0273-2289/00/88/0221/$12.25

Methodological Approach to Development of Enzymatic Technologies for Semisynthetic Betalactam Antibiotic Production

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Abstract

The algorithm for the research and design of optimized processes for synthesis of semisynthetic betalactam antibiotics with the use of peptidases with mechanism of action based on the acylenzyme intermediate formation as a catalyst was formulated.

The applicability of the proposed approach to the development of the processes for enzymatic synthesis of cefoxitin and cefazolin, semisynthetic cephalosporins, as an example, was demonstrated.

Index Entries: Enzymatic synthesis; direct synthesis; acyl transfer synthesis; betalactams; cephalosporins; cefoxitin; cefazolin.

Introduction

The practical implementation of the processes of enzyme engineering requires the design of appropriate technologies and their optimization. Analysis of the literature indicates that selection of a technological scheme is based mainly on an empirical approach. Our experience in development of large-scale enzymatic technologies for manufacturing of the key amino acids (KAA) such as 6-aminopenicillanic, 7-aminodesacetoxycephalosporanic, and 7-aminocephalosporanic *(1)* makes it possible to formulate the algorithm of the scientific research for design and optimization of processes for production of semisynthetic betalactam antibiotics with the use of enzymatic synthesis, catalyzed by an immobilized peptidase with mechanism of action based on the acylenzyme intermediate formation *(2)*. The algorithm includes:

1. Investigation of reversibility of enzymatic synthesis and characterization of main electrochemical properties of acylating agents

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(substrate, S) and nucleophils (N). Statement of the possibility of direct synthesis of antibiotics by comparative analysis of pH profiles of reaction equilibrium constants, enzyme activity, and the rate constant of enzyme inactivation.
2. Elucidation of quantitative relationship between the maximum conversion of the nucleophil, which is an antibiotic equilibrium yield ($\eta_{syn,eq}$) and the operation parameters required for its achievement as a function of a number of thermodynamic parameters.
3. Design of technology for the direct synthesis.

Acyl transfer is an alternative for direct synthesis. We suggest using methyl esters of the corresponding carboxylic acids as the acyl moiety. In the case of acyl transfer, it is necessary:

1. To analyze the kinetic scheme of the processes and derive relationships between maximum values of the kinetically controlled yield ($\eta_{syn,t}$) and the operation parameters required to reach it as a function of kinetic parameters of the processes.
2. To state the required complex of kinetic studies, perform optimization calculation, and design biocatalytic technology on the basis of acyl transfer synthesis.
3. To develop a mathematical model of the process adequate to the kinetic model of enzymatic synthesis within the mechanism of action based on acylenzyme intermediate formation.
4. To determine the whole complex of the kinetic and thermodynamic constants and optimize the processes based either on direct synthesis or acyl transfer.

Along with the step of enzymatic synthesis, biocatalytic technologies include the steps of separation of the reaction mixture components and isolation of the main product (P). Design and optimization of these technologies require consideration of a relationship between the steps and the order of the step variants by their preference. The general criteria for the process efficiency used in technology optimization of biocatalytic processes markedly differ when they are based on enzymatic transformation or enzymatic synthesis.

To produce the KAA, it is possible to use an "ideal" technology including the steps of biotransformation (bt) and isolation of KAA by precipitation. The criterion for the efficiency of this technology is the total yield of the main product *(3)*. The value of the total yield is higher under the conditions of the higher η_{bt}. It should be indicated that η_{bt} also affects the precipitation step since the complex formation with the unreacted substrate increases the minimum solubility of the main product *(4)*.

The maximum yield at the step in the synthesis process for semisynthetic betalactam antibiotics does not always reflect the maximum efficiency of the technology, in contrast to the processes for enzymatic production of KAA. In these processes, the consumption coefficients of

nucleophil and substrate as an integral criterion of the technology efficiency can be used. An optimal technology is characterized by minimum values of the consumption coefficients of the main raw material. When the initial raw material is not consumed completely, its regeneration and reuse in the reaction are required. Such a scheme makes it possible to increase the process productivity, to lower the excess of the acylating agents, and to increase the total efficiency of the technology.

This article demonstrates the applicability of the proposed approach to the design of technology for enzymatic synthesis of cefoxitin and cefazolin, semisynthetic cephalosporins.

Materials and Methods

Cefoxitin was a product of Re Yon Pharmaceutical Co. (Korea). The content of the main substance is 92.6% (HPLC). Cefazolin was a product of Cheil Foods & Chemicals Inc. (Korea). The content of the main substance is 98.5 % (HPLC).

Biocatalyst (BC) preparation. Immobilized cephalosporin acid synthetase from *Escherichia coli*, strain 1787 was used as a BC throughout the study. The strain is from the Culture Collection of the National Research Centre for Antibiotics (Russia). The cultivation and enzyme isolation were carried out as described in ref. *5*. The enzyme was precipitated from a cell-free extract by ammonium sulfate, then modified with glutaraldehyde, and finally entrapped in polyacrylamide gel according to ref. *6*.

The activity of the immobilized enzyme was determined by estimation of the velocities of the substrate and product hydrolysis and the product synthesis. The reaction mixture was assayed for the content of the substrates and reaction products by HPLC (Waters Associates Inc.). Sylasorb C18 with the particle size of 4.5 μm in diameter was applied as a stationary phase in a stainless-steel column (250 × 4.0 mm). The mobile phases were mixtures of methanol and 0.05 *M* phosphate-ammonia buffer, pH 2.5, in different ratios.

Results and Discussion

The investigation of the possibility of direct enzymatic synthesis of cefoxitin and cefazolin based on the formation of acylamine bond from the KAA (7-amino-7-methoxy- 3-carbamoyloxymethyl-cephalosporanic acid for cefoxitin and 7-amino-3-[(2-methyl-1,3,4-thiadiazol-5-yl)-thiomethyl]-cephalosporanic acid, TDA for cefazolin) and carboxylic acid (thienylacetic acid, TAA and 1(H)-tetrazolylacetic acid, TzAA, respectively) was performed. The relationship between $\eta_{syn,eq}$ and the operation conditions of the production process (the initial concentrations of nucleophil ($[N]_o$) and substrate ($[S]_o$), pH, and temperature) can be described by the following equation *(7)*:

$$\eta_{syn,\,eq} = (X+1)/2 - (\sqrt{\{[N]_o \cdot (X+1) + K_{h,\,eq}\}^2 - 4X[N]_o^2} - K_{h,\,eq})/2[N]_o \quad (1)$$

where $K_{h,eq}$ is the equilibrium constant of cefoxitin or cefazolin hydrolysis calculated from the values of the equilibrium concentration of the reaction mixture components, *M*; *X* is the ratio of $[S]_o$ and $[N]_o$.

The direct synthesis is applicable when the pH range overlaps both the region of high activity and stability of the BC and the region of sufficiently high values of the equilibrium constant of synthesis ($K_{syn,eq} = 1/K_{h,eq}$). The comparative analysis of the pH profile of the equilibrium constant of the cefoxitin and cefazolin syntheses and the BC activity and stability under conditions favorable for enzyme functioning (pH 6.0–8.0) showed that synthesis of cefoxitin was thermodynamically advantageous (Fig. 1).

The dependencies of $\eta_{syn,eq}$ on the concentration of the nucleophil and excess of the acylating agent were estimated according to Eq. 1 using the values of $K_{h,eq}$ (Fig. 2). The agreement between the estimates and experimental data was satisfactory. The maximum usage of the nucleophil was achieved with a 2.5–4-fold excess of the acylating agent and pH 6.0. The investigation results showed the possibility of the direct synthesis of cefoxitin and were used for design of technology for cefoxitin production.

It is evident (Fig. 1) that in the case of cefazolin the direct enzymatic synthesis was not realized, because within the pH range from 6.0 to 8.0 hydrolysis but not synthesis of cefazolin is thermodynamically advantageous *(7)*. The cefazolin synthesis with the transfer of the acyl moiety of the activated derivative of TzAA (methyl ester of TzAA, METzAA) to the primary amino group of TDA was studied according to the kinetic scheme presented in *(7,8)*.

The relationship between the maximum conversion of the nucleophil in kinetically controlled synthesis of cefazolin ($\eta^{max}_{syn,\tau}$) and the operation conditions providing it (the initial concentrations of TDA ($[N]_o$) and METzAA ($[S]_o$)) is described by the following equation *(8)*:

$$\eta^{max}_{syn,\tau} = 2[S]_o/(\sqrt{Q^2 - 4[S]_o \cdot [N]_o} + Q) \quad (2)$$

where

$$Q = [S]_o + [N]_o + {}^{(\alpha+1)}/_{\beta} + [N]_o \cdot \gamma \cdot (\alpha + 1) \quad (3)$$

α, β, and γ are the kinetic parameters of the cefazolin synthesis processes proceeding in accordance with the kinetic scheme. The relationships between these parameters and the kinetic constants of the elementary steps of the processes are described in ref. *7*.

The dependence of $\eta^{max}_{syn,\tau}$ on the $[N]_o$ and $X = [S]_o/[N]_o$ was estimated according to Eqs. 2 and 3 with the values of the kinetic parameters determined (Fig. 3). The agreement between the estimates and experimental data was satisfactory. The investigation results were used for design of technology for cefazolin production.

For the technology optimization, the mathematical model of cefazolin enzymatic synthesis was developed. The model consists of the equations describing differences in the concentrations of final product (*P*), nucleophil (*N*), substrate (*S*), and by-product – TzAA (P_2):

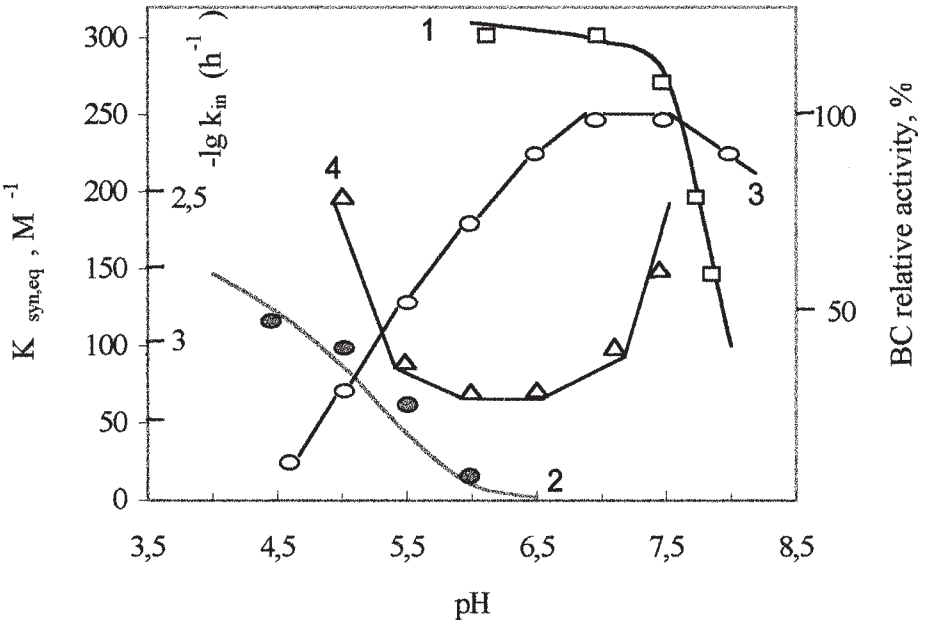

Fig. 1. pH-Profile for the equilibrium constants ($K_{syn,eq}$) of cefoxitin (1) and cefazolin (2) synthesis, the relative BC activity measured by cefazolin hydrolysis (3) and the rate constant of enzyme inactivation (k_{in}, h^{-1}) (4).

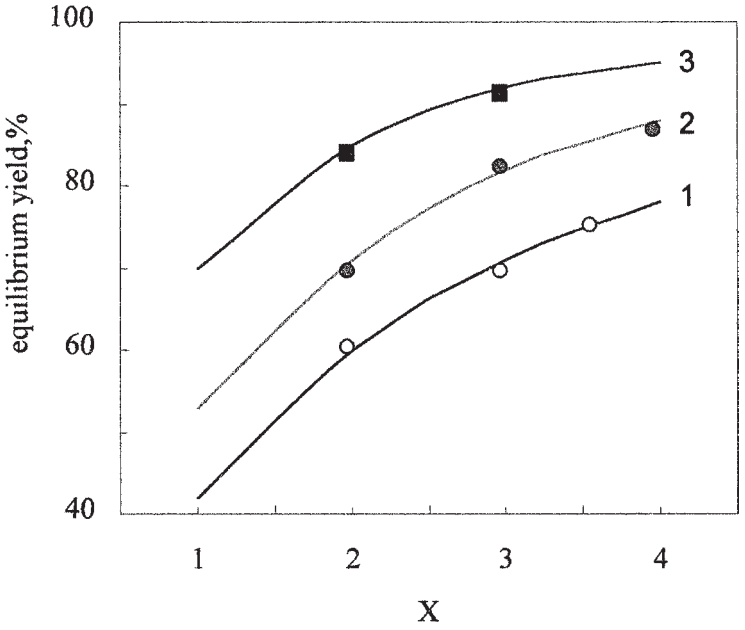

Fig. 2. The dependence of $\eta_{syn,eq}$ of cefoxitin on the molar excess of TAA for three initial concentrations of KAA. Lines are the results of estimation according to Eq. 1, symbols are the experimental data. 1, $[N]_o = 0.004\ M$; 2, $[N]_o = 0.008\ M$; 3, $[N]_o = 0.02\ M$, pH 6.0–6.5, 40°C, [BC] = 2.0 U/g (1 U is equal to 1 μmol of cefoxitin per min).

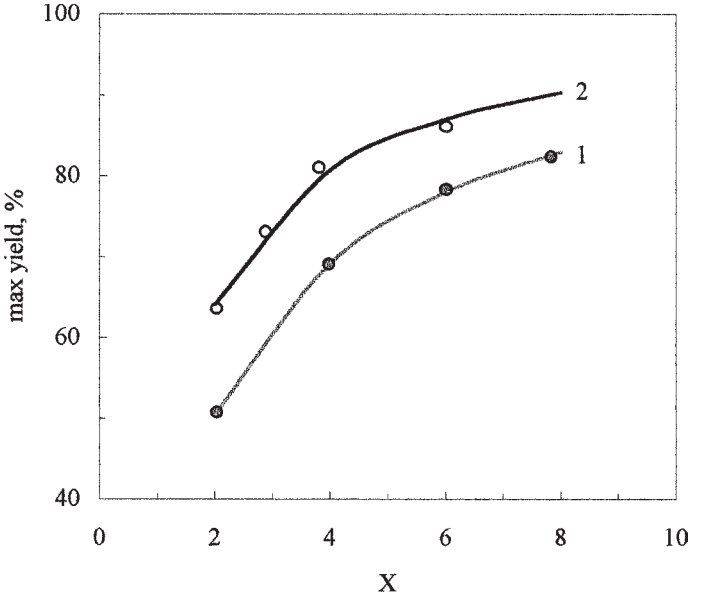

Fig. 3. The dependence of $\eta^{max}_{sym,\tau}$ of cefazolin on the molar excess of METzAA for two initial concentrations of TDA. Lines are the results of estimation according to Eq. 2, symbols are the experimental data. 1, $[N]_o$ = 0.014 *M*; 2, $[N]_o$ = 0.027 *M*, pH 6.2, 40°C, [BC] = 1.5 U/g (1 U is equal to 1 μmol of cefazolin per min).

$$dS/d\tau = -(V_1 + V_3) - k_{sp}S \quad (4A)$$

$$dN/d\tau = V_2 - V_3 - k_{10} \cdot N \quad (4B)$$

$$dP/d\tau = V_3 - V_2 - k_9 \cdot P \quad (4C)$$

$$dP_2/d\tau = V_1 + V_2 \quad (4D)$$

where V_1 is the velocity of the enzymatic hydrolysis of METzAA; V_2 is the velocity of the enzymatic hydrolysis of cefazolin; V_3 is the velocity of the enzymatic synthesis of cefazolin; k_{sp} is the constant of spontaneous hydrolysis of substrate; k_9 and k_{10} are the constants of cefazolin and nucleophil inactivation rates, respectively.

The relationships between V_1–V_3 and the kinetic and thermodynamic parameters of enzymatic synthesis of cefazolin are described in ref. *9*.

It should be noted that the kinetic and thermodynamic scheme of the acyl transfer synthesis of cefazolin and the adequate mathematical model developed by us are the most complete out of those described in the literature. They consider not only the mechanism of the formation and reacylation of the acylenzyme intermediate, but also the inhibition of the acylenzyme complex formation by the nucleophil and by-product, the reversibility of the cefazolin synthesis, the final product and nucleophil inactivation, and the substrate spontaneous hydrolysis.

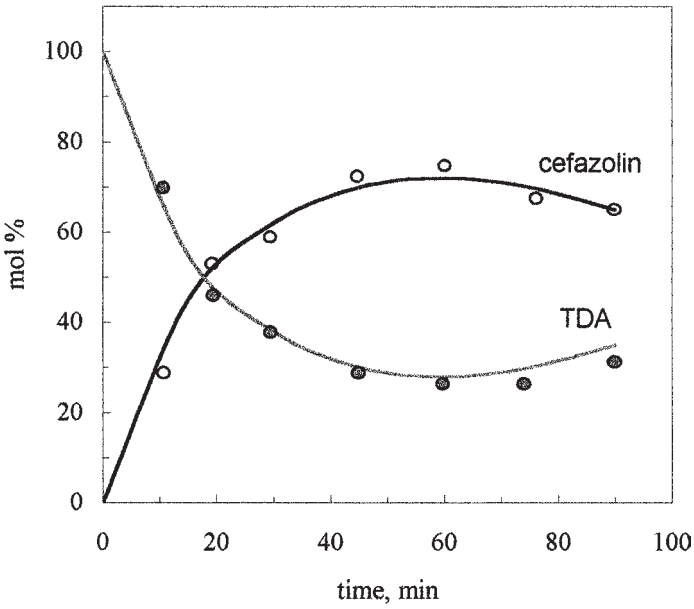

Fig. 4. The changes in the reaction mixture composition in cefazolin synthesis. Lines are the results of estimation according to the mathematical model, Eqs. 4A–D, symbols are the experimental data. $[N]_o$ = 0.058 *M*, *X* = 2, pH 6.2, 40°C, [BC] = 1.5 U/g.

The parameters of the mathematical model were evaluated by the experimental data on the study of the initial velocities of the main processes (cefazolin synthesis or substrate and product hydrolysis). These so-named "model values" were verified by comparison of the experimental kinetic curves of the cefazolin synthesis at low TDA concentrations, and the curves were estimated with the use of the mathematical model and its model values. The model values and verified "operation values" are presented in ref. *9*. The use of the operation values made it possible to show rather precisely the changes in the reaction mixture composition during cefazolin synthesis (Fig. 4).

The operation parameters were optimized within one cycle of the cefazolin synthesis using the mathematical model and its operation values. The pH value as a managing parameter was chosen and the computer program for its estimation was developed.

The mathematical model of the cefazolin synthesis step was supplemented with the equations describing the reaction mixture separation. The fractional precipitation of final product and unreacted TDA was used for this purpose. The pH and temperature values were chosen as operating parameters of the separation step. The values of minimum solubility (S^o) of cefazolin, TDA, and TzAA were estimated ($S^o_P = 6.6 \cdot 10^{-4}$ *M*, $S^o_N = 2.9 \cdot 10^{-4}$ *M*, $S^o_{P_2} = 0.616$ *M*, 20°C). The pH and temperature dependencies of solubility were calculated ($pK_{a,P} = 2.38$, $pK_{a1,N} = 2.46$, $pK_{a2,N} = 5.04$, $pK_{a,P_2} = 2.2$, 20°C). The consumption coefficients of TDA (C_N) and TzAA (C_S) were

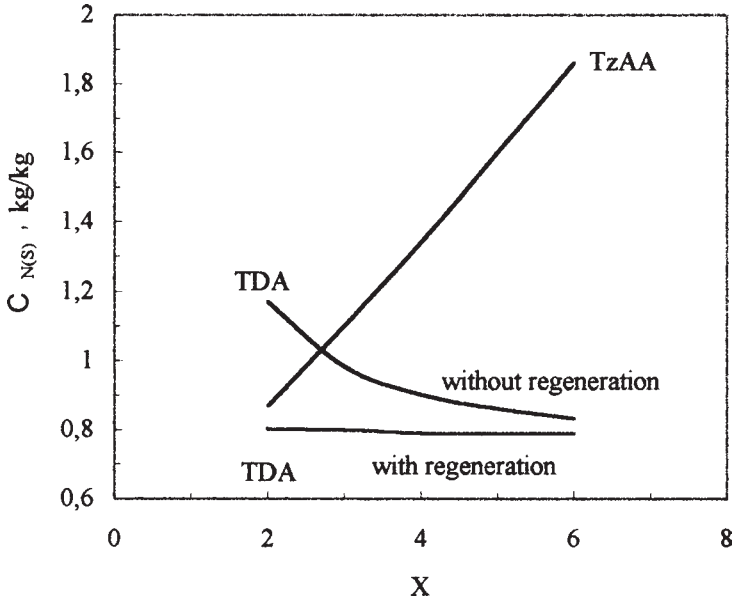

Fig. 5. The dependence of C_N and C_S on the molar excess of METzAA. Lines are the results of estimation according to Eqs. 5 and 6. $[N]_o = 0.07\ M$, $X = 2$, pH 6.2, 40°C [BC] = 1.5 U/g.

expressed in kilogram of the initial reagent per kilogram of the main product and calculated by the following equations:

$$C_N = (C^t_N[1 - (1 - \eta_{syn,\tau})\eta_{N,reg}])/\eta_{syn,\tau} \cdot \eta_{P,isol} \quad (5)$$

$$C_S = (C^t_S[X - (X - \eta_{syn,\tau})\eta_{S,reg}])/\eta_{syn,\tau} \cdot \eta_{P,isol} \quad (6)$$

where

$$C^t_N = MM_N/MM_P$$

and

$$C^t_S = MM_S/MM_P$$

and are theoretical consumption coefficients of N and S, respectively; $\eta_{N,reg}$ and $\eta_{S,reg}$ are yields on the steps of nucleophil and substrate regeneration, respectively; $\eta_{P,isol}$ is the yield on the step of final product isolation.

The minimum value of the consumption coefficients, equal to 0.8 and 0.9 for TDA and TzAA, respectively, can be attained in the framework of the "ideal" technology with 70–75% transformation of the nucleophil ($[N]_o = 7.0 \times 10^{-2}\ M$) due to a twofold excess of the acylating agent, the subsequent fractional precipitation of TDA and cefazolin, and TDA reuse (Fig. 5). This value of C_N proved to be equal to the value corresponding to at least a 90% total yield of the main product.

Conclusion

A methodological approach to development of enzymatic technologies for semisynthetic betalactam antibiotic production, contrary to the traditionally used empirical approach, was proposed. The technologies of the direct cefoxitin and cefazolin syntheses with transfer of acyl moiety were developed in accordance with the formulated algorithm. The mathematical model of cefazolin synthesis supplemented with the equations describing the reaction mixture component separation by fractional precipitation was improved. The optimizing calculations of the whole technology operation parameters with the consumption coefficients of TDA and TzAA as integral criterion of the technology efficiency were fulfilled.

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