Preparation of Diacylglycerol-enriched Rice Bran Oil by Lipase-catalyzed Deacidification in Packed-bed Reactors by Continuous Dehydration

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Abstract: Diacylglycerol-enriched rice bran oil (RBO-DAG) was produced by deacidification of high-acid rice bran oil (RBO) with glycerol (Gly) using Lipozyme RM IM by continuous dehydration by combination of two enzyme columns (column 1 and 3, used for deacidification) with one molecular sieves column (column 2, used for dehydration). The conditions for three columns were respectively optimized. Response surface methodology (RSM) was used to optimize the conditions of column 1. The content of DAG and conversion of free fatty acid (FFA) were used as indicators and the effects of the enzyme load (8-12 g), flow rate (0.3-0.6 mL/min), substrate molar ratio (4-6) and reaction temperature (55-75°C) were investigated. The content of DAG and conversion of FFA were significantly correlated to the flow rate and substrate molar ratio. Most desirable conditions of the reaction with respect to the maximal DAG content and FFA conversion was attained under the residence time of 40 min, substrate molar ratio of 5.52 (Gly: RBO) and temperature of 66°C. The conditions for column 2 were investigated by varying molecular sieves load and flow rate, and the maximal dehydration rate of 85.22% was obtained under the optimal conditions. For column 3, the optimum conditions were obtained as: flow rate, 0.2 mL/min; temperature, 65°C, and the content of DAG and FFA were 38.99% and 3.04%, respectively under these conditions. The catalytic activity of the lipase was stable in twelve continuous operations with 83.22% of its original ability, demonstrating its potential in the continuous packed-bed reactors (PBRs) system. These results showed that packed-bed reactors combined with continuous deacidification and dehydration in one system had great value in industrial production for high-acid RBO with the improved conversion rate.

Key words: diacylglycerol, high-acid rice bran oil, lipase-catalyzed, continuous dehydration, molecular sieves, packed bed reactor, Lipozyme RM IM

1 Introduction

Diacylglycerol (DAG), with isomers of sn-1, 3-DAG and sn-1, 2 (or 2, 3) -DAG, occurs naturally in various edible oils and fats as a minor natural component¹,². Nutritional and functional effects of DAG have been demonstrated in recent studies. DAG, particularly sn-1, 3- DAG, has been identified with the beneficial effects to decrease the post-prandial triacylglycerol(TAG) levels in serum³-⁶ and control body weight⁷,⁸, while having a similar energy value and absorption coefficient to TAG⁹. Labelled with “Generally Recognized as Safe” (GRAS), DAG oil was available to consumers as a “Food for Specified Health Use” (FOSHU) since 1999 by Kao Corporation of Japan¹⁰. Substantial studies have been done on the production of DAG focusing on enzymatic and chemical approach through esterification⁵-¹², glycerolysis¹³,¹⁴ and partial hydrolysis¹⁵.

At present, annual output of rice bran oil (RBO) in China was about 700,000 tons. It contains 4.2% unsaponifiable matter including phytosterols, γ-oryzanol, tocopherols, toco-trienols, polyphenols and squalene, which have been
proved to have the ability of decreasing harmful LDL cholesterol\(^{(10)}\). However, due to a high free fatty acid (FFA) content varying from about 15% to 40% in crude rice bran oil\(^{(16)}\), harsh refining conditions are thus required if traditional refining process such as physical or chemical refining processes are used, which results in the large loss of neutral fat and minor compounds\(^{(17)}\). Therefore, enzymatic deacidification was chosen for high-acid RBO by conversion of FFA into monoacylglycerol (MAG)\(^{(18)}\), DAG\(^{(19, 20)}\) and TAG\(^{(21, 22)}\). Its obvious advantage is to improve the yield and increase the production in a comparable mild condition. Based on previous studies, both batch and packed-bed reactors were used in the enzymatic deacidification. Continuous packed-bed reactors (PBRs) were widely used with obvious benefits compared to batch reactor in protecting the enzyme in a comparable gentle manner. It can also improve the reaction efficiency and supply an efficient and simple method in industrial production. Numerous works have validly confirmed its advantage based on continuous enzyme reaction in production of MAG\(^{(23)}\), DAG\(^{(24)}\), TAG\(^{(24)}\) and human milk fat substitutes\(^{(25)}\). Recycling of the enzyme was also easier in the continuous system. Nonetheless, approach which covers the field of continuous reaction by packed-bed reactors incorporation of continuous dehydration has not been reported to date.

Based on previous findings, the byproduct water played an important role in deacidification during the esterification reaction and a final mixture of high FFA concentration could be resulted by high water content in the reaction system. The reason was that the equilibrium was inhibited due to the accumulation of water, which was responsible for the decrease in the conversion. Moving water out of the system is a critical to improve the conversion rate. However, it is difficult to control the content of water at a desirable level in the packed-bed reactor as it is well-known that water is essential for the enzyme to control its activity. Addition of molecular sieves had been proved to be an efficient method with a good result in the system of esterification reactions\(^{(26, 27)}\). Nonetheless, molecular sieves have not been used in the deacidification of RBO to produce diacylglycerol-enriched rice bran oil (RBO-DAG) in packed-bed reactors.

In this study, RBO-DAG was produced by deacidification of high-acid rice bran oil with glycerol (Gly) using Lipozyme RM IM in a continuous dehydration system by packed-bed reactors with two enzyme columns (column 1 and 3, used for deacidification) and one molecular sieves column (column 2, used for dehydration). The conditions for three columns were respectively optimized. Response surface methodology (RSM) was applied to optimize the conditions of the first deacidification column with the flow rate, substrate molar ratio, and reaction temperature as the parameters and the content of DAG and the conversion of FFA as indicators. The dehydration rate of the second molecular sieves column was also investigated by variation of the molecular sieves load and flow rate, and as for the column 3, flow rate and temperature were chosen as parameters.

2 Materials and methods

2.1 Materials

Crude high-acid rice bran oil was purchased from Dele-kang Food Co., Ltd. (Zhejiang, China), Lipozyme RM IM (immobilized lipase from Rhizomucor miehei, 275 IUN/g catalytic activity) was kindly donated by Novozymes A/S (Bagsvaerd, Denmark), 4Å-sodium molecular sieves (activated in Muffle furnace at 550 ± 10°C for about 2h before using). Glycerol, anhydrous ether, 95% ethanol and acetic acid were obtained from National Pharma-ceutical Group Corporation (Shanghai, China). Acetonitrile and dichloro-methane were of HPLC purity bought from J&K SCIENTIFIFIC LTD. All other reagents used were of analytical grade.

2.2 Experimental Design

A three-level three-factor Box–Behken design was carried out in this study, requiring 17 experiments (Table 1). The three factors and their various levels were flow rate (Fr, 0.3-0.6 mL/min), substrate molar ratio (Sr, 4-6 glycerol and high-acid rice bran oil) and reaction temperature (Rtemp, 55-75°C). The responses were the content of DAG (wt %) and the conversion of FFA. Table 1 conveys the independent factor (Xi), levels and experimental design by using of coded and uncoded parameters.

2.3 Packed Bed Reactor and Continuous Reaction

Crude RBO was pretreated by degumming, dewaxing, and bleaching before enzyme catalyzed reaction. The content of DAG and FFA in the pretreated high-acid RBO was about 0.76% and 18.00%, respectively. A brief description of the united reactors is illustrated in Fig. 1.

The amounts of Lipozyme RM IM and the molecular sieves packed into the jacket columns (3, 6 and 9 in Fig. 1) were 10.0 g, 20.0 g and 10.0 g. The parameters of the columns were 30 cm in length, 1.6 cm in inner and 2.6 cm in outer diameter (designed by Shanghai Xinyi Glasses Instrument Co., Ltd., China). Methods of wet-(used for enzyme columns, 3 and 9 in Fig. 1) and dry-packing (used for molecular sieves column, 6 in Fig. 1) were carried out to fill the columns, respectively. Reactants which composed of glycerol and high-acid rice bran oil was thoroughly mixed under magnetic stirring and preheated to 60-65°C. A series glycerol-to-oil molar ratios between 0.5-6 were chosen for production. Pumps (DHL-A, Shanghai Huxi Analysis Instrument Factory Co., Ltd., China) were occupied to get each mixture of the set temperature downwards the first enzyme column for first-deacidification with the
controlled flow rate in range of 0.3-0.6 mL/min. The temperature of the first enzyme column was maintained at the same level according to the prior mixture by circulating water bath. Therefore, dehydration oil was attained from the first-deacidification product after the adsorption of the molecular sieves at the rate of 0.3-0.7 mL/min, which occurring in the second column. Thereafter the mixture was pumped to the third column at a series rate of 0.2-0.6 mL/min for second-deacidification. The reaction of this operation was similar to the first column with the temperature of 45-75°C. Prior to the sampling, 54 g of product was discarded, which equivalent to three void volumes of the enzyme bed. Furthermore, continuously and consecutively experiments were carried out to validly certify the stability of the condition and the enzyme with the method described above.

Table 1  Experimental design and results of content of DAG and conversion of FFA as affected by the flow rate, substrate molar ratio and reaction temperature.

<table>
<thead>
<tr>
<th>Treatment No. ( ^a )</th>
<th>Flow rate (ml/min)</th>
<th>Substrate molar ratio ( ^b )</th>
<th>Temperature ( ^b ) (ºC)</th>
<th>Content of DAG (%)</th>
<th>Conversion of FFA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0(0.45)</td>
<td>-1(4)</td>
<td>-1(55)</td>
<td>15.97</td>
<td>44.81</td>
</tr>
<tr>
<td>2</td>
<td>0(0.45)</td>
<td>0(5)</td>
<td>0(65)</td>
<td>21.78</td>
<td>55.35</td>
</tr>
<tr>
<td>3</td>
<td>1(0.6)</td>
<td>0(5)</td>
<td>1(75)</td>
<td>14.14</td>
<td>39.05</td>
</tr>
<tr>
<td>4</td>
<td>0(0.45)</td>
<td>0(5)</td>
<td>0(65)</td>
<td>23.36</td>
<td>58.75</td>
</tr>
<tr>
<td>5</td>
<td>1(0.6)</td>
<td>1(6)</td>
<td>0(65)</td>
<td>16.84</td>
<td>44.98</td>
</tr>
<tr>
<td>6</td>
<td>0(0.45)</td>
<td>0(5)</td>
<td>0(65)</td>
<td>21.94</td>
<td>56.22</td>
</tr>
<tr>
<td>7</td>
<td>-1(0.3)</td>
<td>0(5)</td>
<td>-1(55)</td>
<td>19.01</td>
<td>52.46</td>
</tr>
<tr>
<td>8</td>
<td>-1(0.3)</td>
<td>1(6)</td>
<td>0(65)</td>
<td>23.84</td>
<td>62.86</td>
</tr>
<tr>
<td>9</td>
<td>0(0.45)</td>
<td>-1(4)</td>
<td>1(75)</td>
<td>18.34</td>
<td>45.04</td>
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<tr>
<td>10</td>
<td>0(0.45)</td>
<td>1(6)</td>
<td>-1(55)</td>
<td>17.99</td>
<td>49.24</td>
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<tr>
<td>11</td>
<td>0(0.45)</td>
<td>0(5)</td>
<td>0(65)</td>
<td>21.45</td>
<td>53.82</td>
</tr>
<tr>
<td>12</td>
<td>1(0.6)</td>
<td>0(5)</td>
<td>-1(55)</td>
<td>12.81</td>
<td>33.77</td>
</tr>
<tr>
<td>13</td>
<td>0(0.45)</td>
<td>1(6)</td>
<td>1(75)</td>
<td>19.57</td>
<td>50.57</td>
</tr>
<tr>
<td>14</td>
<td>-0(0.3)</td>
<td>0(5)</td>
<td>1(75)</td>
<td>21.65</td>
<td>54.38</td>
</tr>
<tr>
<td>15</td>
<td>1(0.6)</td>
<td>-1(4)</td>
<td>0(65)</td>
<td>13.28</td>
<td>39.71</td>
</tr>
<tr>
<td>16</td>
<td>0(0.45)</td>
<td>0(5)</td>
<td>0(65)</td>
<td>22.47</td>
<td>57.44</td>
</tr>
<tr>
<td>17</td>
<td>-1(0.3)</td>
<td>-1(4)</td>
<td>0(65)</td>
<td>22.41</td>
<td>56.23</td>
</tr>
</tbody>
</table>

\( ^a \) Treatments were run in randomized order

\( ^b \) Substrate molar ratio (Glycerol: RBO)

2.4 Analysis of Glycerides

RBO-DAG profiles was analyzed by a reversed-phase high performance liquid chromatography (RP-HPLC, Waters, America) system with an evaporative light-scattering detector (ELSD, Alltech, USA) setting at 75°C. Separation of the samples were carried on a Lichrospher C18 column (250×4.6 mm i.d., 5 μm, Hanbon Science & Technology Co., Ltd., Jiangsu, China) with the selected temperature of 40°C through gradient elution. Mobile phases combined of A (acetonitrile: acetic acid = 99.95:0.05, v/v), B (dichloromethane) and C (water) were applied at the flow rate of 1 mL/min. Flow rate of the nitrogen nebulizer gas was 1.7 L/min. Sample, concentration of 5 mg/mL in acetone, was automatically injected after filtration (13×0.22 μm) and the injection volume was 10 μL. The details of the gradient were described previously by Zhong et al. The process lasts for 42 min and responses of ELSD were transformed into the content of the separated MAG, DAG and TAG, as area percent of the whole sample. Duplicate evaluations were made.

2.5 Determination of the Content of FFA and Water

The content of FFA and water (wt.%) were determined according to AOCS official methods. Analyses were made in duplicate.

2.6 Statistical Analysis

The selection of the combination of values on independent parameters and their levels were based on the Box–Behnken design. The experiments were performed in a totally randomized order with the actual variables encoded. Regression analysis, the coefficient of determination ($R^2$) and analysis of variance (ANOVA) were evaluated to see the fitting of the quadratic polynomial mode. A software package of Design Expert (Ver. 8.0.5b, State-Ease Inc. Statistics Made Easy, Minneapolis, Minnesota) was used to analyze the experimental dates. The polynomial equation was represented as:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j$$

Where $Y$ is predicted response (% yield), $X_i$ and $X_j$ are the independent variables, $\beta_0, \beta_i, \beta_{ij}$ and $\beta_{ij}$ are constant regression coefficients.

3 Results and Discussion

3.1 Optimization of Deacidification Conditions for Column 1

Lipozyme RM IM has been proved to have a better ability to enrich DAG when MAG was used as acyl acceptor in deacidification of high-acid RBO compared with Novozyme 435 and Lipozyme TL IM. However, Kristensen, J. B. emphasized that glycerol was a better acyl acceptor instead of MAG to produce DAG oil. The content of DAG and FFA in the pretreated high-acid RBO was about 0.76% and 18.00%, respectively. Effects of the four selected factors on the content of DAG and FFA in the final RBO-DAG are shown in Fig. 2. The content of FFA decreased with the increase of enzyme load and substrate molar ratio, while it was proportional to flow rate. When it came to the temperature of 65°C, a minimum content of FFA was attained under the conditions of flow rate of 0.45 mL/min and substrate molar ratio of 5. We observed a decrease in FFA concentration in the range of 45-65°C and it increased during the temperature of 65 to 75°C. The reason for the variation could be explained by the fact that the catalytic performance was affected by the temperature and a higher temperature than 65°C may lead to the decreasing of the catalytic performance. Effects of the enzyme load and substrate molar ratio on the content of DAG were contrary to the content of FFA. It increased as the increasing of enzyme load and substrate molar ratio. The content of DAG in the product decreased with the increase of the flow rate due to the lack of reaction time. A maximal DAG concentration was found at the temperature of 65°C, which was similar to the relationship between temperature and the content of FFA. Therefore, response surface methodology was used to optimize the conditions of column 1 and intervals which were based on the efficiency and desirability of the content of DAG and FFA were selected as follows: flow rate, 0.3-0.6 mL/min; substrate molar ratio, 4-6; temperature, 55-75°C.

3.2 Model Fitting and ANOVA

On the basis of the results of the first-deacidification conditions of column 1, RSM was used to further acquire the optimal conditions. The results that obtained based on the experiments are shown in Table 1. Under the conditions, the DAG content and FFA conversion varied from 1.28% to 23.84%, and 33.77% to 62.86%, respectively. Multiple regression technique was used to develop the quadratic polynomial model for the content of DAG and conversion of FFA:

$$Y_1 = -22.20 - 3.37X_1 + 1.03X_2 + 0.99X_3 + 0.53X_1X_2 - 0.33X_1X_3 - 0.2X_2X_3 - 2.09X_1^2 - 1.02X_2^2 - 3.21X_3^2$$  \(Y_1\)

$$Y_2 = 56.31 - 8.55X_1 + 3.11X_2 + 1.47X_3 - 0.34X_1X_2 + 0.84X_1X_3 - 0.47X_2X_3 - 3.56X_1^2 - 1.81X_2^2 - 7.84X_3^2$$  \(Y_2\)

$Y_1$ and $Y_2$ are the predicted values for the content of DAG (in percent) and the conversion of FFA, respectively, and $X_1$, $X_2$, and $X_3$ are the coded variables as described in Table 1.
Fig. 2  Effects of the enzyme load, flow rate (residence time), substrate molar ratio and reaction temperature on the content of DAG and the content of FFA. a. flow rate, 0.45 ml/min; substrate molar ratio, 5; temperature, 65°C. b. enzyme load, 10 g; substrate molar ratio, 5; temperature, 65°C. c. enzyme load, 10 g; flow rate, 0.45 mL/min; temperature, 65°C. d. enzyme load, 10 g; flow rate, 0.45 mL/min; substrate molar ratio, 5.

Table 2  Regression analysis of variance for response surface quadratic model (ANOVA) pertaining to the predicted content of DAG and the conversion of FFA.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean of square</th>
<th>F value</th>
<th>Prob &gt; F²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content of DAG (%)</td>
<td>201.58</td>
<td>9</td>
<td>22.40</td>
<td>47.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>3.34</td>
<td>7</td>
<td>0.48</td>
<td></td>
<td></td>
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<tr>
<td>Lack of fit</td>
<td>1.11</td>
<td></td>
<td>0.37</td>
<td>0.67</td>
<td>0.6157</td>
</tr>
<tr>
<td>Pure error</td>
<td>2.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>204.91</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient of variation = 3.59% (R² = 0.9837)</td>
<td></td>
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</tr>
</tbody>
</table>

Conversion of FFA (%)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean of square</th>
<th>F value</th>
<th>Prob &gt; F²</th>
</tr>
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<tr>
<td>Model</td>
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<td>9</td>
<td>114.98</td>
<td>52.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>15.33</td>
<td>7</td>
<td>2.19</td>
<td></td>
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<tr>
<td>Lack of fit</td>
<td>1.02</td>
<td></td>
<td>0.34</td>
<td>0.095</td>
<td>0.9587</td>
</tr>
<tr>
<td>Pure error</td>
<td>14.31</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>total</td>
<td>1050.11</td>
<td>16</td>
<td></td>
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<tr>
<td>Coefficient of variation = 2.95% (R² = 0.9854)</td>
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</table>
of lack of fit is 1.64, indicating that lack of fit is not significant relative to pure error. To examine the fitting of the model, the regression equation and determination coefficient ($R^2$) were analyzed. The values of the determination coefficient ($R^2$) were 0.9837 and 0.9854, which means that 98.8% of the sample variation in the RBO-DAG production was attributed to the independent variables and only less than 2% of the total variations were not explained by the model. The signal to noise ratio of 20.418, which was larger than 4, indicates an adequate signal and the reliability of the model. The value of the adjusted determination coefficient (Adj. $R^2 = 0.9628$) indicates a high significance of the model.33 Furthermore, a comparable lower value of the coefficient of variation (CV = 3.59%) also indicates a better dependability and precision of the experiments.

3.3 Optimization of Reaction and Model Verification

Based on the results of RSM analysis, desirable combination of the variables was predicted with respect to the content of DAG and the conversion of FFA by the use of the models. The obtained optimal conditions were described below: flow rate, 0.3 mL/min; molar ratio of 5.52; temperature at 66.1°C, with the DAG yield and FFA conversion of 23.93% and 62.54%, respectively. Verification tests were carried out to evaluate the reliability of the design. Products that contained high DAG yield of 23.93% were obtained at the optimum conditions and meanwhile the conversion of FFA was up to 62.41%. The results were in good correlation with predicted values and certified acceptability of the finding.

3.4 Optimization of Dehydration Conditions for Column 2

In the present study, we examined the approach of adding another column packed with molecular sieves to move out the water from the first-deacidification product and enhanced the conversion in the following reaction. The effects of the flow rate and amount of the 4Å-sodium molecular sieves on dehydration rate were shown in Fig. 3. With the addition of the 4Å-sodium molecular sieves up to 20.0g, a dehydration rate up to 74.32% and a final product with the water content of 0.143% was obtained. A series dehydration rates were obtained by varying the flow rate. Among the different flow rates, 0.3ml/min was selected as the optimal one with a water content decreasing from 0.839% to a satisfactory level of 0.143%. This observation confirmed the truth of 4Å-sodium molecular sieves as a distinguished absorbing agent by using of continuous packed bed reactor.

3.5 Optimization of Secondary Deacidification Conditions for Column 3

With the view to enrich the content of DAG and lower the content of FFA, a further step for deacidification was performed. The product from column 2 was pumped to column 3 and the results were shown in Fig. 4. A higher flow rate might damage the structure of the enzyme in the continuous system.34 Hence, we chose the flow rate varied from 0.2 to 0.6 mL/min for secondary deacidification by the fact that the first-deacidification RBO had a comparable low FFA content. In general, increased DAG and MAG contents were observed with prolonged residence time. The maximum content of DAG of the product was 39.55 wt%. During the final 30 min, the content of DAG was increased only 1.5 wt%. However, the content of FFA decreased from 4.03% to 3.16%. Therefore, a flow rate of 0.2 mL/min was beneficial to reach a satisfactory product from the practical point of view. The content of DAG increased to a maximal point at the temperature of 65°C and then decreased thereafter. The content of FFA was contrary to the DAG concentration and the lowest FFA content was obtained at 65°C.

Therefore, the optimal conditions of the first column were follows: flow rate at 0.3 mL/min; molar ratio of 5.52; temperature at 66.10°C. The content of DAG and the...
conversion of FFA were 23.93%, 62.54%, respectively. When it came to the second column, the optimal conditions for dehydration were the flow rate of 0.3 mL/min with molecular sieves of 20.0 g. The conditions for the second deacidification enzyme column with the highest content of DAG yield of 39.55 wt% and the lowest FFA content of 3.16% were obtained at 65°C with a flow rate of 0.2 mL/min. Compared to the results of previous workers\(^{20}\), the content of DAG was enrich 20% with a shorten time and the yield was about 10.5 g of RBO-DAG in an hour.

3.6 Evaluation of Lipozyme RM IM Stability in Packed-Bed Reactor

Operational stability and reusability of the commercial immobilized lipzyme RM IM were important parameters in the continuous system. For this purpose, consecutive packed-bed reactions were implemented under the optimized conditions by estimating the conversion of FFA and the results were shown in Fig. 5. Products were sampled during the consecutive reactions. The immobilized lipase was operated 12 times (one time for 16 hours) with excellent stability under the optimal conditions. The content of DAG was up to 19.92% and the FFA concentration was 8.72% when it came to the 12th reaction with 83% of its original catalytic activity, which proved the outstanding stability of the lipase. The characteristic may benefit from the mild condition in packed-bed reactors which supply a comparable better situation as the enzyme was prevented from direct and intensive magnetic stirring compared to batch reactor. Similar observation was also found in the study of Song et al.\(^{20}\), who confirmed the fact that the enzyme was stable after 10 times without significant loss of activity in batch reactor.

In this study, RBO-DAG was produced by Lipozyme RM IM catalyzed deacidification of high-acid RBO with glycerol by continuous dehydration by combination of packed-bed columns. Column 1 and column 3 were enzyme columns that were used for the first and second deacidification, respectively. Column 2 was occupied for dehydration of the first-deacidification RBO filled with molecular sieves. RSM was successfully applied to model and optimize the first-deacidification column and the optimal conditions were: flow rate, 0.3 mL/min; molar ratio, 5.52; temperature, 66.10°C. Byproduct water can be efficiently moved out by column 2 packed with simple dry 4Å- sodium molecular sieves. The column could obtain maximal dehydration rate with the value of 85.22% at the conditions of molecular sieves load of 20.0 g and flow rate of 0.3 mL/min. The conditions of secondary deacidification were also optimized as 0.2 mL/min, 65°C and molar ratio of 5. Under these conditions, the product contained 39.55 wt% DAG and 3.16 wt% FFA. With respect to economic reasons, consecutive reaction tests were carried out to packed-bed reactors, which

![Fig. 4](image-url)

**Fig. 4** Effects of the flow rate (residence time) and temperature on the content of DAG and FFA. a. temperature, 65°C. b. flow rate, 0.3 mL/min.

![Fig. 5](image-url)

**Fig. 5** Effect of enzyme stability and reusability on the conversion of FFA: flow rate at 0.3 mL/min; substrate molar ratio of 5.52; temperature at 66.09°C.
showed the immobilized lipase could be operated 12 times with excellent stability under the optimal conditions. These results showed packed-bed reactors combined with continuous dehydration in one system to improve the conversion rate are advantageous for high-acid RBO, which has great potential industrial production.

Notes
The authors declare no competing financial interest.

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