Research Article

Modeling, Simulation, and Kinetic Studies of Solvent-Free Biosynthesis of Benzyl Acetate

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Solvent-free biosynthesis of benzyl acetate through immobilized lipase-mediated transesterification has been modeled and optimized through statistical integrated artificial intelligence approach. A nonlinear response surface model has been successfully developed based on central composite design with transesterification variables, namely, molarity of alcohol, reaction time, temperature, and immobilized lipase amount as input variables and molar conversion (%) as an output variable. Statistical integrated genetic algorithm optimization approach results in an optimized molar conversion of 96.32% with the predicted transesterification variables of 0.47 M alcohol molarity in a reaction time of 13.1 h, at 37.5°C using 13.31 U of immobilized lipase. Immobilized lipase withstands more than 98% relative activity up to 6 recycles and maintains 50% relative activity until 12 recycles. The kinetic constants of benzyl acetate, namely, \( K_m \) and \( V_{max} \) were found to be 310 mM and 0.10 mmol h\(^{-1}\) g\(^{-1}\), respectively.

1. Introduction

Flavour esters of short chain carboxylic acids and alcohols are among the most important and versatile components of natural flavors and fragrances and have a wide range of applications in food, beverage, cosmetic, and pharmaceutical sectors [1]. Knowing the health prospects of natural flavours, most of the customers are inclining towards the daily commodities which utilize biologically derived flavours (considered as “Natural Flavours”) instead of chemical ones [2]. Among different biotechnological processes, immobilized lipase-mediated transesterification under solvent-free conditions is one of the economically viable clean technology for flavor ester production with continuous mode of operation [3, 4]. The specificity of lipase, usage of mild reaction tempeartures, and solvent-free conditions making this process as immense commercial of interest and replacing the existed chemical catalyzed flavour ester synthesis [5]. Benzyl acetate is a short chain flavor ester found in plants such as jasmine, hyacinth, gardenias, and azaleas with the commercial value in flavour, food, and chemical industries. The global demand for benzyl acetate ranges between 5000 and 10,000 tons per annum. Covalent immobilization of lipases to insoluble polymeric supports aids in the industrial process economics by facilitating easy enzyme recovery from the reaction mixture [6]. The regio- and stereospecificities of immobilized lipase-mediated transesterification reactions enable the production of certain flavour compounds which are difficult to synthesize by chemical means in a sustainable manner and the products are considered as “natural.” Several researchers reported the utilization of these catalysts (immobilized lipases) for flavour ester synthesis. Some attempts have been also made for the synthesis of benzyl acetate in solvent-associated approach through chemical and biotechnological approaches. Amarnath et al. 2004 [7] reported the synthesis of benzyl acetate using polyaniline salts (Sb/Pd ratio of 0.7). Majumder et al. 2006 [8] utilized hexane as solvent, Lipozyme RM IM for the synthesis of benzyl acetate and investigated the effect of enzyme amount and substrate molar ratio on benzyl acetate yield. Synthesis of benzyl acetate under solvent-free conditions facilitate, the simplification of the downstream processing and reduced environmental hazards, and reduction of separation costs due to the nonrequirement of solvent recovery [9, 10]. In lipase-mediated synthesis of flavour esters,
the transesterification variables play an important role on
the final ester yield. Moreover, the reaction side product
water hampers the maximum ester yield by promoting the
reverse reaction, hydrolysis. To attain the optimal yields of
these flavour esters, having the knowledge of individual and
interaction effects of transesterification variables on ester
yield will be helpful to model and optimize the process.

In recent days, several researchers acknowledged the bet-
ter solvable approaches of statistical integrated evolutionary
optimization techniques in modeling and optimization of
different bioprocesses. This integrated approach works on
the statistical-based response surface methodology (RSM)
and artificial intelligence-based Genetic algorithm (GA)
approach [11]. RSM is a powerful mathematical modeling
approach which does not need the explicit expressions of
the physical meaning of the system or process under invest-
igation and develops nonparametric regression model. This
model approximates the functional relationships between
transesterification variables (input) and the molar conversion
(output and response) of the process using experimental data
[12] and helpful to estimate the optimal settings of input
variables to maximize the response. Artificial intelligence
based GA, developed by Holland, uses the Darwinian evolu-
tion concept of “survival of the fittest” to overcome the
local optima obstacle by attaining the global optima quickly
[13]. There have been a few attempts of using only statistical
techniques in lipase-mediated synthesis of flavour esters to
model and optimize the process but no attempts have been
found using statistical integrated evolutionary approaches.
A statistical approach was utilized to optimize the lipase.
Palatase catalyzed biosynthesis of flavour-active octanoic acid
esters from coconut cream and fuel oil as the biocatalyst with
the influential significance of temperature, time, and enzyme
amount on the ester yield [14, 15]. Esterification reactions
of butyl acetate and isoamyl acetate synthesis catalyzed by
Candida antarctica lipase B (Novozym 435) were also
optimized using response surface methodology [16, 17].

Hence, in the present study, the statistical integrated
artificial intelligence was utilized for the first time in mod-
eling and optimization of the immobilized lipase catalyzed
synthesis of food flavour ester under solvent-free condi-
tions. The significance of individual and combined effects
of various transesterification variables, namely, molarity of
alcohol, reaction time, temperature and immobilized enzyme
amount on molar conversion was studied through response
surface methodology and optimal conditions of the process
was determined through GA approach. The reusability and
kinetics of immobilized lipase in solvent-free system were
also investigated.

2. Materials and Methods

2.1. Microorganism and Chemicals. A well-known lipolytic
fungal strain Rhizopus oryzae 3562 was isolated from the local
soil of IIT Kharagpur and maintained on Potato Dextrose
Agar (PDA) medium. p-nitrophenyl pannitate (p-NPP) and
benzyl acetate standards were purchased from Sigma (USA).
All chemicals used were of AR grade and were procured from
Merck, Qualigens, and Himedia, India.

2.2. Production and Immobilization of Lipase. Lipase was
produced using wheat bran as a substrate through solid-state
fermentation [18]. Lipase immobilization was carried out
using covalent attachment technique using activated silica
as immobilization matrix and was explained in our previous
work [19].

2.3. Lipase Assay and Protein Determination. Lipase assay
was done spectrophotometrically using pNPP as the substrate
[20]. One unit (U) of enzyme is defined as the amount of
enzyme that liberates one micromole of p-nitrophenol per
minute under the assay conditions. Total protein was esti-
mated using bovine serum albumin (BSA) as standard. [21].

2.4. Transesterification Reaction. Transesterification reaction
was carried out in screw-capped vials containing benzyl
alcohol in vinyl acetate (0.3–0.7 M), where vinyl acetate acts
as an acyl donor. Reaction was initiated by the addition of
immobilized R. oryzae 3562 lipase (8–16 U). Samples were
placed in an orbital shaker at 200 rpm and temperatures (30–
50°C) for a time interval of 8–16 h, along with the respective
controls (based on the central composite design and one
variable at time experiments).

2.5. GC Analysis. Aliquots of reaction mixture were with-
drawn periodically from reaction mixture and centrifuged at
1747 g for 10 min to remove the immobilized enzyme then
diluted with n-hexane (10 times). Synthesis of benzyl acetate
was analyzed by injecting the diluted aliquots of the reaction
mixture in a gas chromatograph (Agilent 6820). The column
temperature was kept at 100°C for 1 min, thereafter raised
to 180°C at the rate of 15°C/min and maintained at this
temperature for 2 min. The temperatures of both the injector
detector were set at 250°C. Nitrogen was used as a carrier
gas. The retention time of benzyl alcohol and benzyl acetate
was 3.2 min, and 4.4 min respectively.

2.6. Modelling Studies through RSM. Response surface meth-
odology (RSM) is a statistical model approach for empirical
modeling which evaluates the effect of individual and inter-
action effects of the process parameters on the corresponding
response value. These effects can be approximated by the
quadratic model equation through a sequence of designed
experiments [22]. Transesterification reaction variables such
as molarity of alcohol (M), reaction time (h), temperature
(°C), and immobilized enzyme amount (U) play an important
role in solvent-free synthesis of Benzyl acetate. The individual
and interaction effects of these parameters on the flavor ester
yield in terms of molar conversion (%) were studied by means
of a central composite design (with four variables at three
levels) of RSM using MINITAB 14 software. Range of these
transesterification was chosen through the one variable at a
time experiments and tabulated in Table 1. The set of experi-
ments executed was shown in Table 2. Results were analyzed
through the MINITAB 14 programs through the analysis
of variance (ANOVA) and significance tests, and obtained
RSM model was checked for adequacy through coefficient
of determination ($R^2$ and adjusted $R^2$) and deleted resid-
uals.
Table 1: Transesterification variables and their levels.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Notation</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol molarity (M)</td>
<td>$X_1$</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>Reaction time (h)</td>
<td>$X_2$</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>$X_3$</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Enzyme activity (U)</td>
<td>$X_4$</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

2.7. Artificial Intelligence-Based Optimization Approach. Simulation of the transesterification process through GA approach provides an accurate predicted result against the usual gradient-based optimization approaches. The parallel search pattern of GA makes the optimization task of choice for various industrial problems. In the present approach of binary coded GA (works on the mechanics of coping of the strings), three operations, namely, reproduction, crossover ($p_c$), and mutation ($p_m$) play an important role in creating a better solution. At first, the optimization approach starts with a randomly generated initial population ($N$) of strings (in the form of binary coded individuals). The resultant fitness of each individual string after each generation is evaluated with respect to the given molar conversion (objective function) using the reproduction operator. At the end of each cycle, a mating pool of good strings was selected from the fitness value of each population using reproduction operation. During crossover operation, the randomly selected mating pairs exchange properties between the two parents to form two children solutions. Mutation operation is used to avoid the local minima problem, which brings a local change to the solution. The sequence of these steps will repeat until the termination criteria met [13]. In the present attempt of maximization of benzyl acetate molar conversion (%), utilizing the duality concept the maximization problem was converted to minimization problem. An overall 40 bits (10-bits of each variable) GA-string was utilized in the search pattern.

3. Results and Discussion

In lipase-catalyzed transesterification reactions, different transesterification variables, namely, molarity of alcohol, reaction time, temperature, and enzyme amount play an important role on the final molar conversion [4]. In this study, central composite design of RSM including five factors with three levels was used to obtain a proper model for the immobilized lipase-mediated synthesis of flavour esters.

3.1. Model Development, Statistical Analysis, and Validation. Modeling task of immobilized lipase-mediated solvent-free synthesis of benzyl acetate has been carried out through running of suggested set of experimental runs of central composite design. The complete set of 27 experimental design matrix and its responses based on experimental runs and its predicted values of molar conversion proposed by CCD are given in Table 2, which facilitates in evaluating relationship between controllable experimental factors and observed results [23]. Based on these results, an empirical relationship between the responses and independent variables of immobilized lipase-catalyzed biosynthesis of benzyl acetate has been expressed using the following second-order polynomial equation in coded form:

$$
MC(\%) = 94.9260 - 0.4533X_1 + 3.3772X_2 
- 0.3994X_3 + 5.4272X_4 - 0.2244X_1X_2 
+ 0.5719X_1X_3 - 0.4806X_1X_4 - 0.5044X_2X_3 
- 2.5869X_2X_4 - 0.3331X_3X_4 - 2.1741X_1^2 
- 4.8891X_2^2 - 1.8891X_3^2 - 6.8891X_4^2
$$

where $X_1$, $X_2$, $X_3$, and $X_4$ represent the input process namely molarity of alcohol (M), reaction time (h), temperature (°C), and immobilized enzyme amount (U), respectively.

The $P$ values of significance test (Table 3) for $X_1$, $X_4$, $X_1^2$, $X_2^2$, $X_3^2$ and $X_2X_3$, are found to be less than 0.05 and are considered to have significant impact on the molar conversion (%) by considering 95% ($α = 0.05$) as a level of confidence. The $P$ value of the factors $X_1$, $X_3$, $X_1^2$, $X_2X_3$, $X_1X_3$, $X_1X_4$, $X_2X_3$ and $X_1X_4$ is found to be more than the confidence level (0.05), but their square terms $P$ value is found to be less than the confidence level which indicates its nonlinear relationship with the response. The significant contribution of the linear, square, and interaction terms towards the response has been also depicted through the ANOVA results (Table 4), where the $P$ values were seen to be less than the significance value level ($α = 0.05$) for all terms. Moreover, the good prediction accuracy and generalization ability of the predicted model has been visualized through close agreement of predicted and experimental values of the RSM design. The coefficient of multiple regression $R^2$ values and the adjusted $R^2$ values was found to be 98.6%, and 97.1% respectively, which indicate the fitness and adequacy of the model [24]. As there is no dramatic difference between $R^2$ and adj $R^2$, it can be assumed that the nonsignificant terms have not been included in the model. Furthermore, the analysis of deleted residuals showed that the deleted residuals were well within the acceptable limits of $+3$ and $-3$ with only one observation beyond the acceptable limits which can be considered as a case of outlier (Table 2).

The response surface plots (Figure 1) were analyzed to understand the interaction and influence of different transesterification variables on the molar conversion (%) of flavour ester. The response surface plots in Figures 1(a), 1(c), and 1(d) were both part of a parabolic cylinder, exhibiting a minimum and maximum ridge, respectively, in the investigated domain which indicates the nonlinear relationship of interaction variables on molar conversion. In these plots, the optimum values of both variable factors, such as the reaction time & alcohol molarity (Figure 1(a)), enzyme activity & alcohol molarity (Figure 1(c)), and enzyme activity & temperature (Figure 1(f)) could be analyzed by the saddle point or by determining the maxima formed by the $x$- and $y$-coordinates. Interaction effects of temperature with alcohol molarity & reaction time and enzyme activity & reaction time on molar
Table 2: Central composite design with the experimental, predicted responses and its R-studentized residuals.

<table>
<thead>
<tr>
<th>Run order</th>
<th>Input variables</th>
<th>Response (MC(%))</th>
<th>Predicted</th>
<th>R-studentized residual</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MA RT Temp EA</td>
<td>Exp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.3 12 40 12</td>
<td>90.98</td>
<td>93.21</td>
<td>−2.49</td>
</tr>
<tr>
<td>2</td>
<td>0.7 8 30 8</td>
<td>64.96</td>
<td>66.94</td>
<td>−2.77</td>
</tr>
<tr>
<td>3</td>
<td>0.3 16 30 8</td>
<td>80.67</td>
<td>80.96</td>
<td>−0.32</td>
</tr>
<tr>
<td>4</td>
<td>0.5 12 40 12</td>
<td>96.00</td>
<td>94.93</td>
<td>0.74</td>
</tr>
<tr>
<td>5</td>
<td>0.7 16 50 8</td>
<td>78.00</td>
<td>79.43</td>
<td>−1.73</td>
</tr>
<tr>
<td>6</td>
<td>0.3 16 50 8</td>
<td>79.56</td>
<td>78.68</td>
<td>0.99</td>
</tr>
<tr>
<td>7</td>
<td>0.5 12 40 8</td>
<td>82.00</td>
<td>82.61</td>
<td>−0.55</td>
</tr>
<tr>
<td>8</td>
<td>0.7 8 50 8</td>
<td>70.24</td>
<td>68.96</td>
<td>1.52</td>
</tr>
<tr>
<td>9</td>
<td>0.3 8 50 16</td>
<td>83.52</td>
<td>83.63</td>
<td>−0.12</td>
</tr>
<tr>
<td>10</td>
<td>0.5 12 50 12</td>
<td>92.00</td>
<td>92.64</td>
<td>−0.57</td>
</tr>
<tr>
<td>11</td>
<td>0.3 8 30 16</td>
<td>85.46</td>
<td>85.23</td>
<td>0.24</td>
</tr>
<tr>
<td>12</td>
<td>0.3 16 30 16</td>
<td>88.45</td>
<td>88.27</td>
<td>0.19</td>
</tr>
<tr>
<td>13</td>
<td>0.5 12 40 12</td>
<td>96.00</td>
<td>94.93</td>
<td>0.74</td>
</tr>
<tr>
<td>14</td>
<td>0.7 16 50 16</td>
<td>84.00</td>
<td>83.48</td>
<td>0.57</td>
</tr>
<tr>
<td>15</td>
<td>0.7 8 50 16</td>
<td>82.45</td>
<td>82.71</td>
<td>−0.39</td>
</tr>
<tr>
<td>16</td>
<td>0.3 12 40 12</td>
<td>96.00</td>
<td>94.93</td>
<td>0.74</td>
</tr>
<tr>
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<td>93.41</td>
<td>−1.37</td>
</tr>
<tr>
<td>18</td>
<td>0.5 12 40 16</td>
<td>93.00</td>
<td>93.36</td>
<td>−0.42</td>
</tr>
<tr>
<td>19</td>
<td>0.5 8 40 12</td>
<td>87.00</td>
<td>86.66</td>
<td>0.30</td>
</tr>
<tr>
<td>20</td>
<td>0.7 16 30 8</td>
<td>81.00</td>
<td>79.42</td>
<td>1.97</td>
</tr>
<tr>
<td>21</td>
<td>0.3 8 30 8</td>
<td>68.52</td>
<td>67.58</td>
<td>1.07</td>
</tr>
<tr>
<td>22</td>
<td>0.5 12 30 12</td>
<td>93.00</td>
<td>93.44</td>
<td>−0.39</td>
</tr>
<tr>
<td>23</td>
<td>0.3 16 50 16</td>
<td>85.43</td>
<td>84.65</td>
<td>0.86</td>
</tr>
<tr>
<td>24</td>
<td>0.7 8 30 16</td>
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<td>82.67</td>
<td>0.63</td>
</tr>
<tr>
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<td>67.31</td>
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</tr>
<tr>
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<td>84.00</td>
<td>84.81</td>
<td>−0.90</td>
</tr>
<tr>
<td>27</td>
<td>0.7 12 40 12</td>
<td>93.45</td>
<td>92.29</td>
<td>1.08</td>
</tr>
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</table>

Table 3: Results of significance test on the nonlinear model coefficients, standard errors, T statistics, and P values for the lipase activity (coded form).

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Terms</th>
<th>Standard coefficient</th>
<th>Error coefficient</th>
<th>T</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Constant</td>
<td>94.9260</td>
<td>0.5609</td>
<td>169.225</td>
<td>0.000</td>
</tr>
<tr>
<td>2</td>
<td>$X_1$</td>
<td>−0.4533</td>
<td>0.3588</td>
<td>−1.264</td>
<td>0.230</td>
</tr>
<tr>
<td>3</td>
<td>$X_2$</td>
<td>3.3772</td>
<td>0.3588</td>
<td>9.413</td>
<td>0.000</td>
</tr>
<tr>
<td>4</td>
<td>$X_3$</td>
<td>−0.3994</td>
<td>0.3588</td>
<td>−1.113</td>
<td>0.287</td>
</tr>
<tr>
<td>5</td>
<td>$X_4$</td>
<td>5.4272</td>
<td>0.3588</td>
<td>15.127</td>
<td>0.000</td>
</tr>
<tr>
<td>6</td>
<td>$X_1^2$</td>
<td>−2.1741</td>
<td>0.9492</td>
<td>−2.290</td>
<td>0.041</td>
</tr>
<tr>
<td>7</td>
<td>$X_2^2$</td>
<td>−4.8891</td>
<td>0.9492</td>
<td>−5.150</td>
<td>0.000</td>
</tr>
<tr>
<td>8</td>
<td>$X_4^2$</td>
<td>−1.8891</td>
<td>0.9492</td>
<td>−1.990</td>
<td>0.070</td>
</tr>
<tr>
<td>9</td>
<td>$X_1X_2$</td>
<td>−6.8891</td>
<td>0.9492</td>
<td>−7.257</td>
<td>0.000</td>
</tr>
<tr>
<td>10</td>
<td>$X_1X_3$</td>
<td>−0.2244</td>
<td>0.3805</td>
<td>−0.590</td>
<td>0.566</td>
</tr>
<tr>
<td>11</td>
<td>$X_2X_3$</td>
<td>0.5719</td>
<td>0.3805</td>
<td>1.503</td>
<td>0.159</td>
</tr>
<tr>
<td>12</td>
<td>$X_1X_4$</td>
<td>−0.4806</td>
<td>0.3805</td>
<td>−1.263</td>
<td>0.231</td>
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<tr>
<td>13</td>
<td>$X_2X_4$</td>
<td>−0.5044</td>
<td>0.3805</td>
<td>−1.325</td>
<td>0.210</td>
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<tr>
<td>14</td>
<td>$X_3X_4$</td>
<td>−2.5869</td>
<td>0.3805</td>
<td>−6.798</td>
<td>0.000</td>
</tr>
<tr>
<td>15</td>
<td>$X_1X_4$</td>
<td>−0.3331</td>
<td>0.3805</td>
<td>−0.875</td>
<td>0.399</td>
</tr>
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</table>

SS = 1.522, R-Sq = 98.6%, R-Sq(adj) = 97.1%.
Table 4: Results of ANNOVA-lipase activity.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sequential SS</th>
<th>Adjusted SS</th>
<th>Adjusted MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>Regression</td>
<td>14</td>
<td>2027.81</td>
<td>2027.81</td>
<td>144.844</td>
<td>62.51</td>
<td>0.000</td>
</tr>
<tr>
<td>Linear</td>
<td>4</td>
<td>742.06</td>
<td>742.06</td>
<td>185.514</td>
<td>80.07</td>
<td>0.000</td>
</tr>
<tr>
<td>Square</td>
<td>4</td>
<td>1163.10</td>
<td>1163.10</td>
<td>290.775</td>
<td>125.49</td>
<td>0.000</td>
</tr>
<tr>
<td>Interaction</td>
<td>6</td>
<td>122.65</td>
<td>122.65</td>
<td>20.442</td>
<td>8.82</td>
<td>0.001</td>
</tr>
<tr>
<td>Residual error</td>
<td>12</td>
<td>27.80</td>
<td>27.80</td>
<td>2.317</td>
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<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>10</td>
<td>27.80</td>
<td>27.80</td>
<td>2.780</td>
<td></td>
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<tr>
<td>Pure error</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>2055.62</td>
<td></td>
<td></td>
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</table>

Figure 1: Surface plots of molar conversion (%) with: (a) alcohol molarity and reaction time, (b) alcohol molarity and temperature, (c) alcohol molarity and enzyme activity, (d) reaction time and temperature, (e) reaction time and enzyme activity, (f) temperature and enzyme activity.
conversion have been depicted through response surface plots of Figures 1(b), 1(d), and 1(e). The convex nature of these plots is clearly indicating the significance of variable interactions on the molar conversion. The response equation can be written in the uncoded form as follows:

\[
\text{MC(\%)} = -111.473 + 51.2227X_1 + 10.7627X_2 + 1.57960X_3 + 14.2641X_4 - 0.280469X_1X_2 + 0.285937X_1X_3 - 0.60781X_1X_4 - 0.0126094X_2X_3 - 0.161680X_2X_4 - 0.00832813X_3X_4 - 54.3519X_1^2 - 0.305567X_2^2 - 0.0188907X_3^2 - 0.430567X_4^2,
\]

(2)

where \(X_1, X_2, X_3, \) and \(X_4\) represent the input process parameters of transesterification reaction.

The significant effect of temperature, reaction time, and enzyme amount on ester yield were also reported in case of lipase-mediated synthesis of flavour-active octanoic acid esters acetate [14]. Martins et al. 2011 [16] utilized central composite design of RSM for modeling the esterification reaction of butyl acetate synthesis catalyzed by Candida antarctica lipase B (Novozym 435) and reported the significance effects of transesterification variables such as temperature, alcohol molar ratio, and enzyme content on ester conversion yields. The obtained uncoded nonlinear response equation based on CCD has been taken as objective function for simulation studies of genetic algorithm for enhanced molar conversion (%).

3.2. Artificial Intelligence-Based Optimization Approach. The present optimization approach of binary coded GA has been aimed to enhance the molar conversion of flavor ester were tested in triplicate experimental runs using 0.47 M molarity of alcohol and 13.31 U 13.1h, at 37.5 \(^\circ\)C, and 13.31 U for molarity of alcohol, reaction time, temperature, and immobilized lipase amount, respectively. The optimized set of transesterification variables and the corresponding maximum molar conversion of flavor ester were tested in triplicate experimental runs using 0.47 M molarity of alcohol and 13.31 U 13.1h, at 37.5 \(^\circ\)C in 13.1h. Under these results, the molar conversion seen to be equal to 96.32\% (10% enhancement compared to one variable at a time selection approach), with the close agreement of GA-predicted value. Several researchers acknowledged the efficient searching abilities of artificial intelligence such as GA in different biochemical studies. Giordano et al. 2011 [26] reported the efficient problem solving approach of GA-coupled Plackett-Burman methodology in enzymatic hydrolysis of lignocellulosic residues. An optimized result has also been reported for the optimization of biocatalytic transglycosylation processes using GA approach [27]. Moreover the enhanced optimizing capability of genetic algorithm has been acknowledged in case of industrial enzymes production. Evolutionary optimization of extraction from fermented broth has been reported for fungal lipase production [28]. Similar enhanced results were reported in case of fermentation medium optimization for the production of glucansucrase [29] and halogenase enzymes [30].

3.3. Reusability of the Biocatalyst. At the end of each cycle, the immobilized lipases were removed from the reaction medium and washed with hexane to remove any substrate or product retained on the matrix. After drying at room temperature, the immobilized lipases were again introduced into fresh medium. The residual activity measured for the immobilized lipase after each cycle is shown in Figure 3. The remaining activity of immobilized lipase after 4 uses accounts for more than 98\% of the initial activity, which reduces to 50\% after 12 cycles of use. The development of an attractive biocatalyst requires high stability that allows repeated use of the catalytic material. The reduction in the conversion measured after the fifth use of immobilized lipase may be a consequence of a combined effect of deactivation or desorption of lipase and the loss of biocatalyst material due to high stirring speeds and repeated manipulation operations that is, filtration/drying/addition to a new substrate mixture [31]. Karra-Chaabouni et al. 2006 [32] reported that approximately 29\% of the initial activity was retained after 10 cycles of use for the hexyl acetate synthesis by immobilized Staphylococcus simulans lipase. The Rhizopus sp. lipase immobilized on celite retains its high activity only until the second cycle, while most of its activity was lost after four cycles [33].

3.4. Kinetics of Benzyl Acetate Synthesis. In the reaction, initially when the product concentrations are zero, the expression for initial reaction rate is

\[
\nu = \frac{V_{\text{max}}}{1 + (Km_A/ [A]) + (Km_C/ [C])},
\]

(3)

where \(\nu\) is the initial reaction rate, \(V_{\text{max}}\) is the maximum reaction rate, \([A]\) and \([C]\) are the respective concentrations of vinyl acetate and benzyl alcohol and \(Km_A\) and \(Km_C\) are the kinetic constants for the vinyl acetate and benzyl alcohol, respectively.

In this solvent-free reaction for benzyl acetate synthesis, the concentration of vinyl acetate can be regarded as constant. So the initial reaction rate equation can be expressed simply as

\[
\nu = \frac{V_{\text{max}} [C]}{[C] + Km_{\text{Capp}}},
\]

(4)

where \(\nu\) is the initial reaction rate, \(V_{\text{max}}\) the maximum initial reaction rate and \(Km_{\text{Capp}}\) the apparent Michaelis constant.
In the present investigation, kinetic parameters for benzyl acetate synthesis were calculated by varying the benzyl alcohol concentration (125–1000 mM). The kinetic constants \( V_{\text{max}} \) and \( K_m \) were calculated from the reciprocal plot shown in Figure 4. The \( K_m \) and \( V_{\text{max}} \) value for benzyl acetate synthesis was 310 mM and 0.10 mmol h\(^{-1}\) g\(^{-1}\), respectively. Previous studies have shown that the esterification and transesterification reactions using immobilized lipases could be described by the Ping-Pong kinetic models [34, 35]. As the synthesis reaction studied here was carried out at excess of vinyl acetate, so the concentration of vinyl acetate can be regarded as constant. Similar approaches have been applied previously for kinetic studies of lipase-mediated ester synthesis [36, 37].

4. Conclusion

In the present study, the individual and interaction effects of transesterification variables on molar conversion of benzyl acetate were studied and optimized through the statistical integrated artificial intelligence approach under solvent-free conditions catalyzed by immobilized lipase. The obtained results indicate that a maximal molar conversion of 96.32% was achieved in 13.17 h with the 0.47 M benzyl alcohol in vinyl acetate and an immobilized enzyme amount of 13.31 U, at 37.58°C and 200 rpm. The immobilized lipase was reusable for four cycles with retaining the relative activity of more than 98%. The kinetic parameters, \( K_m \) and \( V_{\text{max}} \) values of the benzyl acetate synthesis were found to be 310 mM.
and 0.10 mmol h⁻¹ g⁻¹, respectively. The results indicate that the statistical integrated artificial intelligence aspect was an efficient and systematic approach for optimization of immobilized lipase-catalyzed flavour ester synthesis.

**Authors’ Contribution**

Vijay Kumar Garlapati and Annapurna Kumari equally contributed as a first author.

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**References**


