Optimization of cephalosporin C acylase immobilization using crosslinked enzyme aggregates technique

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Abstract: Cephalosporin C acylase (CCA) is an essential enzyme for the one-step conversion of cephalosporin C into 7-aminocephalosporanic acid (7-ACA), an intermediate compound used to synthesize various semi-synthetic cephalosporin antibiotics. The industrial process prefers to use enzymes in immobilized form rather than soluble. A crosslinked enzyme aggregate (CLEAs) is a potential matrix-less enzyme immobilization technique to produce stable immobilized enzymes with high activity and low production costs. This study aimed to optimize the CCA immobilization using the CLEAs technique with Chitosan as a co-aggregate. The CCA lysate was obtained from harvesting CCA fermentation broth using a mutant strain of Escherichia coli through cell separation and lysis steps. Partially purified CCA by ammonium sulfate addition was conducted to obtain an active fraction of 20-60% saturation, followed by co-aggregation with Chitosan to form physical CCA aggregates. The aggregates were then immobilized using a crosslinking technique using glutaraldehyde to form CLEAs-CCA. Optimization of the immobilization process was carried out by Response Surface Methodology in three steps, (i) screening of the influencing factors, (ii) determining the level of the significant factors, and (iii) optimizing the immobilization condition. CLEAs-CCA activity was used as a response parameter. Under optimum conditions, CLEAs-CCA activity obtained was 85.91 Ug⁻¹.

Key words: 7-aminocephalosporanic acid, cephalosporin C acylase, Chitosan, crosslinked enzyme aggregates, response surface methodology.

Introduction

Cephalosporins are antibiotics with a broad antibacterial spectrum and almost cover 50% of β-lactam antibiotics humans use1,2. Most of them are derived from 7-aminocephalosporanic acid (7-ACA), a precursor compound generated from hydrolysis of cephalosporin C (CPC), a natural antibacterial obtained from Acremonium chrysogenum fermentation3. Therefore, it is essential to produce 7-ACA efficiently.

The industrial demands for efficient and environmentally friendly 7-ACA production have shifted from chemical to enzymatic processes. Initially, a two-step bioconversion using D-amino acid oxidase and glutaryl-7-aminocephalosporanic acid acylase was developed4. Recently, one-step bioconversion using cephalosporin C acylase (CCA, EC 3.5.1.11) has paid the attention of researchers because it is more efficient5.

The industrial application of enzymes is preferred in the immobilized rather than soluble form due to the more efficient bioconversion process6. Immobilization of enzymes on a matrix is a popular method. However, crosslinking the enzyme with low superficial lysine residues with glutaraldehyde may cause weak enzyme binding, making it quickly released from its CLEAs13. The addition of co-aggregates, such as bovine serum albumin, poly-ethyleneimine, and poly-lysine, could form larger aggregates size and higher stability9,11,12. Chitosan is a relatively inexpensive biopolymer (polyaminosaccharides, copolymers of N-acetyl-D-glucosamine and N-amino-D-glucosamine) with high availability rich in reactive amino groups, potentials to mediate the formation crosslinks between the aggregates8,10,15.

In this research, optimization of the CCA immobilization by CLEAs technique using Chitosan as a co-aggregate was studied. Optimization was conducted with Response Surface Methodology (RSM), as it is an effective and widely used method for optimization in bioprocesses, including enzyme immobilization16-18. CLEAs-CCA (Ug⁻¹) activity was used as a response. The experiments were carried out in three steps, namely, (i) screening of the influencing factors using 2²-1 fractional factorial design (FFD), (ii) determining the level of significant fac-

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tors using the steepest ascent method, and (iii) optimization of immobilization condition using RSM with Central Composite Design (CCD). CLEAs-CCA obtained were also evaluated for pH and temperature stability.

**Materials and methods**

*Escherichia coli* mutant as the CCA producer was provided by The National Research and Innovation Agency (BRIN), previously named as Biotech Center, Agency for the Assessment and Application of Technology - Indonesia. The culture was maintained in Miller Luria Bertani (LB) agar medium (Hi-media, Mumbai, India) pH 7.0, containing 100 µgmL-1 ampicillin. *E. coli* was selected and inoculated into 2 mL of fresh LB medium pH 7.0 containing ampicillin 100 µgmL-1 and incubated at 37°C with shaking at 120 rpm in a water bath. After two hours incubation, the seed culture was then prepared by suspending 0.5 mL of the seed culture into 2 mL of fresh 50 mL LB pH 7.0 containing ampicillin of 100 µgmL-1 and incubated at 37°C with shaking at 170 rpm. The seed culture was then prepared by suspending 0.5 mL of the overnight culture to 2 mL fresh LB medium pH 7.0 containing ampicillin of 100 µgmL-1 and incubated at 37°C with shaking at 170 rpm in water bath. After two hours incubation (OD500 ± 0.6), 0.5 mL of the seed culture was inoculated into a fresh 50 mL LB, pH 7.0 containing ampicillin of 100 µgmL-1 and incubated for 200 rpm until an OD600 of 0.6-0.8 was reached. The culture was then induced with lactose with a final concentration of 200 µmL-1 in a 250 mL Erlenmeyer flask and incubated at 37°C, shaken at 200 rpm. Variation in suspension pH (x4), crosslinking time (x5), and crosslinking temperature (x6) were screened using the FFD experimental design. The CCA production method was adopted from Martius et al.21. A single colony of *E. coli* was selected and inoculated into 2 mL of fresh LB medium pH 7.0 containing ampicillin 100 µgmL-1 and cultivated at 37°C overnight with shaking at 170 rpm. The seed culture was then prepared by suspending 0.5 mL of the overnight culture to 2 mL fresh LB medium pH 7.0 containing ampicillin of 100 µgmL-1 and incubated at 37°C with shaking at 170 rpm in water bath. After two hours incubation (OD500 ± 0.6), 0.5 mL of the seed culture was inoculated into a fresh 50 mL LB, pH 7.0 containing ampicillin of 100 µgmL-1 and incubated for 200 rpm until an OD600 of 0.6-0.8 was reached. The culture was then induced with lactose with a final concentration of 0.2% (w/v), and then cultivation was continued at 25°C with shaking at 200 rpm for 12 hours for CCA expression. The cells were harvested by centrifugation at 6,000 g, 4°C, for 10 minutes when the fermentation was finished. The pellets were then washed twice with a working buffer (0.1 M phosphate buffer pH 8.0) and then suspended in the same buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). PMSF was used to inactivate the protease in cells. Cells were lysed by sonication at 20 kHz, 25% amplitude, with a mode of 5 seconds “on” and 20 seconds “off” for 12 cycles using Misonix Sonicator XL2020 (Misonix, United States) in an ice bath. Cell debris was separated by centrifugation at 12,000 g, 4°C, for 5 minutes. CCA lysate was then stored at -20°C for further experiment.

**Chitosan and glutaraldehyde solution preparation**

Chitosan solution was prepared by Arsenault et al. method21. Chitosan powder was dissolved in 0.1 M HCl to obtain a specific concentration, stirring for 30 minutes, and then sonicated for one hour to dissolve Chitosan completely. The pH of the solution was adjusted to 5.5 by adding 1 N NaOH. Glutaraldehyde solution with a particular concentration (% w/v) was prepared by weighing 50% glutaraldehyde solution and diluted with a working buffer.

**CCA partial purification**

Simple partial purification of CCA was carried out by precipitation technique using ammonium sulphate (AS)22. AS powder was added slowly to 10 mL CCA lysate until it reached 20% saturation. The mixture was stirred continuously at a low speed at 4oC for 3 hours. The precipitate was separated by centrifugation at 10,000 g at 4°C for 15 minutes, and the supernatant was used for further fractionation. Precipitation was continued by adding AS to 60% saturation. The CCA aggregates of 20-60% saturation were separated by centrifugation and then resuspended in 40 mL of a working buffer. The suspension was used for CLEAs formation.

**CLEAs-CCA formation method**

The CLEAs formation method was adopted from Mageed et al.23 with slight modifications. A 0.5 mL chitosan solution was added to 1 mL of CCA aggregates suspension to obtain a final chitosan concentration in the range 1-5 mgmL-1 (x1) according to the FFD factor level (Table 1). Chitosan is insoluble at pH above 6.5 and will aggregate with CCA aggregates. The aggregation was conducted by shaking at 150 rpm for 30-90 minutes (x2). The co-aggregation allows CCA aggregates and Chitosan to interact electrostatically or hydrogen bonding. Furthermore, 0.5 mL of glutaraldehyde solution was added to obtain a final concentration in the range of 1-5 mgmL-1 (x1) according to the FFD factor level (Table 1). In such a way, glutaraldehyde crosslinks of primary amine groups of enzyme and Chitosan. After the reaction was completed, the CLEAs-CCA was filtered using filter paper and washed with working buffer three times. The CLEAs-CCA was stored at -20°C for further analysis.

**CLEAs-CCA activity determination**

The CLEAs-CCA activity was determined using a micro-scale procedure according to Fernandez-Arrojo et al.24 with minor modifications. Twenty milligrams of CLEAs-CCA were added to a microtube containing 60 µL of CPC solution with a concentration of 20 µgmL-1 in a working buffer (0.1 M phosphate buffer pH 8.5). The mixture was incubated in an incubator shaker at 37°C for 5 minutes. After the reaction was completed, 20 µL of the reactant was taken and mixed with 280 µL of stop solution (the solution consists of 0.5% p-DAB in absolute methanol; 0.05 M NaOH: 20% acetic acid glacial with ratio 1:2:4). The solution was left for 10 minutes at room temperature to develop color, and then the absorbance was measured at 415 nm.

**Screening of factors**

In the first step, factors affecting CLEAs-CCA immobilization were screened using the FFD experimental design. The factor screening plays an important role when many potential factors influence the response while the resource availability is limited25. FFD screened out the influencing factors based on

**CLEAs-CCA activity was calculated using equation (1).**

\[
\text{CLEAs – CCA activity (Ug}^{-1}) = \frac{\mu \text{mol of 7-ACA x dilution factor}}{\text{reaction time x wet weight of CLEAs – CCA (g)}}
\]
their main effects and excluded their interaction. By preliminary literature review, six independent factors and their levels were selected, and the screening experiment design is presented in Table 1. The FFD experiments consist of 22 runs (16 runs of two-level fractional factorial (2^n-2) and 6 center point runs). The experimental design and the results are shown in Table 2.

Steepest ascent method

The level of significant factors obtained from the FFD experiment was initially optimized using the steepest ascent method to determine the center point of each factor. Sequential experiments were carried out by applying various influencing factors along the direction of the maximum increase in the response as a rapid and efficient method for approaching the optimum neighborhood. Two points flanking the maximum response point of each factor are selected for the next optimization step.

Table 1. Independent factors and their levels were used in the FFD experiment.

<table>
<thead>
<tr>
<th>Factors</th>
<th>symbol</th>
<th>Factor levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan concentration (mg/mL)</td>
<td>x₁</td>
<td>+1 0 -1</td>
</tr>
<tr>
<td>Co-aggregation time (minutes)</td>
<td>x₂</td>
<td>5 3 1</td>
</tr>
<tr>
<td>Glutaraldehyde concentration (% v/v)</td>
<td>x₃</td>
<td>+1 -1 +1</td>
</tr>
<tr>
<td>pH suspension</td>
<td>x₄</td>
<td>9 8 7</td>
</tr>
<tr>
<td>Cross-linking time (minutes)</td>
<td>x₅</td>
<td>240 150 60</td>
</tr>
<tr>
<td>Cross-linking temperature (°C)</td>
<td>x₆</td>
<td>30 25 20</td>
</tr>
</tbody>
</table>

The CCD with RSM was employed to optimize the two independent significant factors (glutaraldehyde concentration and crosslinking time). The other factors which have an insignificant effect were kept constant at the minimum level. The CCD and the result of the experiments are presented in Table 3.

The effect of significant factors was modeled with a second-order polynomial equation as given by equation (2).

\[ Y = b_0 + b_i x_i + b_j x_j + b_{ij} x_i x_j + b_{ii} x_i x_i + b_{jj} x_j x_j + \epsilon \] (2)
Where \( Y \) represents the response predicted value, \( b_0 \) is a constant, \( b_i \) is the linear coefficient, \( b_{ij} \) is the interaction coefficient, \( b_{ii} \) and \( b_{jj} \) is the quadratic coefficient, and \( x_i, x_j \) are the coded level of the factors. Validation of optimum conditions obtained by the model was carried out by experiment with three replications. The relative error between predicted and experimental values was calculated by equation (3):

\[
\text{Relative error (\%) } = \left( \frac{\text{mean experimental value} - \text{predicted value}}{\text{mean experimental value}} \right) \times 100\%
\]

\[
\text{Immobilization yield (\%) } = \left( \frac{\text{initial enzyme activity (U) - filtrate activity(U)}}{\text{initial enzyme activity (U)}} \right) \times 100\%
\]

\[
\text{Activity yield (\%) } = \left( \frac{\text{CLEAs activity (U)}}{\text{initial enzyme activity (U) - filtrate activity(U)}} \right) \times 100\%
\]

\[
\text{Residual activity (\%) } = \left( \frac{\text{activity of CLEAs - CCA}}{\text{activity of CLEAs - CCA}_{\text{max}}} \right) \times 100\%
\]

\[
Y = 21.33 - 8.60x_3 - 5.19x_5
\]
model due to the model inadequacy, is 0.055, which implies that the lack of fit is insignificant. The value of 'Adequate Precision' 27.097 indicates an adequate signal. A value of 'Adequate Precision' > 4 is desirable.

A quadratic model equation fitted to the experimental data, given as coded factors, was shown in equation (8):

\[
Y = 84.77 + 11.94x_3 - 4.24x_5 + 9.35x_3x_5 - 31.03x_3^2 - 14.93x_5^2
\]  

(8)

The 3D surface plot of the quadratic model helps to investigate the desired response value and find the optimal operating conditions (Fig. 2). The peak of the curve is the maximum response obtained from the effect of significant factors and their interactions under optimal conditions. According to the quadratic model, the optimum condition for CLEAs-CCA immobilization was \( x_3 = 0.18 \) and \( x_5 = -0.09 \), or in actual value, glutaraldehyde concentration was 0.20 \( \% \) (v/v) and crosslinking time was 119 minutes, with optimum predicted CLEA activity was 86.02 Ug\(^{-1}\).

Validation of the model

A three-replicate experiment validated the prediction of CLEAs-CCA activity obtained under optimum conditions. As shown in Table 6, the experimental data were in good agreement with the predicted value in CLEAs-CCA activity. The relative error between predicted and experimental values fell at 0.13\%. It means that the results are within the confidence interval of 95\%, confirming that the model is valid.

Stability of CLEAs-CCA

The thermal stability tests showed that free CCA had a slight decrease of activity (2.82\%) after incubation at 45°C compared to 35°C. However, at 55°C, its activity decreased sharply and only retained 4.57\% activity. CLEAs-CCA showed better thermal stability and even showed an increase after incubation at 45°C. However, the CLEAs-CCA activity decreased after incubation at 55°C and retained only 25.87\% activity. The experimental results are depicted in Figure 3a.

The pH stability tests also showed that CLEAs-CCA had better stability than free CCA. After incubation at pH 4.5 for 30 minutes, the CLEAs-CCA activity retained was 62.87\%, while free CCA was 45.87\%. The experimental results are presented in Figure 3b.

Discussion

A simple immobilization technique and high enzyme stability are critical for enzymes in their production process.
mobilizing enzymes using the CLEAs technique is a potential method to fulfill those demands. This technique involves only a simple preparation and does not require high purity of enzymes. In comparison, immobilization of CCA on the epoxy matrix was preceded by enzyme purification using immobilized metal affinity chromatography to obtain high purity enzyme \(4,26\). On the other hand, immobilization by the CLEAs technique does not require pure enzyme. The effect of enzyme purity on the CLEAs-PGA was studied by Rajendhran and Gunasekaran \(27\). The CLEAs-PGA generated from partially purified enzymes (enzyme purity 10 times higher than the initial crude enzymes) had 1.5 times higher activity than purified by the Ni-NTA chromatography technique (purity 31 times higher than the initial crude enzyme) \(27\). Therefore, complex, high-cost, and time-consuming enzyme purification steps are unnecessary for CLEAs preparation.

In general, CLEAs formation involves two critical steps, i.e., enzyme precipitation to induce the physical enzyme aggregates formation and aggregates crosslinking to form permanently insoluble enzymes through covalent coupling of the aggregates while preserving their globular structure \(7,28\). The physical enzyme aggregates formation could be carried out by adding salts, organic solvents, or non-ionic polymers to enzyme solutions, as commonly used in protein purification. Partial purification of CCA using the salting-out method with the addition of AS was used in this study due to it being a simple method and relatively small effect on enzyme activity \(8\). The active fraction of 20-60% saturation was used for CCA immo-

<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>
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<td>5</td>
<td>1834.49</td>
<td>99.72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glutaraldehyde concentration (x5)</td>
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<td>1</td>
<td>1140.69</td>
<td>62.01</td>
<td>0.0001</td>
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<tr>
<td>Cross-linking times (x5)</td>
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<td>7.81</td>
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<tr>
<td>xxx</td>
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<td>349.97</td>
<td>19.02</td>
<td>0.0033</td>
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<td>x5</td>
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<td>1</td>
<td>6699.86</td>
<td>364.29</td>
<td>&lt;0.0001</td>
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<td>x5²</td>
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<td>1550.93</td>
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<td>Residual</td>
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<td>7</td>
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<td>Lack of fit</td>
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<td>35.34</td>
<td>6.21</td>
<td>0.0550*</td>
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<tr>
<td>Pure error</td>
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</tr>
<tr>
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<td></td>
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<td>R²</td>
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<td>Adjusted R²</td>
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<td>PRESS</td>
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<td>Adequate Precision</td>
<td>27.097</td>
<td></td>
</tr>
</tbody>
</table>

\*insignificant at 95% confidence level, df: Degrees of freedom, PRESS: Predicted residual error sum of squares

Table 5. ANOVA for response surface quadratic model.

**Figure 1.** The steepest ascent experiment to determine the new level of significant factors is used in optimization.
bilitation. The partially CCA purification aimed to remove the contaminant protein in the lysate, such as esterase and β-lactamase, which are co-expressed when E. coli was used as an enzyme expression system\(^2^9\). Both enzymes could hydrolyze CPC resulted in decreasing in yield of 7-ACA\(^3\).

The number of lysine residues on the enzyme surface influences the effectiveness of crosslinking between cross-linkers and enzyme molecules. The amount of lysine residues also affects protein functionalization and enzyme immobilization\(^3^0,3^1\). Lysine is the amino acid with an NH\(_2\) (amino) side chain, the most reactive residue on the enzyme surface. Luo et al.\(^3^2\) reported that CCA contains only low (nine) superficial

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**Figure 2.** The 3D surface plot of the quadratic model.

**Table 6.** Experimental data at optimum conditions for model validation.

<table>
<thead>
<tr>
<th>Optimum factors level</th>
<th>Predicted value (Ug(^{-1}))</th>
<th>Experimental value (Ug(^{-1}))</th>
<th>Experimental mean value (Ug(^{-1}))</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>x(_1): 0.20%</td>
<td>86.02</td>
<td>93.04</td>
<td>81.34</td>
<td>85.91</td>
</tr>
<tr>
<td>x(_2): 119 minutes</td>
<td></td>
<td></td>
<td></td>
<td>0.13</td>
</tr>
</tbody>
</table>

---

**Figure 3.** (a) Thermal stability test results; (b) pH stability test results; ■: optimized CLEAs-CCA; □: free CCA. Data are presented as mean values ± S.E.
lysine residues. Therefore, a co-aggregate compound rich in free amino groups, such as Chitosan, must be added before crosslinking. Chitosan as a co-aggregate can increase the effectiveness of crosslinking of CLEAs formation for various enzymes. In this study, the Chitosan with high DD (>75%) was mixed with the enzyme to provide an adequate number of free amino groups for crosslinking. DD is the ratio of N-amino-D-glucosamine to N-acetyl-D-glucosamine.

Glutaraldehyde is a widely applied bi-functional reagent for many CLEAs preparations since it is an efficient crosslinker with low cost and high availability. In this study, glutaraldehyde crosslinking was carried out at pH > 6.5. Under these conditions, the deprotonated amino group of Chitosan becomes more reactive as a nucleophile agent. Glutaraldehyde acts as an amino fixing arm to form an imine bond between the primary amino group of Chitosan and the enzyme, creating networks among them. The reaction of primary amino groups of superficial lysine residues from neighboring enzyme molecules to glutaraldehyde can form inter- and intra-molecular aldim aldol condensates, thereby increasing the stability of biocatalyst activity. However, the CLEAs formation using excessive glutaraldehyde would lead to enzyme inactivation due to its high reactivity and small size, allowing it to penetrate the enzyme’s active site and access all essential amino acids involved in catalytic activity, leading the changes in intrinsic enzyme properties. In addition, more severe inactivation may occur for enzymes that have reactive amino acid residue in the vicinity of their active site. CCA has an essential N-terminal serine in its active site, which is susceptible to reacting with glutaraldehyde. He et al. reported that crosslinking CCA with glutaraldehyde causes activity loss by 18% due to its excessive crosslinking. Therefore, adding Chitosan is necessary for crosslinking when glutaraldehyde is used. It could form reticulated covalent bonds between enzyme–enzyme, enzyme–polymer, and polymer–polymer, thereby increasing the number of crosslinks and reducing enzyme inactivation as well as increasing aggregate size. In this study, the particle size of CLEAs-CCA without chitosan addition was tiny and increased along with chitosan addition. The particle size of CLEAs is essential to facilitate mass transfer rate, product separation as well as its reusability after enzymatic processing. Chitosan could also act as a coating agent for enzyme-CCA. As a poly-ionic biopolymer, Chitosan can interact with enzymes, preventing enzyme dissociation. Nevertheless, under certain conditions, such as low pH or high temperature, the biopolymer can be desorbed and lose its function as a protective layer. As a crosslinker agent, glutaraldehyde stabilizes CLEAs structure by crosslinking the biopolymer to the enzyme surface and among the biopolymers, avoiding the enzyme being re-dissolved during the buffer rinsing and their application.

This study used CCD for response surface optimization with two process variables (glutaraldehyde concentration and crosslinking time). It revealed that the optimized conditions were achieved at glutaraldehyde concentration 0.2% (v/v) and crosslinking time 119 minutes. The other factors were set at a minimum value: chitosan concentration 1 mgmL⁻¹, co-aggregation time 30 minutes, pH suspension 7, and crosslinking temperature 20°C. The maximum CLEAs-CCA activity achieved was 85.91 Ug⁻¹ (Table 6). Theoretically, at low glutaraldehyde concentration, and glutaraldehyde molecule reacts with a few amino groups of enzymes. Excessive interaction between residual amino acids on protein surface with glutaraldehyde must be avoided to maintain the catalytic activity of the immobilized enzyme. In addition, relatively low crosslinking temperatures were used in this study to avoid CCA degradation. Within the temperature range used, maximum CLEAs activity was achieved in 2 hours.

The maximum activity of CLEAs-CCA obtained from this experiment was comparable and even exhibited slightly higher than that of immobilized CCA with matrix-binding obtained by Zhu et al. and Wei et al., i.e., 81 Ug⁻¹ and 60 Ug⁻¹, respectively. However, the immobilization yield of CLEAs-CCA was 60.31%, and the activity yield was only 13.89%. Enzyme activity can be partially lost upon the precipitation and glutaraldehyde crosslinking step caused by the changes in intrinsic enzyme properties. Besides, the issues of diffusion and transport would arise since the pore sizes of the CLEAs may be small. However, the immobilization processes could promote enzyme resistance to pH or temperature changes and its repetitive use, making it more efficient than a free enzyme.

The poly-ionic biopolymer chitosan is essential to form larger particles and contributes to a stronger bond between the enzyme aggregates to obtain stable CLEAs. However, it becomes a part of the CLEAs non-catalytic structure, resulting in diluting the enzyme activity. Immobilized enzyme results from the original CLEAs (without co-aggregate addition) was claimed as almost 100% undiluted, containing only enzyme and a tiny fraction of crosslinker. Therefore, the dilution of CLEAs-CCA could be defined as the ratio of the bound enzyme and the quantity of Chitosan used. The addition of Chitosan as co-aggregate contributed to the enzyme dilution of 44.85%. However, this dilution effect is much lower than that of the matrix-bound immobilized enzyme, which causes the enzyme dilution up to 99.57.

The thermal stability of the immobilized enzyme influences its industrial application. The rigid structure of CLEAs obtained by crosslinking is the reason for their increased stability. In addition, the thermal stability of the CLEAs can be generated by the effect of enzyme coating by Chitosan. In this study, chitosan solution was prepared at pH 5.5 (pKa of Chitosan is around 6.5); therefore, Chitosan has a low negative charge. Electrostatic interaction and/or hydrogen bonding between the N-acetyl-D-glucosamine hydroxyl group of Chitosan and positively charged amino acid residues on the enzyme surface may occur, thereby increasing the thermal stability of CLEAs. In addition, during the conversion CPC to 7-ACA, the pH of the solution tends to decrease due to the by-products accumulation, resulting in a decrease in the hydrolysis rate and enzyme stability. The CLEAs-CCA showed good stability at low pH up to 4.5; therefore, product inhibition related to low pH could be minimized.

Conclusions

The maximum CLEAs-CCA activity of 85.91 26 Ug⁻¹ was obtained with the optimized immobilization conditions: glutaraldehyde concentration 0.2% (v/v) and crosslinking time 119 minutes. Chitosan as co-aggregate increases the number of crosslinks, which is indicated by the formation of larger particles size and increasing CLEAs-CCA stability. CLEAs-CCA immobilization also succeeded in reducing enzyme dilution up to 55.15%.

Author Contributions

Conceptualization and methodology, J.J. and A.W.; performing experiments and analyzing data, J.J.; writing—original draft preparation, J.J.; writing—review and editing, A.W. and K.S.; supervision, A.W. and K.S. All authors have read and
agreed to the published version of the manuscript.

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**Conflicts of Interest**
The authors declare no conflict of interest.

**Bibliographic references**


