td>
<td>2 – 4</td>
</tr>
</tbody>
</table>

The ANOVA result of the model that only considers variables having a p-value of < 0.05 is shown in Table 5. Fig.5a shows the Pareto chart of this model. The variables that were omitted from the model, from the least to the most significant, were E, F, C, BD, D, and A (a description of these variables is given in Table 4). This led to a substantial model having a p-value of 0.0001. The plot of predicted vs. actual esterification activity of the samples is shown in Fig.5b. Clearly, the model could predict the esterification activity relatively well.

Table 5. ANOVA results of the variables screening in which the esterification activity was analyzed using GCMS.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
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<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
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<td>9</td>
<td>1.547E-5</td>
<td>36.83</td>
<td>0.0001</td>
</tr>
<tr>
<td>B-V PBS for washing celite</td>
<td>3.182E-5</td>
<td>1</td>
<td>3.182E-5</td>
<td>75.74</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G-t drying</td>
<td>3.845E-5</td>
<td>1</td>
<td>3.845E-5</td>
<td>91.52</td>
<td>&lt;0.0001</td>
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<td>AB</td>
<td>2.605E-6</td>
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</tr>
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<td>AC</td>
<td>6.057E-6</td>
<td>1</td>
<td>6.057E-6</td>
<td>14.42</td>
<td>0.0090</td>
</tr>
<tr>
<td>AD</td>
<td>7.420E-6</td>
<td>1</td>
<td>7.420E-6</td>
<td>17.66</td>
<td>0.0057</td>
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<td>AE</td>
<td>1.424E-5</td>
<td>1</td>
<td>1.424E-5</td>
<td>33.89</td>
<td>0.0011</td>
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<tr>
<td>AF</td>
<td>1.125E-5</td>
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<td>1.125E-5</td>
<td>26.78</td>
<td>0.0021</td>
</tr>
<tr>
<td>AG</td>
<td>3.507E-6</td>
<td>1</td>
<td>3.507E-6</td>
<td>8.35</td>
<td>0.0277</td>
</tr>
<tr>
<td>ABD</td>
<td>2.391E-5</td>
<td>1</td>
<td>2.391E-5</td>
<td>56.91</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
The most significant variables affecting the esterification activity of the immobilized CRL were the drying time (variable G) and the buffer volume used for washing the celite (variable B). As can be seen from the Pareto chart (Fig.5a), drying time had a negative effect (blue bar) on the esterification activity. This means the shorter the drying time, the higher the esterification activity of the immobilized CRL. On the other hand, buffer volume (variable B) gave a positive effect that increasing buffer volume (within the range studied) for washing celite increased the esterification activity of the immobilized enzyme. These indicate that the higher activity is related to the higher water content in the immobilized CRL. In fact, enzymes need a certain amount of hydration water to remain active. The required amount of hydration water can not be generalized. However, with both lower and higher water content than the amount necessary, enzyme activity would decrease [27].

The buffer pH used for washing celite (variable A) appeared to be insignificant on its own. However, its interaction with all of the other variables (AB, AC, AD, AE, AF, AG, and ABD) was significant. Hence, all of the variables were indeed significant in affecting the esterification activity of the immobilized CRL. However, since titration was not very accurate due to the reasons described in the previous section, the significant variable appeared different in each data set because of the noises between the data.

4 Conclusions

In this study, titration analyses were shown to be inaccurate and inconsistent in determining significant variables affecting the esterification activity of immobilized CRL. The inaccuracy of titration-based analysis might be pronounced due to the non-aqueous nature of the esterification reaction. Possible causes have been discussed. When NaOH solution (water as the solvent) was used as the titrant, reverse reaction and or partition of the butyric acid between the phases might occur due to the non-aqueous nature of the esterification mixture. When the titrant was ethanolic NaOH, it might have been caused by the change in NaOH concentration during the study. Using GC-MS analysis, optimization of the immobilization procedure could be performed. More independent variables possibly affecting the esterification activity of the immobilized CRL could thus be considered using the optimized method. The most significant variables were the buffer volume used to prewash the celite and the time required to dry the immobilized samples. Interactions between buffer pH used to prewashed celite and all the other variables (AB, AC, AD, AE, AF, AG, and ABD) were also significant in affecting the esterification activity of the immobilized CRL.
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References


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