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# Enhanced Expression of Recombinant Cephalosporin Acylase in *Escherichia coli* via Autoinduction Medium

(Pengekspresan Sefalosporin Asilase Rekombinan yang Dipertingkatkan dalam Escherichia coli melalui Medium Autoinduksi)

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ABSTRACT

The large-scale synthesis of β-lactam antibiotics, particularly cephalosporins, relies heavily on the industrial production of recombinant cephalosporin acylase (CCA). This study aimed to identify the most suitable *Escherichia coli* host strain for CCA production. The selected strain was then used for optimizing medium components and culture conditions using an autoinduction medium in shaking flask fermentation. Initially, three *E. coli* strains were evaluated: BL21(DE3), BL21(DE3) RIPL, and B(DE3) Origami, to determine the most efficient host for recombinant CCA expression. Among the tested conditions, the autoinduction medium proved particularly effective, contributing to a significant improvement in enzyme yield and serving as the basis for subsequent optimization experiments. To identify the key factors influencing CCA production, a Plackett-Burman design was employed to screen medium components and fermentation parameters, with *E. coli* BL21(DE3) as the most optimal expression host. The significant variables were then optimized using a Central Composite Design, which resulted in a maximum enzyme activity of 53.3 U/mL under the following conditions: 0.78 g/L glucose monohydrate, 1.12 g/L glycerol, 6.21 mL/L trace elements, and an enzyme expression time of 19.48 h. The optimized conditions led to a 3.8-fold increase in CCA activity compared to unoptimized conditions. These findings provide valuable insights into efficient recombinant CCA production and have important implications for its industrial application in antibiotic synthesis.

Keywords: Central composite design; enzyme characterization; enzyme kinetics; enzyme purification; optimization

ABSTRAK

Sintesis berskala besar antibiotik β-laktam, terutamanya sefalosporin sangat bergantung kepada penghasilan industri sefalosporin asilase (CCA) rekombinan. Kajian ini bertujuan untuk mengenal pasti strain perumah *Escherichia coli* yang paling sesuai untuk pengeluaran CCA. Strain terpilih kemudiannya digunakan untuk mengoptimumkan komponen medium dan keadaan kultur menggunakan medium autoinduksi dalam penapaian kelalang goncang. Pada mulanya, tiga strain *E. coli* telah dinilai: BL21(DE3), BL21(DE3) RIPL dan B(DE3) Origami, untuk menentukan hos yang paling berkesan untuk pengekspresan CCA rekombinan. Antara keadaan yang diuji, medium autoinduksi terbukti sangat berkesan, menyumbang

kepada peningkatan ketara dalam hasil enzim dan berfungsi sebagai asas untuk uji kaji pengoptimuman seterusnya. Untuk mengenal pasti faktor utama yang mempengaruhi pengeluaran CCA, reka bentuk Plackett-Burman telah digunakan untuk menapis komponen sederhana dan parameter fermentasi dengan E. coli BL21(DE3) sebagai hos pengekspresan yang paling optimum. Pemboleh ubah ketara kemudiannya dioptimumkan menggunakan reka bentuk komposit pusat yang menghasilkan aktiviti enzim maksimum 53.3 U/mL di bawah keadaan berikut: 0.78 g/L glukosa monohidrat, 1.12 g/L gliserol, 6.21 mL/L unsur surih dan masa pengekspresan enzim selama 19.48 jam. Keadaan yang dioptimumkan membawa kepada peningkatan 3.8 kali ganda dalam aktiviti CCA berbanding keadaan yang tidak dioptimumkan. Penemuan ini memberikan pandangan berharga tentang pengeluaran CCA rekombinan yang cekap dan mempunyai implikasi penting dalam aplikasi industri, terutamanya sintesis antibiotik.

Kata kunci: Kinetik enzim; pencirian enzim; pengoptimuman; penulenan enzim; reka bentuk komposit pusat

## INTRODUCTION

The production of β-lactam antibiotics, particularly cephalosporins, remains a cornerstone of the pharmaceutical industry due to their broad-spectrum efficacy against a wide range of bacterial pathogens. Cephalosporins block key enzymes needed for bacterial cell wall formation, weakening the structure and causing bacterial lysis (Bui, Patel & Preuss 2024). Central to the biosynthesis of these antibiotics is cephalosporin acylase (CCA), an enzyme that hydrolyzes the acyl side chains of cephalosporin C to produce 7-aminocephalosporanic acid (7-ACA), a crucial intermediate in the synthesis of semi-synthetic cephalosporins (Figure 1). This enzyme consists of two subunits, α and β, with molecular weights of approximately 25 kDa and 58 kDa, respectively (Martius, Wibisana & Ardiyani 2018). Its activation involves a biphasic post-translational processing mechanism. Initially, an intramolecular autocatalytic cleavage mediated by a nucleophilic Ser or Thr residue generates a novel N-terminal catalytic domain (Kim et al. 2002). Subsequently, a secondary intermolecular cleavage eliminates the residual spacer peptide, culminating in the maturation of the functional enzyme (Kim & Kim 2001).

One of the major challenges in producing recombinant proteins in *Escherichia coli* is achieving high yields of soluble and functional protein. Under non-optimized overexpression conditions, proteins often accumulate as insoluble aggregates, known as inclusion bodies, within the host cells. In general, protein misfolding is the primary cause of these biologically inactive aggregates. Therefore, the efficient production of recombinant CCA is of substantial industrial importance, particularly for meeting the growing demand for semi-synthetic β-lactam antibiotics. This growing demand is underscored by the global cephalosporin market, which reached a valuation of USD14.1 billion in 2023 and is expected to continue its upward trajectory with a projected compound annual growth rate of 3.1% through 2032 (Faizullabhoy & Wani 2024).

Cephalosporin-class antibiotics hold significant promise for continued pharmaceutical development; however, their industrial production faces persistent challenges, particularly in optimizing the expression and functionality of CCA. One major obstacle is the limited structural compatibility of the enzyme with cephalosporin C substrates, along with host-related issues - especially in *E. coli*, where overexpression frequently results in the formation of inclusion bodies and inactive protein. To address these challenges, a wide array of molecular and process engineering strategies has been explored. These include optimizing host organisms to enhance expression levels (Conti et al. 2014), designing high-efficiency expression vectors to boost transcriptional output (Sun et al. 2021; Zhang et al. 2005), and employing both site-directed and random mutagenesis to improve enzyme activity and substrate specificity (Conti et al. 2014; Li et al. 2023; Wang et al. 2012). In parallel, advances in fermentation engineering have improved cultivation conditions and facilitated scale-up for industrial application (Gaurav, Kundu & Kundu 2010). Although these strategies provide a solid foundation, further experimental validation is necessary to establish an optimized, integrated expression system capable of meeting industrial demands (Rasyidah, Sismindari & Purwanto 2024).

The successful production of recombinant proteins, including CCA, is largely dependent on the selection of an appropriate host system. *E. coli* is extensively utilized for this purpose, owing to its rapid growth, well-characterized genetics, and ease of genetic manipulation (Shahzadi 2021). Among the various *E. coli* strains employed, *E. coli* strains BL21(DE3), BL21-CodonPlus(DE3) RIPL, and B(DE3) Origami are particularly notable due to their distinct advantages in recombinant protein production. *E. coli* BL21 is recognized for its robustness and capacity for high-level expression; *E. coli* B(DE3) Origami provides an oxidative cytoplasmic environment conducive to disulfide bond formation, which is essential for the correct folding of certain proteins; and *E. coli* BL21(DE3) RIPL is optimized to supply tRNAs for rare codons, thereby enhancing the expression of proteins encoded by genes with rare codon usage (Falak, Sajed & Rashid 2022).

In addition to the choice of host strain, the optimization of medium components and culture conditions plays a critical role in maximizing recombinant protein yield. Conventional induction methods using isopropyl β-D-1-thiogalactopyranoside (IPTG) are not only toxic to cells

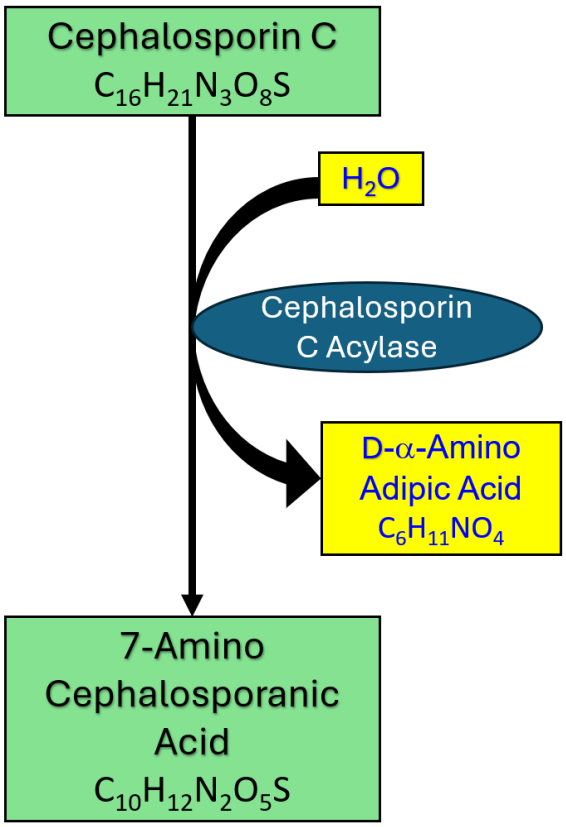



FIGURE 1. Enzymatic conversion of cephalosporin C to 7-aminocephalosporanic acid (7-ACA) by cephalosporin C acylase (CCA) in a one-step process. The reaction involves the hydrolytic removal of the D-α-aminoadipic acid side chain, yielding 7-ACA, a key intermediate in the synthesis of semi-synthetic cephalosporin antibiotics (Adapted from Pollegioni, Rosini & Molla 2013)

but also costly and labor-intensive, often requiring precise timing for effective induction (Khani & Bagheri 2020; Li et al. 2011). Autoinduction media offer a valuable alternative, facilitating simultaneous growth and induction of protein expression without the need for external inducers. This approach exploits a metabolic shift that occurs during the transition from the exponential to the stationary phase, triggering protein expression and simplifying the fermentation process. The use of autoinduction medium has been reported to increase the yield of target proteins compared to conventional media (Briand et al. 2016; Isakova et al. 2023; Kataoka & Takasu 2020; Nikolova et al. 2021).

This study aims to evaluate the efficiency of different *E. coli* strains—BL21(DE3), BL21-CodonPlus(DE3) RIPL, and B(DE3) Origami - for the expression of recombinant CCA. Among these, the highest CCA-producing strain was selected for subsequent optimization of medium components and culture conditions using an autoinduction medium to achieve high-yield production. Furthermore, the produced enzyme will be characterized to assess its activity, stability, and kinetic parameters, providing insights into the optimal conditions for industrial-scale production. The findings of this research are expected to significantly contribute to the efficient and cost-effective production of recombinant CCA, thereby supporting its application in the large-scale manufacturing of cephalosporin antibiotics.

## MATERIALS AND METHODS

### PLASMID AND STRAINS

The gene sequence of the S12 variant of cephalosporin C acylase (CCA), derived from *Pseudomonas* sp. SE83, includes six-point mutations (V121A, G139S, F58N, I75T, I176V, and S471C) and was synthesized and codon-optimized for *E. coli* expression by GenScript, USA. The full nucleotide and amino acid sequences of the S12 mutant are described by Shin et al. (2009) as SEQ ID NO: 6. The gene was cloned into the pET21a(+) expression vector between the *NdeI* and *XhoI* restriction sites, following the method of Martius, Wibisana and Ardiyani (2018). The

recombinant plasmid, designated pET21a(+)-S12, was transformed into three *E. coli* strains, namely BL21(DE3), BL21-CodonPlus(DE3) RIPL, and B(DE3) Origami, to identify the optimal host for recombinant CCA expression.

### *E. coli* STRAIN SELECTION

The three different *E. coli* strains - BL21(DE3), BL21-CodonPlus(DE3) RIPL, and B(DE3) Origami - were evaluated for optimal expression of recombinant CCA. Each recombinant strain, harbouring the CCA-encoding gene, was cultured in an unoptimized autoinduction medium in a 250 mL Erlenmeyer flask with 50 mL working volume. Strain selection was based on the ability to produce active CCA, as assessed by enzyme activity assays and protein expression analysis via SDS-PAGE. The strain exhibiting the highest expression and enzymatic activity, *E. coli* BL21(DE3), was subsequently chosen for further optimization of the autoinduction medium and fermentation conditions to enhance CCA production.

### CULTIVATION AND OPTIMIZATION MEDIA

Seed cultivation agar medium (LB agar plate) was prepared using 1.5% agar and LB (Luria-Bertani) medium containing 0.5% NaCl, 1% tryptone, and 0.5% yeast extract. Seed cultivation liquid medium (LB broth) was prepared without agar, using the same LB formulation. For the subsequent optimization stage, the Plackett-Burman Design (PBD) method was applied using an autoinduction medium adapted from Li et al. (2011) in 250 Erlenmeyer flasks with 50 mL working volume. This unoptimized medium consisted of tryptone, yeast extract, $(NH_4)_2SO_4$, $KH_2PO_4$, $Na_2HPO_4 \cdot 2H_2O$, glucose monohydrate, lactose, glycerol, $MgSO_4 \cdot 7H_2O$, and trace elements (2.5 mg/L $Na_2MoO_4 \cdot H_2O$, 2.5 mg/L $CoCl_2 \cdot 6H_2O$, 1.5 mg/L $CuCl_2 \cdot 2H_2O$, 3 mg/L $H_3BO_3$, 8 mg/L $Zn(CH_3COO)_2 \cdot 2H_2O$, 8.4 mg/L Titriplex III, 12.3 mg/L $MnCl_2 \cdot 2H_2O$, and 60 mg/L Fe(III) citrate). The detailed composition of the medium variations used in the subsequent PBD optimization is presented in Table 1.

### ENZYME PRODUCTION

Seed cultures were prepared by inoculating a single colony of recombinant *E. coli* from LB agar plates into 5 mL of LB broth supplemented with 100 µg/mL ampicillin. The culture was incubated overnight at 250 rpm and 37 °C. For enzyme production, 2% (v/v) of the seed culture was transferred into 50 mL of LB broth containing ampicillin in a 250 mL Erlenmeyer flask. The culture was initially incubated at 37 °C and 250 rpm for 3 h, followed by a temperature shift to 25 °C for an additional 21 h to induce CCA expression. After fermentation, the cells were harvested by centrifugation at 10,000 rpm and 4 °C for 10 min. The resulting cell pellets were stored at -20 °C until further processing for protein extraction and analysis. Autoinduction medium was used to enable high-level expression without IPTG. A temperature shift from 37 °C to 25 °C aimed to improve protein solubility and reduce inclusion body formation. A 21-h enzyme expression period allowed adequate expression while minimizing degradation. These conditions ensured consistent comparison across host strains.

### CRUDE ENZYME EXTRACTION

For routine analysis, CCA was prepared by aliquoting 1 mL of the culture and harvesting the cells by centrifugation at 10,000 rpm and 4 °C for 10 min. The cell pellets were

TABLE 1. Variables used in Plackett-Burman experimental design

| Variables | Code | Units | Low level (-1) | High level (+1) |
|---|---|---|---|---|
| Tryptone | $X_1$ | g/L | 30 | 40 |
| Yeast extract | $X_2$ | g/L | 15 | 25 |
| $(NH_4)_2SO_4$ | $X_3$ | g/L | 3 | 3.5 |
| $KH_2PO_4$ | $X_4$ | g/L | 6.5 | 7.2 |
| $Na_2HPO_4.2H_2O$ | $X_5$ | g/L | 6.8 | 7.4 |
| Glucose monohydrate | $X_6$ | g/L | 0.25 | 0.75 |
| Lactose | $X_7$ | g/L | 1.5 | 3 |
| Glycerol | $X_8$ | g/L | 0 | 4 |
| $MgSO_4.7H_2O$ | $X_9$ | g/L | 0.1 | 0.2 |
| Trace element 10× | $X_{10}$ | mL/L | 3 | 8 |
| Expression temperature | $X_{11}$ | °C | 25 | 30 |
| Inoculum quantity | $X_{12}$ | % (v/v) | 1 | 5 |
| Expression time | $X_{13}$ | hours | 18 | 22 |
| Propagation time | $X_{14}$ | hours | 2 | 3 |

washed twice with 0.1 M potassium phosphate buffer (pH 8.0) and then resuspended in the same buffer. Half a millilitre of the cell suspension was supplemented with PMSF to a final concentration of 1 mM, followed by homogenization using a TOMY Micro Smash MS 100 in a 2 mL tube containing 50% (w/v) of 0.5 mm silica beads. Cell disruption was performed at 5500 rpm for 10 cycles of 30 s on and 2 min off. The suspension was centrifuged at 15,000 rpm for 10 min, and the supernatant was collected as the crude enzyme in fresh microtubes.

### ENZYME ASSAY

The enzyme activity was tested following the method described by Sun et al. (2021), with slight modifications. The substrate solution was prepared by dissolving CPC in 0.1 M potassium phosphate buffer (pH 8.5) to a concentration of 20 mg/mL. Twenty microliters of enzyme solution were mixed with 20 µL of substrate solution and incubated at 37 °C for 5 min. The reaction was terminated by adding 200 µL of stop solution (50 mM NaOH and 20% glacial acetic acid, in a 1:2 ratio). The mixture was centrifuged at 12,000 rpm for 5 min, and 200 µL of the supernatant was mixed with 50 µL of 0.5% (w/v) pDAB methanol solution. After standing at room temperature for 10 min, the absorbance at 415 nm was measured using a spectrophotometer. The CCA activity was calculated based on the absorbance value and a standard curve. One unit of CCA activity was defined as the amount of enzyme required to produce 1 µmol of 7-ACA per minute at 37 °C and pH 8.5. Each sample was measured in triplicate.

### PLACKETT-BURMAN DESIGN (PBD)

The Plackett-Burman Design (PBD) was employed to evaluate the effects of various autoinduction medium components, fermentation conditions (expression temperature, expression time, and propagation time), and inoculum quantity on enzyme activity produced by the selected strain only (*E. coli* BL21(DE3)). The variables tested included 10 medium components and four fermentation conditions, as detailed in Table 1. Each variable was tested at two levels: low (-1) and high (+1). A total of 20 experimental runs, with two repetitions each, were performed, and the average results are summarized in Table 2. Enzyme activity was measured in U/mL, and the PBD analysis was based on the first-order polynomial model (1):

$$Y = \beta_o + \sum \beta_i X_i \quad (1)$$

TABLE 2. Plackett-Burman experimental design and result

| Run order | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $X_6$ | $X_7$ | $X_8$ | $X_9$ | $X_{10}$ | $X_{11}$ | $X_{12}$ | $X_{13}$ | $X_{14}$ | Enzyme activity (U/mL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | 18.4 |
| 2 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 14.6 |
| 3 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | 18.6 |
| 4 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | 15.2 |
| 5 | -1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 12.4 |
| 6 | -1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 14.8 |
| 7 | -1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | 28.2 |
| 8 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | -1 | 28.0 |
| 9 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 26.6 |
| 10 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 1 | 35.9 |
| 11 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 14.8 |
| 12 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 13.1 |
| 13 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 27.4 |
| 14 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | 13.3 |
| 15 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 30.4 |
| 16 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | -1 | 27.6 |
| 17 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 7.6 |
| 18 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | 7.9 |
| 19 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | 19.3 |
| 20 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | 12.2 |

where $Y$ represents the enzyme activity response; $\beta_0$ is the model intercept; $\beta_i$ are the linear coefficients; and $X_i$ are the levels of the independent variables. Analysis of variance (ANOVA) was used to determine p-values and $R$ coefficients, assessing the significance and fit of the regression model. Variables with significant effects ($p<0.05$) on enzyme activity were identified and selected for further optimization using the Central Composite Design (CCD) of Response Surface Methodology (RSM).

The composition of trace elements used in this experiment was adapted from Li et al. (2011), with minor modifications. The formulation included the following components: 2.5 mg/L sodium molybdate dihydrate ($Na_2MoO_4 \cdot H_2O$), 2.5 mg/L cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 1.5 mg/L copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), 3 mg/L boric acid ($H_3BO_3$), 8 mg/L zinc acetate dihydrate [$Zn(CH_3COO)_2 \cdot 2H_2O$], 8.4 mg/L Titriplex III (EDTA), 12.3 mg/L manganese(II) chloride dihydrate ($MnCl_2 \cdot 2H_2O$), and 60 mg/L ferric citrate [Fe(III) citrate]. A 10× concentrated stock solution of the trace element mixture was prepared and added in appropriate volumes as specified in Table 1, to prepare a final fermentation medium of 50 mL in a 250 mL flask.

## PATH OF STEEPEST ASCENT

To determine a fit level for the selected variables, the method of steepest ascent was employed. This approach involves moving in the direction that maximizes the increase in enzyme activity. Based on the PBD results, key factors and their step changes were identified. Regression coefficients were used to determine the direction of ascent, prioritizing factors with higher positive coefficients and minimizing the influence of negative ones.

## CENTRAL COMPOSITE DESIGN (CCD)

The Central Composite Design (CCD) was utilized to assess the main, interaction, and quadratic effects of significant independent variables identified from the PBD. Experimental design and statistical analysis were performed using Design Expert version 7.0 (Stat-Ease Inc., Minneapolis, USA), incorporating six repetitions at centre points for robustness. The independent variables selected for CCD included glucose monohydrate concentration (g/L; $X_6$), glycerol concentration (g/L; $X_8$), trace element concentration (mL/L; $X_{10}$), and expression time (hours; $X_{13}$). These variables were assessed at three levels, as shown in Table 3. Thirty experimental permutations, including six centre point duplicates, were conducted, and CCA activity was measured as the response variable (Y).

A multiple regression analysis was performed to create an empirical model relating the response to the independent variables, using a second-order polynomial equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \; i, j = 1, 2 \ldots k \quad (2)$$

where $Y$ represents the response variable (specific enzyme activity); $\beta_0$ is the interception coefficient; $\beta_i$ is the coefficient for the linear effects; $\beta_{ii}$ is the coefficient for the quadratic effects; $\beta_{ij}$ are interaction coefficients; and $X_i$ and $X_j$ are the coded independent variables. Data were analysed by one-way ANOVA with Tukey's multiple comparison tests ($p<0.05$) using the Design Expert software.

## VERIFICATION EXPERIMENT

To validate the optimal fermentation conditions, three parallel experiments were conducted under the optimal conditions. The average results from these experiments were used to assess the reliability of the model and finalize the optimization parameters.

## ENZYME PURIFICATION

Cell lysis for enzyme purification was performed by sonication, following the method described by Julkipli, Syamsu and Wibisana (2022). Enzyme purification was conducted using Ni-NTA resin according to the manufacturer's protocol (Novex). The cell lysate, prepared in lysis buffer, was applied to a Ni-NTA agarose column pre-equilibrated with the same buffer (50 mM $NaH_2PO_4$, pH 8.0, 500 mM NaCl). The column was washed with four bed volumes of wash buffer (100 mM phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole) and eluted with elution buffer (100 mM phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole). Eluted fractions containing cephalosporin acylase were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl to exchange the buffer and remove contaminants. Samples were subsequently analyzed for enzyme activity, protein concentration, and molecular weight using SDS-PAGE.

## ENZYME CHARACTERIZATION

All characterizations were conducted using purified CCA with CPC as the substrate. The optimal temperature was determined by reacting the enzyme and substrate in a buffer over a temperature range of 30-50 °C in a water bath for 5 min. The optimal pH was established by testing over a pH range of 4.0-9.0 using phosphate buffer at 37 °C for 5 min. Thermostability was assessed by measuring residual enzyme activity in 100 mM phosphate buffer (pH 8.5) at temperatures ranging from 30-60 °C. Samples were taken at various time points and rapidly cooled in an ice-water bath. pH stability was evaluated by incubating the enzyme in buffers with pH values ranging from 5.0 to 10.0. The buffers used included acetate buffer (50 mM, pH 5.0), phosphate buffer (100 mM, pH 6.0-8.5), and ammonium chloride buffer (50 mM, pH 9.0-10.0). Enzyme aliquots were maintained at 37 °C for 1 h, after which residual activity was determined using the previously described enzyme assay. Kinetic parameters, including Km and Vmax, were determined using the Lineweaver-Burk plot by measuring the initial reaction rate of CCA with CPC at concentrations ranging from 0.2 to 2.0 mg/mL.

TABLE 3. CCD-RSM for CCA production optimization

| Run | Coded levels | | | | Uncoded levels | | | | Enzyme activity (U/mL) |
|---|---|---|---|---|---|---|---|---|---|
| | $X_6$ | $X_8$ | $X_{10}$ | $X_{13}$ | $X_6$ | $X_8$ | $X_{10}$ | $X_{13}$ | |
| 1 | -1 | -1 | -1 | -1 | 0.4 | 1 | 5 | 19 | 29.8 |
| 2 | 1 | -1 | -1 | -1 | 0.8 | 1 | 5 | 19 | 48.9 |
| 3 | -1 | 1 | -1 | -1 | 0.4 | 3 | 5 | 19 | 25.6 |
| 4 | 1 | 1 | -1 | -1 | 0.8 | 3 | 5 | 19 | 32.6 |
| 5 | -1 | -1 | 1 | -1 | 0.4 | 1 | 8 | 19 | 14.5 |
| 6 | 1 | -1 | 1 | -1 | 0.8 | 1 | 8 | 19 | 41.1 |
| 7 | -1 | 1 | 1 | -1 | 0.4 | 3 | 8 | 19 | 16.3 |
| 8 | 1 | 1 | 1 | -1 | 0.8 | 3 | 8 | 19 | 24.5 |
| 9 | -1 | -1 | -1 | 1 | 0.4 | 1 | 5 | 23 | 26.3 |
| 10 | 1 | -1 | -1 | 1 | 0.8 | 1 | 5 | 23 | 29.2 |
| 11 | -1 | 1 | -1 | 1 | 0.4 | 3 | 5 | 23 | 26.1 |
| 12 | 1 | 1 | -1 | 1 | 0.8 | 3 | 5 | 23 | 10.6 |
| 13 | -1 | -1 | 1 | 1 | 0.4 | 1 | 8 | 23 | 22.4 |
| 14 | 1 | -1 | 1 | 1 | 0.8 | 1 | 8 | 23 | 35.4 |
| 15 | -1 | 1 | 1 | 1 | 0.4 | 3 | 8 | 23 | 13.6 |
| 16 | 1 | 1 | 1 | 1 | 0.8 | 3 | 8 | 23 | 11.6 |
| 17 | -2 | 0 | 0 | 0 | 0.2 | 2 | 6.5 | 21 | 14.8 |
| 18 | 2 | 0 | 0 | 0 | 1 | 2 | 6.5 | 21 | 33.4 |
| 19 | 0 | -2 | 0 | 0 | 0.6 | 0 | 6.5 | 21 | 39.5 |
| 20 | 0 | 2 | 0 | 0 | 0.6 | 4 | 6.5 | 21 | 28.8 |
| 21 | 0 | 0 | -2 | 0 | 0.6 | 2 | 3.5 | 21 | 26.5 |
| 22 | 0 | 0 | 2 | 0 | 0.6 | 2 | 9.5 | 21 | 13.8 |
| 23 | 0 | 0 | 0 | -2 | 0.6 | 2 | 6.5 | 17 | 36.8 |
| 24 | 0 | 0 | 0 | 2 | 0.6 | 2 | 6.5 | 25 | 11.5 |
| 25 | 0 | 0 | 0 | 0 | 0.6 | 2 | 6.5 | 21 | 59.0 |
| 26 | 0 | 0 | 0 | 0 | 0.6 | 2 | 6.5 | 21 | 51.1 |
| 27 | 0 | 0 | 0 | 0 | 0.6 | 2 | 6.5 | 21 | 45.7 |
| 28 | 0 | 0 | 0 | 0 | 0.6 | 2 | 6.5 | 21 | 47.0 |
| 29 | 0 | 0 | 0 | 0 | 0.6 | 2 | 6.5 | 21 | 50.1 |
| 30 | 0 | 0 | 0 | 0 | 0.6 | 2 | 6.5 | 21 | 52.0 |

### CONVERSION OF CPC TO 7-ACA

The conversion of CPC to 7-ACA was evaluated to assess enzyme performance. CPC solutions at concentrations of 0.2%, 0.5%, 1.0%, and 2.0% were prepared in 0.1 M phosphate buffer (pH 8.5) and mixed with CCA to achieve a total enzyme activity of 10 U. The mixture was incubated at 37 °C for 90 min. At specific intervals, 20 µL samples were withdrawn, and the reaction was halted by adding 200 µL of stop solution (5% acetic acid with NaOH). The samples were then analysed by HPLC using a Shimadzu 20A HPLC system with a Luna® C8 column (5 µm, 4.6 × 150 mm). The mobile phase consisted of 25 mM $KH_2PO_4$ solution (pH 3.0) with 5% acetonitrile, at a flow rate of 1 mL/min. Absorbance was measured using a UV detector at $\lambda = 254$ nm.

### SDS-PAGE ANALYSIS

The lysate was resuspended in 2× SDS sample buffer (0.004% bromophenol blue, 10% 2-mercaptoethanol, 20% glycerol, 4% SDS, 0.125 M Tris-HCl; Sigma) with the buffer volume calculated as [resuspension volume (mL) = $OD_{600}$ / 6]. Samples were boiled for 5 min, incubated on ice, and analysed by SDS-PAGE.

### STATISTICAL ANALYSES

Statistical analysis was performed using Stat-Ease software for optimization studies, while Microsoft Excel was used to compare the experimental results under optimal conditions during model validation using a t-test.

### RESULTS AND DISCUSSION

Enzyme activity analysis confirmed the functional expression of the recombinant protein, with volumetric CCA activities of 16.63, 9.48, and 9.06 U/mL for *E. coli* strains BL21(DE3), BL21-CodonPlus(DE3) RIPL, and B(DE3) Origami, respectively (Figure 2). The higher enzyme yield and activity observed in *E. coli* BL21(DE3), compared to RIPL(DE3), suggest that codon usage in BL21(DE3) is already optimized for efficient translation. This observation is consistent with the findings of Correddu et al. (2020), who reported that codon optimization and the substitution of clustered rare codons significantly enhanced CCA expression relative to expression in *E. coli* BL21(DE3) RIPL. In our case, the additional rare tRNAs provided by RIPL(DE3) appeared redundant and did not confer a significant advantage for CCA expression.

Similarly, the use of the Origami B strain resulted in a lower yield of active CCA. *E. coli* Origami is engineered to enhance disulfide bond formation through an oxidizing intracellular environment (de Marco 2009), which benefits proteins requiring disulfide bonds for proper folding. However, CCA may not require extensive disulfide bonding or post-translational modifications. Consequently, the Origami strain did not provide any notable advantage for CCA expression, and the engineered environment may have introduced additional stress or misfolding issues, leading to lower enzyme activity compared to BL21(DE3).

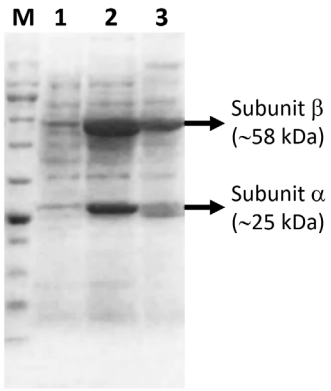
Production yields and protein activity are influenced not only by the bacterial strain used but also by the specific protein being expressed. In this case, the presence of additional tRNAs in the RIPL(DE3) strain and the ability of the Origami strain to promote disulfide bond formation could have imposed a higher metabolic load. This increased burden may have diverted cellular resources away from protein synthesis, resulting in lower enzyme expression and activity.

### PLACKETT-BURMAN EXPERIMENTAL DESIGN

A Plackett-Burman design (PBD) was employed to screen and identify key factors influencing the biosynthesis of CCA in *E. coli* during fermentation. Based on preliminary host strain evaluation, *E. coli* BL21(DE3) was selected for further optimization experiments aimed at enhancing CCA expression. The PBD analysis showed that glucose, glycerol, trace element concentration, and enzyme expression time had a statistically significant effect on enzyme activity (Tables 2 & 4), with a high coefficient of determination ($R^2$ = 0.9355), indicating a good fit of the model. In contrast, factors such as inoculum volume and propagation time were not found to significantly influence enzyme production. CCA activity varied substantially across the 20 experimental runs, ranging from 7.6 to 35.9 U/mL, reflecting the impact of the tested variables.

To further improve enzyme yield, only the significant factors identified through PBD were selected for subsequent optimization, while the non-significant factors were maintained at minimal or fixed levels. ANOVA for the factorial model is presented in Table 4. The model exhibited an F-value of 5.18 and a p-value below 0.0500, confirming its statistical significance. Specifically, glucose

A




B

| Parameter | B(DE3) Origami | BL21 (DE3) | BL21-CodonPlus (DE3) RIPL |
|---|---|---|---|
| Volumetric CCA activity (U/mL) | 9.06 ±0.07 | 16.63 ± 1.14 | 9.48 ± 0.71 |
| CCA yield (U/OD) | 1.32 ± 0.04 | 3.15 ± 0.2 | 1.66 ± 0.09 |

FIGURE 2. SDS-PAGE analysis of recombinant CCA expression in three *E. coli* hosts (A) and its hydrolytic activities (B). Lane M: marker; lanes 1–3: B(DE3) Origami, BL21(DE3), and BL21-CodonPlus(DE3) RIPL, respectively. Each well was loaded with an equal protein concentration (25 µg) of soluble CCA. All assays were performed in duplicates

TABLE 4. ANOVA of the Plackett-Burman design matrix for screening medium and fermentation conditions

| Source | Sum of squares | df | Mean square | F value | p-value |
|---|---|---|---|---|---|
| Model | 1021.64 | 14 | 72.97 | 5.18 | 0.0398* |
| $X_1$-Tryptone (g/L) | 0.06 | 1 | 0.06 | 0.00 | 0.9487 |
| $X_2$-Yeast extract (g/L) | 0.04 | 1 | 0.04 | 0.00 | 0.9575 |
| $X_3$-$(NH_4)\cdot 2SO_4$ (g/L) | 47.35 | 1 | 47.35 | 3.36 | 0.1262 |
| $X_4$-$KH_2PO_4$ (g/L) | 0.02 | 1 | 0.02 | 0.00 | 0.9721 |
| $X_5$-$Na_2HPO_4\cdot 2H_2O$ (g/L) | 0.87 | 1 | 0.87 | 0.06 | 0.8131 |
| $X_6$-Glucose monohydrate (g/L) | 306.56 | 1 | 306.56 | 21.76 | 0.0055* |
| $X_7$-Lactose (g/L) | 8.18 | 1 | 8.18 | 0.58 | 0.4804 |
| $X_8$-Glycerol (g/L) | 125.24 | 1 | 125.24 | 8.89 | 0.0308* |
| $X_9$-$MgSO_4.7H_2O$ (g/L) | 17.80 | 1 | 17.80 | 1.26 | 0.3120 |
| $X_{10}$-Trace element 10× (mL) | 141.63 | 1 | 141.63 | 10.05 | 0.0248* |
| $X_{11}$-Temperature (°C) | 83.10 | 1 | 83.10 | 5.90 | 0.0595 |
| $X_{12}$-Inoculum quantity (%) | 18.59 | 1 | 18.59 | 1.32 | 0.3027 |
| $X_{13}$-Enzyme expression time (h) | 183.08 | 1 | 183.08 | 12.99 | 0.0155* |
| $X_{14}$-Propagation time (h) | 89.10 | 1 | 89.10 | 6.32 | 0.0535 |

*: $p<0.05$; $R^2 = 0.9355$

monohydrate (X6), glycerol (X8), trace elements 10× (X10), and enzyme expression time (X13) had p-values less than 0.0500, indicating their strong influence on CCA expression. These variables were thus chosen for further optimization in the next experimental phase.

## PATH OF STEEPEST ASCENT

The analysis of the steepest ascent path, as detailed in Table 5, showed an initial increase in enzyme activity with higher concentrations of glucose, enzyme expression time, trace elements, and glycerol. However, beyond a certain point, enzyme activity began to decline. The highest conditions for maximum enzyme activity were identified: glucose concentration of 0.6 g/L, enzyme expression time of 20 h, trace element concentration of 6.0 mL/L, and glycerol concentration of 1.0 g/L. These conditions were selected as the central values for subsequent response surface experiments.

## CENTRAL COMPOSITE DESIGN AND RESPONSE SURFACE METHODOLOGY

Upon determining the new lower and upper limits of the four critical variables - glucose, trace elements, glycerol concentration, and enzyme expression time - a response surface analysis was performed. The 4-factor Central Composite Design (CCD) matrix generated by the software, along with the experimental data for CCA production, is summarized in Table 3. The best model fit with the data was evaluated based on several criteria, including $R^2$, Adjusted $R^2$, Predicted $R^2$, and PRESS (Predicted Residual Error Sum of Squares). A summary of the statistics for different models used in the regression analysis is presented in Table 6. A quadratic model that relates enzyme activity to the independent variables was found to provide the best fit with the experimental data.

The correlation coefficient ($R^2$) used to determine the relationship between the experimental (actual) and predicted responses was 0.9598, as established by the model, which indicated a good fit between predicted values and the experimental data points (Figure 3). This suggests that 95.98% of the variations for CCA production are explained by the independent variables, and this also means that the model could not explain only 4.02% of variations. The predicted $R^2$ of 0.8323 was in reasonable agreement with the adjusted $R^2$ of 0.9223. Adequate precision measures the signal-to-noise ratio, in which a ratio greater than 4 is desirable. In this work, a ratio of 14.079 indicates the reliability of the experiment data.

ANOVA was used to further assess the adequacy of the model. Table 7 presents the results of the quadratic response surface model fitting in the form of ANOVA. An F-value of 25.58 indicates that the variation explained by the model is substantially greater than that of the residuals. Since the p-value associated with the model is less than 0.0001, the F-value of 25.58 is considered highly significant. This suggests that the factors included in the model have a statistically significant effect on the response (CCA production). Moreover, the significance of each model term was evaluated using the probability of error (Prob > F). In Table 7, Prob > F values less than 0.0500 indicate significant terms. It was found that $X_6$, $X_8$, $X_{10}$,

$X_{13}$, $X_6X_8$, $X_6X_{10}$, $X_6X_{13}$, $X_6^2$, $X_8^2$, $X_{10}^2$, and $X_{13}^2$ were significant model terms, while $X_8X_{10}$, $X_8X_{13}$, and $X_{10}X_{13}$ were insignificant for CCA production. Additionally, the lack-of-fit F-value of 0.860592 indicates that the lack of fit is insignificant relative to the pure error. A non-significant lack of fit is desirable, as it implies that the model fits well. The second-order polynomial model equation for coded variables is provided in Eq. (3), with coefficients having p-values greater than 0.05 removed.

$$Y\ (U/mL) = +47.93 + 4.03X_6 - 4.49X_8 - 3.14X_{10} - 4.53X_{13} - 4.00X_6X_8 + 2.01X_6X_{10} - 3.90X_6X_{13} - 5.97X_6^2 - 3.46\ X_8^2 - 6.97X_{10}^2 - 5.97X_{13}^2 \quad (3)$$

Figure 4 shows the 3D surface plot of the combined effect of two significant parameters on CCA production, with the other variables held constant. Figure 4(a) depicted that increasing the glucose concentration positively affected enzyme activity, particularly at higher glycerol concentrations. Glucose is a preferred carbon source for *E. coli*. When present in the medium, it inhibits the expression of the lac operon via catabolite repression. This prevents the induction of protein expression in the early stages of growth, allowing the cells to grow to a high density before protein production begins (Mühlmann et al. 2017). In this experiment, the highest final $OD_{600}$ was 12.12 at the end of fermentation and was in agreement with several reports

TABLE 5. Experimental design and results of steepest ascent path

| Run | $X_6$ | $X_8$ | $X_{10}$ | $X_{13}$ | Enzyme activity (U/mL) |
|---|---|---|---|---|---|
| 1 | 1.0 | 22 | 8.0 | 2.0 | 40.6 |
| 2 | 0.8 | 21 | 7.0 | 1.5 | 48.9 |
| 3 | 0.6 | 20 | 6.0 | 1.0 | 52.9 |
| 4 | 0.4 | 19 | 5.0 | 0.5 | 52.4 |
| 5 | 0.2 | 18 | 4.0 | 0.0 | 48.9 |

TABLE 6. Model summary statistics

| Source | Std. Dev. | Adjusted $R^2$ | Predicted $R^2$ | $R^2$ | PRESS | Comment |
|---|---|---|---|---|---|---|
| Linear | 11.77 | 0.3162 | 0.2068 | 0.1255 | 4432.86 | |
| 2FI | 12.19 | 0.4434 | 0.1504 | 0.0109 | 5013.44 | |
| Quadratic | 3.69 | 0.9598 | 0.9223 | 0.8323 | 850.05 | Suggested |
| Cubic | 3.40 | 0.9840 | 0.9338 | 0.8044 | 991.64 | Aliased |

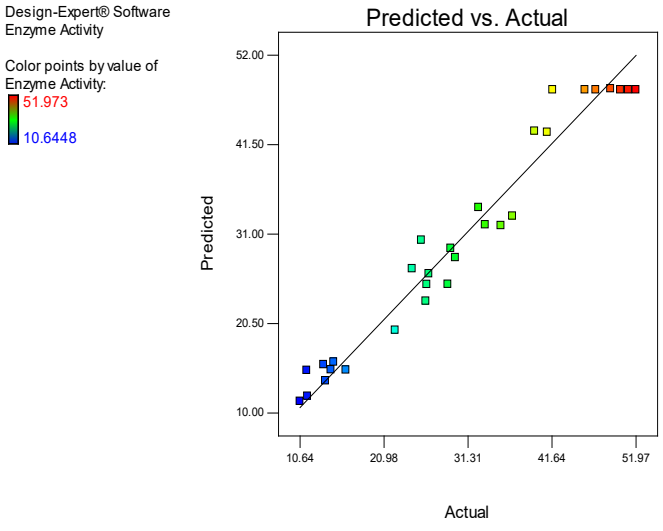



FIGURE 3. Plot of predicted vs actual enzyme activity values

(Isakova et al. 2023; Li et al. 2011; Nikolova et al. 2021). Glycerol also enhanced enzyme activity, with a significant increase observed at higher glucose levels. As glucose is depleted, glycerol consumption begins, supporting continued growth and the onset of protein expression. Trace elements are also a significant variable, as they are essential minerals required in very small amounts for the proper functioning of biological processes in organisms, including bacteria such as *E. coli*. Furthermore, enzyme production reached its maximum after incubation for CCA expression under specific conditions and time. In this experiment, 19.48 h were required to achieve maximum CCA expression.

## MODEL VERIFICATION

Based on the quadratic model presented in Table 7, the optimal conditions for maximum CCA activity were predicted to be 0.79 g/L glucose monohydrate, 1.09 g/L glycerol, 6.29% (v/v) trace elements (10×), and an enzyme expression time of 19.48 h. Under these conditions, the model estimated an enzyme activity of 53.4 U/mL. This predicted value represents a 3.8-fold increase compared to the unoptimized level and surpasses the activities reported in previous studies (Table 8) (Gaurav, Kundu & Kundu 2010; Jobanputra & Vasait 2015; Niwa et al. 1994; Sun et al. 2021). To validate the model, experiments were performed in triplicate under the predicted optimal conditions, yielding an average CCA activity of 57.7 U/mL. A two-tailed t-test at a 5% confidence level produced a p-value of 0.087, indicating no statistically significant difference between the predicted and experimental results. This confirms the reliability of the model in predicting optimal enzyme production conditions.

## ENZYME PURIFICATIONS

The purification of CCA from *E. coli* was achieved through a two-step process involving Ni-NTA affinity chromatography followed by dialysis, resulting in a 3.8-fold increase in specific activity and a final yield of 63.1% (Table 9). Initially, the crude extract contained 195 U of total activity and 93.1 mg of total protein, with a specific activity of only 2.1 U/mg. After IMAC purification, the specific activity increased to 3.0 U/mg, and further dialysis elevated it to 8.0 U/mg, reflecting successful enrichment of the target enzyme. SDS-PAGE analysis (Figure 5) confirmed the purification process by showing a reduction in background proteins across the fractions and the appearance of two distinct bands in the purified fraction, corresponding to the α-subunit (~25 kDa) and β-subunit (~58 kDa) of the CCA precursor (83 kDa total), consistent with its expected molecular structure. These results indicate that the applied purification strategy effectively isolates and concentrates CCA with minimal activity loss and good purity for downstream applications.

TABLE 7. ANOVA of central composite design for CCA optimization

| Source | Sum of squares | df | Mean square | F value | p-value Prob > F |
|---|---|---|---|---|---|
| Model | 4865.068 | 14 | 347.5048 | 25.58575 | < 0.0001* |
| $X_6$-Glucose monohydrate | 389.5923 | 1 | 389.5923 | 28.68453 | < 0.0001* |
| $X_8$-Glycerol | 484.5166 | 1 | 484.5166 | 35.67352 | < 0.0001* |
| $X_{10}$-Trace element 10× | 236.6821 | 1 | 236.6821 | 17.4262 | 0.0008* |
| $X_{13}$-Enzyme expression time | 491.8231 | 1 | 491.8231 | 36.21148 | < 0.0001* |
| $X_6X_8$ | 255.5036 | 1 | 255.5036 | 18.81197 | 0.0006* |
| $X_6X_{10}$ | 64.85809 | 1 | 64.85809 | 4.775309 | 0.0451* |
| $X_6X_{13}$ | 243.308 | 1 | 243.308 | 17.91405 | 0.0007* |
| $X_8X_{10}$ | 4.31445 | 1 | 4.31445 | 0.31766 | 0.5813 |
| $X_8X_{13}$ | 16.14476 | 1 | 16.14476 | 1.188691 | 0.2928 |
| $X_{10}X_{13}$ | 60.72759 | 1 | 60.72759 | 4.471193 | 0.0516 |
| $X_6^2$ | 979.0737 | 1 | 979.0737 | 72.0863 | < 0.0001* |
| $X_8^2$ | 328.2479 | 1 | 328.2479 | 24.16792 | 0.0002* |
| $X_{10}^2$ | 1331.684 | 1 | 1331.684 | 98.04797 | < 0.0001* |
| $X_{13}^2$ | 977.3608 | 1 | 977.3608 | 71.96019 | < 0.0001* |
| Residual | 203.7295 | 15 | 13.58197 | | |
| Lack of Fit | 128.8615 | 10 | 12.88615 | 0.860592 | 0.6088 |
| Pure Error | 74.86798 | 5 | 14.9736 | | |
| Cor Total | 5068.797 | 29 | | | |

*: $p<0.05$; $R^2 = 0.9355$

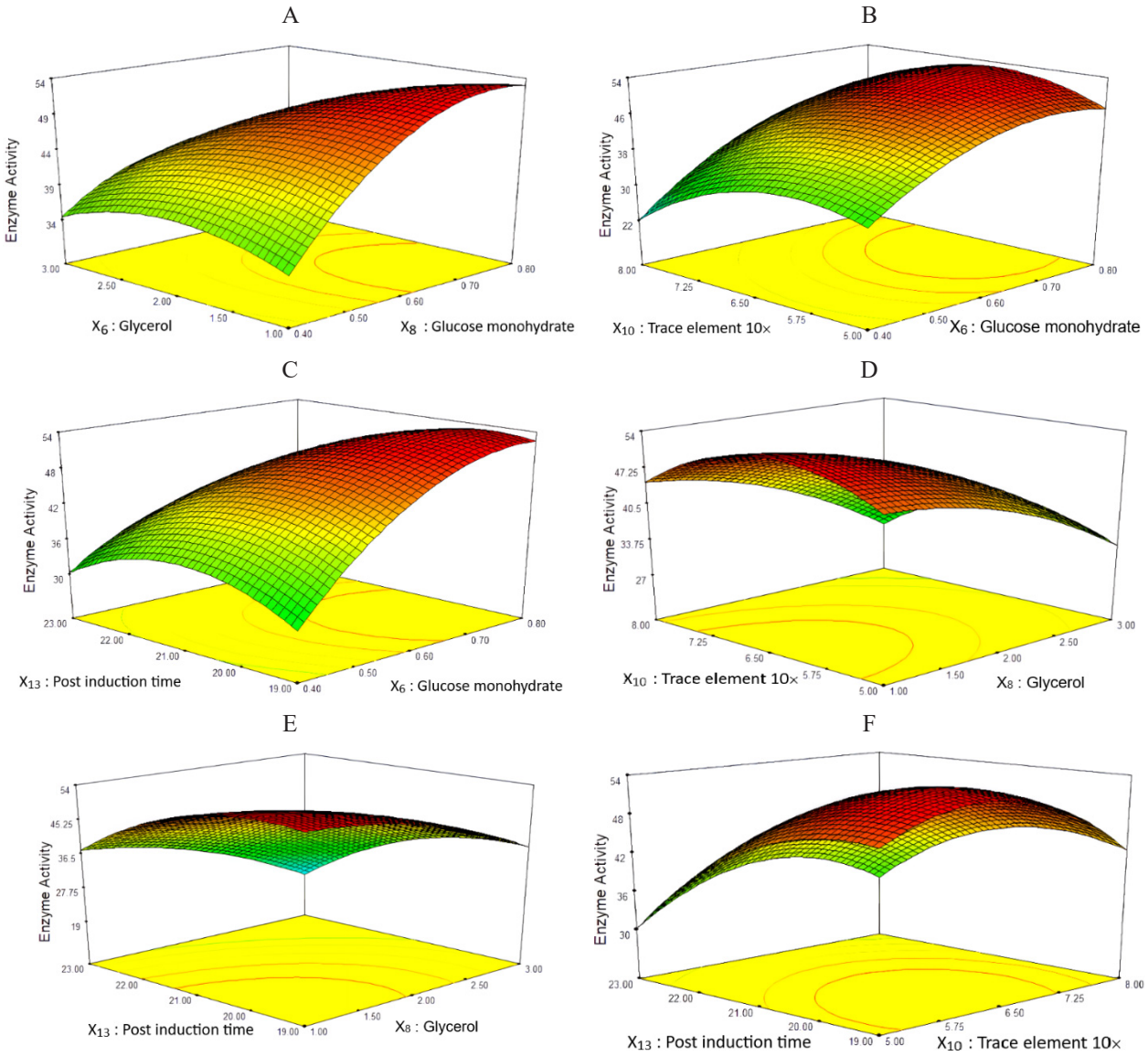



FIGURE 4. Response surface showing the effects of significant variables on the activity of CCA produced by *E. coli*. Interaction between glucose and glycerol concentration (a), glucose and trace element concentration (b), glucose concentration and enzyme expression time (c), glycerol and trace element concentration (d), glycerol concentration and enzyme expression time, and trace element concentration and enzyme expression time

TABLE 8. Reported CCA activities from various microbial sources under different cultivation and assay conditions

| Microbial source | Reported activity | Conditions | Reference |
|---|---|---|---|
| *E. coli* S12 | 53.3 U/mL | Cultivated in an optimized autoinduction medium; activity measured using CPC substrate | This work |
| *Pseudomonas* species | 33.1-42.9 U/mL | Activity measured using CPC and GpNA substrates; peak activity observed in lysate after 72 h incubation | Jobanputra & Vasait (2015) |
| *Micrococcus luteus* | 7.65 U/mL | Grown in optimized medium; activity assessed using CPC substrate | Gaurav, Kundu & Kundu (2010) |
| *E. coli* JM109 | 41.1 U/mL | Cultured in N-3 broth; activity measured in lysate supernatant | Niwa et al. (1994) |
| *E. coli* BBa_J23105 | 12.772 U/mL | Cultivated in optimized medium; activity measured using CPC substrate | Sun et al. (2021) |

TABLE 9. Purication of CCA from *E. coli*

| Purification step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 195.0 | 93.1 | 2.1 | 1.0 | 100.0 |
| Imac Ni-NTA | 134.4 | 44.2 | 3.0 | 1.5 | 68.9 |
| Dialysis | 123.1 | 15.45 | 8.0 | 3.8 | 63.1 |

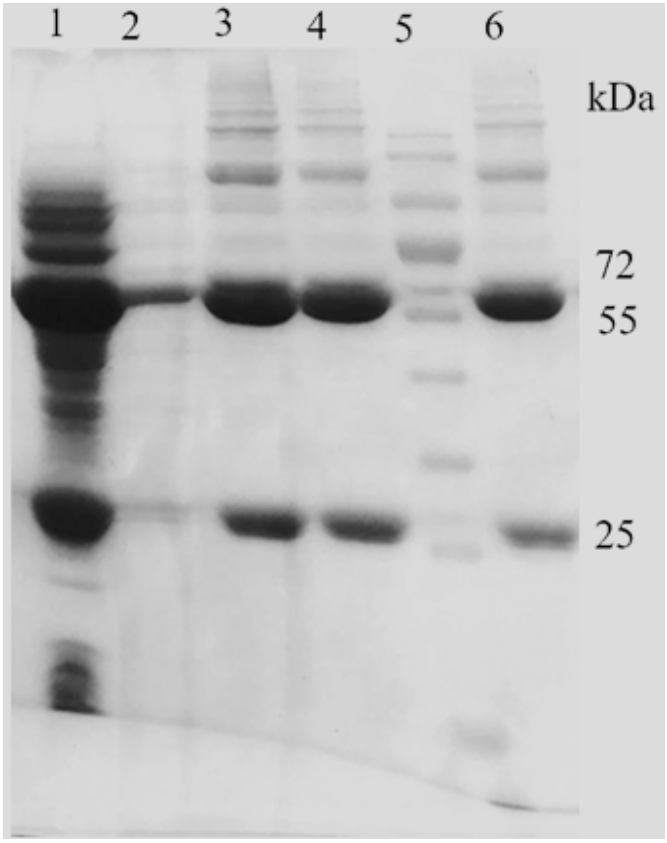



FIGURE 5. SDS-PAGE analysis of crude extract and purified CCA obtained via one-step purification using a nickel-NTA column. Lanes: (1) crude extract; (2) fraction 4; (3) fraction 5; (4) fraction 6; (5) protein marker; (6) fraction 7. The precursor of S12 has an approximate molecular weight of 83 kDa, comprising an α-subunit (25 kDa) and a β-subunit (58 kDa)

## ENZYMATIC PROPERTIES

The CCA enzyme exhibits optimal activity at 45 °C and pH 8.5, making it well-suited for moderately thermophilic and alkaline conditions (Figure 6(A)-6(B)). Enzyme activity increases steadily from 30 °C, reaching a peak at 45 °C (~350 U/mL), followed by a slight decline at 50 °C, indicating moderate thermal tolerance. Activity is minimal at acidic pH but rises sharply above pH 7, with a maximum observed at pH 8.5 (~180 U/mL). Regarding stability, the enzyme retains over 90% of its activity between 30 °C and 40 °C but undergoes a sharp decline beyond 45 °C, dropping to ~10% at 60 °C (Figure 6(C)). Its pH stability is robust, maintaining over 85% activity between pH 6 and 9, with optimal stability around pH 8-8.5, although a decrease is noted at pH 10 (Figure 6(D)). These characteristics suggest that the enzyme is both catalytically efficient and relatively stable under neutral to mildly alkaline conditions and temperatures up to 45 °C, supporting its applicability in diverse biotechnological processes.

The kinetic properties of cephalosporin C (CPC) acylase were investigated, with particular emphasis on the enzyme's substrate affinity, typically expressed as Km and Vmax. The Km and Vmax values for CPC acylase were determined using CPC as the substrate. Initial reaction

rates were measured across a range of CPC concentrations (0.2 to 2.0%) and analysed using a Lineweaver-Burk plot (Figure 7). The determined Km for CPC was 5.273 mM, and the Vmax was 0.978 U/mL.

Bioconversion experiments were conducted at several substrate concentrations while maintaining a constant enzyme concentration in its free form. These experiments were performed under conditions mimicking industrial applications, specifically in 0.1 M phosphate buffer at pH 8.5 and a temperature of 37 °C. As shown in Figure 8, at 0.2% CPC, the reaction rapidly reaches a plateau, suggesting that the enzyme operates below its optimal substrate concentration, resulting in a slower rate of 7-ACA formation. Higher substrate concentrations (0.5% to 2.0% CPC) exhibited increased rates of 7-ACA formation, indicating a more optimal substrate concentration range. However, at 2% CPC, the reaction begins to level off slightly earlier, potentially indicating mild substrate or product inhibition or enzyme saturation at higher concentrations. Furthermore, after 30 min, a plateau starts to appear at all CPC concentrations. Since substrate remains available (particularly in the cases of 1% and 2% CPC), this plateau suggests that the rate of 7-ACA formation is nearing equilibrium, in addition to the potential effects of substrate or product inhibition or enzyme saturation mentioned earlier.

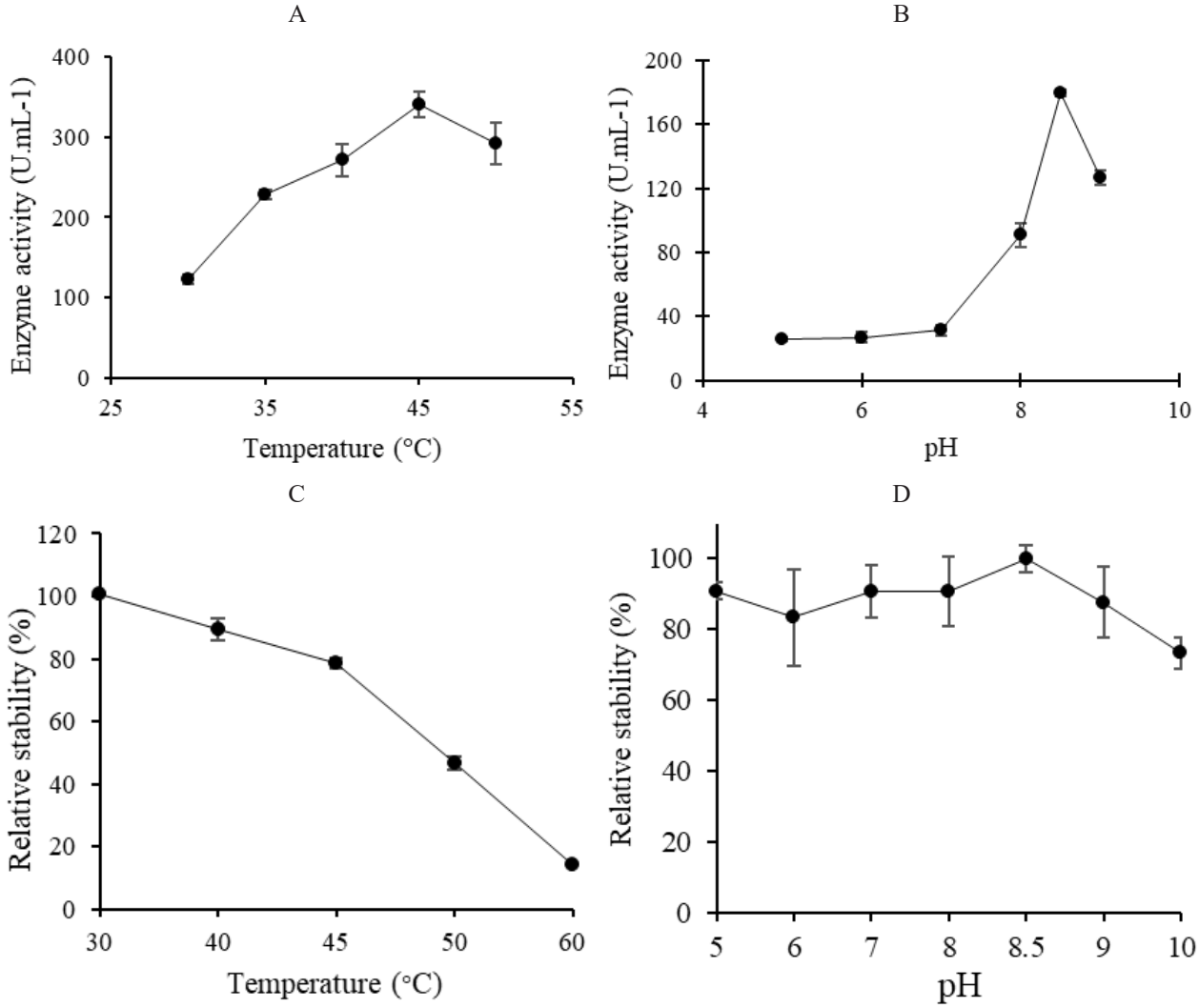



FIGURE 6. CCA characteristics: optimum temperature (A), optimum pH (B), temperature stability (C), and pH stability (D)

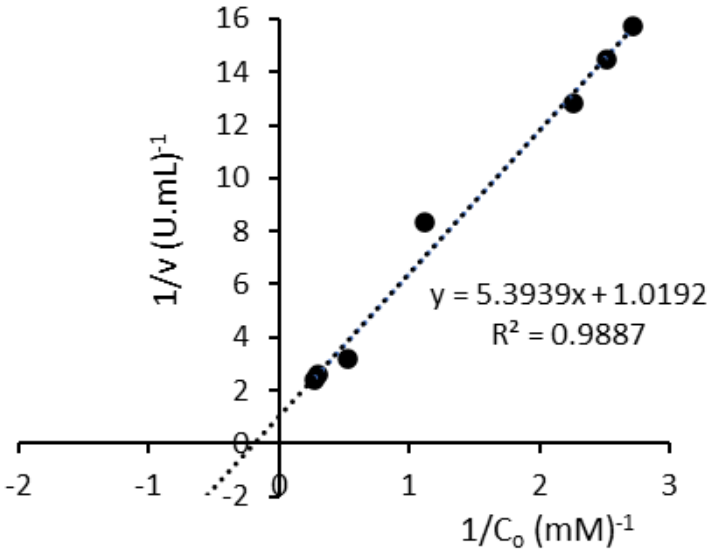



FIGURE 7. Lineweaver-Burk plot showing the effect of substrate concentration on enzyme activity

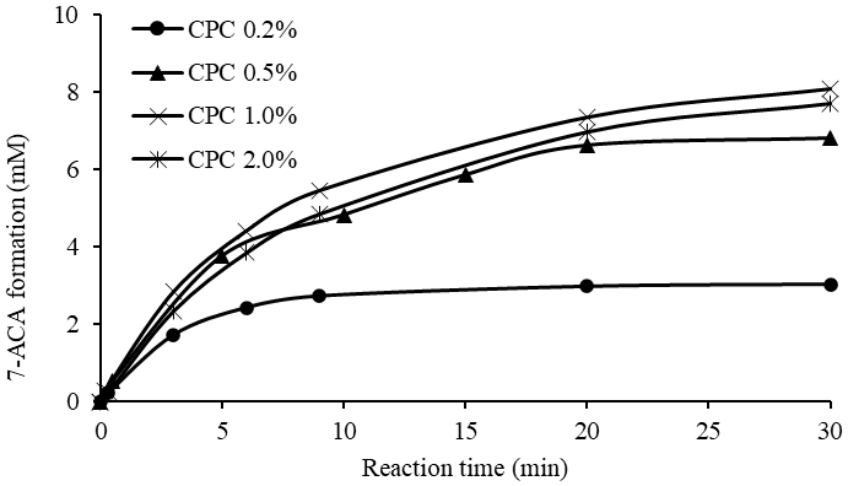



FIGURE 8. Time course of 7-ACA formation at various substrate concentrations

## CONCLUSION

The optimization of recombinant CCA production in various *E. coli* strains using an autoinduction medium resulted in significant enhancements in enzyme yield. Through initial screening with the Plackett-Burman design, key medium components and fermentation conditions - such as glucose monohydrate, glycerol, trace elements, and enzyme expression time - were identified as critical factors. Further optimization using the Central Composite Design led to a maximum enzyme activity of 53.3 U/mL, representing a 3.8-fold increase compared to non-optimized conditions. The optimal parameters included 0.78 g/L glucose, 1.12 g/L glycerol, 6.21 mL/L trace elements, and a enzyme expression time of 19.48 h. These findings highlight the potential for significantly improving CCA production, which has important implications for the large-scale synthesis of β-lactam antibiotics, particularly cephalosporins. This study provides a robust framework for optimizing recombinant protein production for industrial applications.

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REFERENCES


Briand, L., Marcion, G., Kriznik, A., Heydel, J.M., Artur, Y., Garrido, C., Seigneuric, R. & Neiers, F. 2016. A self-inducible heterologous protein expression system in *Escherichia coli*. *Scientific Reports* 6: 33037.

Bui, T., Patel, P. & Preuss, C.V. 2024. Cephalosporins. In *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK551517/ (Accessed on 21st May 2025).

Conti, G., Pollegioni, L., Molla, G. & Rosini, E. 2014. Strategic manipulation of an industrial biocatalyst - Evolution of a cephalosporin C acylase. *FEBS Journal* 281: 2443-2455.

Correddu, D., Montaño López, J.D.J., Angermayr, S.A., Middleditch, M.J., Payne, L.S. & Leung, I.K. 2020. Effect of consecutive rare codons on the recombinant production of human proteins in *Escherichia coli*. *IUBMB Life* 72(2): 266-274.

de Marco, A. 2009. Strategies for successful recombinant expression of disulfide bond-dependent proteins in *Escherichia coli*. *Microbial Cell Factories* 8: 1-18.

Faizullabhoy, M. & Wani, G. 2024. Cephalosporin drugs market - by generation (first, second), indication (respiratory, UTI, STI, gastrointestinal infection), route of administration (oral, parenteral, topical), type (generic, branded), distribution channel, global forecast 2024–2032. *Global Market Insights* Report ID: GMI8194. https://www.gminsights.com/industry-analysis/cephalosporin-drugs-market (Accessed on 21st May 2025).

Falak, S., Sajed, M. & Rashid, N. 2022. Strategies to enhance soluble production of heterologous proteins in *Escherichia coli*. *Biologia* 77: 893-905.

Gaurav, K., Kundu, K. & Kundu, S. 2010. Biosynthesis of cephalosporin-C acylase enzyme: Optimal media design, purification, and characterization. *Artificial Cells, Blood Substitutes, and Biotechnology* 38(5): 277-283.

Isakova, A., Artykov, A., Vorontsova, Y., Dolgikh, D., Kirpichnikov, M., Gasparian, M. & Yagolovich, A. 2023. Application of an autoinduction strategy to optimize the heterologous production of an antitumor bispecific fusion protein based on the TRAIL receptor-selective mutant variant in *Escherichia coli*. *Molecular Biotechnology* 65(4): 581-589.

Jobanputra, A.H. & Vasait, R.D. 2015. Cephalosporin C acylase from *Pseudomonas* species: Production and enhancement of its activity by optimization of process parameters. *Biocatalysis and Agricultural Biotechnology* 4(4): 465-470.

Julkipli, J., Syamsu, K. & Wibisana, A. 2022. Optimization of cephalosporin C acylase immobilization using crosslinked enzyme aggregates technique. *Bionatura* 7(1): 9.

Kataoka, K. & Takasu, A. 2020. IPTG-independent autoinduction of extracellular matrix proteins using recombinant *E. coli* as the expression host. *Polymer Journal* 53(2): 385-391.

Khani, M. & Bagheri, M. 2020. Protein expression and purification skimmed milk as an alternative for IPTG in induction of recombinant protein expression. *Protein Expression and Purification* 170: 105593.

Kim, S. & Kim, Y. 2001. Active site residues of cephalosporin acylase are critical not only for enzymatic catalysis but also for post-translational modification. *Journal of Biological Chemistry* 276(51): 48376-48381.

Kim, Y., Kim, S., Earnest, T.N. & Hol, W.G. 2002. Precursor structure of cephalosporin acylase: Insights into autoproteolytic activation in a new N-terminal hydrolase family. *Journal of Biological Chemistry* 277(4): 2823-2829.

Li, Z., Kessler, W., Van Den Heuvel, J. & Rinas, U. 2011. Simple defined autoinduction medium for high-level recombinant protein production using T7-based *Escherichia coli* expression systems. *Applied Microbiology and Biotechnology* 91(4): 1203-1213.

Li, X., Wang, J., Su, W., Li, C., Qu, G., Yuan, B. & Sun, Z. 2023. Characterization and engineering of cephalosporin C acylases to produce 7-Aminocephalosporanic acid. *Molecular Catalysis* 550: 113595.

Martius, E., Wibisana, A. & Ardiyani, Y. 2018. The optimization of soluble cephalosporin C acylase expression in *E. coli*. *IJES* 7: 29-34.

Mühlmann, M., Forsten, E., Noack, S. & Büchs, J. 2017. Optimizing recombinant protein expression via automated induction profiling in microtiter plates at different temperatures. *Microbial Cell Factories* 16(1): 220.

Nikolova, G., Georgieva, Y., Atanasova, A., Radulova, G. & Kapogianni, A. 2021. Autoinduction as means for optimization of the heterologous expression of recombinant single - chain Fv (scFv) antibodies. *Molecular Biotechnology* 63(11): 1049-1056.

Niwa, M., Saito, Y., Sasaki, H. & Ishii, Y. 1994. Cephalosporin C acylase. *U.S. Patent No. 5,336,613*. Washington, DC: U.S. Patent and Trademark Office.

Pollegioni, L., Rosini, E. & Molla, G. 2013. Cephalosporin C acylase: Dream and(/or) reality. *Applied Microbiology and Biotechnology* 97: 2341-2355.

Rasyidah, M.A., Sismindari, S. & Purwanto, P. 2024. Cephalosporin C acylase: Important role, obstacles, and strategies to optimize expression in *E. coli*. *Journal of Applied Pharmaceutical Science* 14(10): 15-24.

Shahzadi, I. 2021. Scale-up fermentation of *Escherichia coli* for the production of recombinant endoglucanase from *Clostridium thermocellum*. *Scientific Reports* 11(1): 7145.

Shin, Y.C., Jeon, J.Y., Jung, K.H., Park, M.R. & Kim, Y. 2009. Cephalosporin C acylase mutant and method for preparing 7-ACA using same. *U.S. Patent No. 7,592,168*. Washington, DC: U.S. Patent and Trademark Office.

Sun, H., Liu, T., Luo, H., Nie, Z., Chang, Y., Yu, H. & Zhongyao, S. 2021. Optimization of cephalosporin C acylase expression in *Escherichia coli* by high-throughput screening a constitutive promoter mutant library. *Applied Biochemistry and Biotechnology* 193: 1056-1071.

Wang, Y., Yu, H., Song, W., An, M., Zhang, J., Luo, H. & Shen, Z. 2012. Overexpression of synthesized cephalosporin C acylase containing mutations in the substrate transport tunnel. *Journal of Bioscience and Bioengineering* 113(1): 36-41.

Zhang, W., Liu, Y., Zheng, H., Yang, S. & Jiang, W. 2005. Improving the activity and stability of GL-7-ACA acylase CA130 by site-directed mutagenesis. *Appl. Environ. Microbiol.* 71(9): 5290-5296.

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