ENZYMATIC TRANSFORMATION OF BETALACTAM ANTIBIOTICS. TRENDS OF DEVELOPMENT AND APPROACHES TO PRACTICAL IMPLEMENTATION

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The general approaches to enzymatic synthesis of semisynthetic betalactam antibiotics were developed. The principal scheme includes the enzymatic transformation of natural antibiotics to key amino acids and following their acylation to produce the semisynthetic betalactams. Ten various biocatalysts are enough for production of all semisynthetic betalactams according to the scheme. The physico-chemical approaches to development of biocatalytic processes of betalactam antibiotic transformation and synthesis as well to design of biocatalysts of these processes are discussed.

One of the most actual trends of the pharmaceutical industry is the development of efficient approaches to the transformation of natural betalactam antibiotics to new semisynthetic analogous with use of the enzymatic methods instead of the chemical ones. The unique specificity and high reactivity of enzymes are very important for such labile compounds as betalactams since they ensure the proceeding of reactions in mild conditions and make it possible to exclude toxic reagents, large volumes of organic solvents and low temperatures.

One of the first world industrial processes of enzyme engineering was the production of 6-aminopenicillanic acid (6-APA) from penicillin (Pen G or Pen V), practically implemented by all companies in the end of 70-s. Nowadays the world industry uses the immobilized enzymes also for production of the key amino acids, such as 7-aminodesacetoxycephalosporanic (7-ADCA) and 7-aminocephalosporanic acid (7-ACA). The enzymatic synthesis of the betalactam antibiotics on the base of key amino acids is the perspective field of biotechnology. The mechanisms of action of enzymes, used in the synthesis and transformation of betalactam antibiotics, as well as the kinetics and thermodynamics of processes, catalysed by peptidohydrolases from different microorganisms, are the subject for studying of many researches [1–11]. However nowdays there are only single examples of industrial enzymatic synthesis of betalactam antibiotics, such as semisynthetic penicillin—amoxicillin [12], and cephalosporin—cephalexin [13].

The general approaches to enzymatic synthesis of semisynthetic betalactam antibiotics were developed by us on the base of the literature data as well the own long-term experience in the field of enzymatic synthesis and transformation of betalactams (Fig. 1). The principal scheme includes the enzymatic transformation of natural antibiotics, such as Pen G, cephalosporin C (Ceph C) and cephamycin, to key amino acids—6-APA, 7-ADCA, 7-ACA, desacetyl-7-ACA, and 7-α-methoxy-ACA, with use of specific biocatalysts (BC). The following acylation of key amino acids with use of BC_2 which is synthetase from *Xanthomonas sp.*, specific to aminophenylacetic acid derivatives, results in obtaining of aminopenicillins and aminocephalosporins (for example, ampicillin, cephalexin, and cefaclor), and with use of BC_3 on the base of synthetase from *E. coli* results in obtaining of parenteral cephalosporins-acids (for example, cefazolin and cefoxitin). The use of acylases from various microorganisms (*Achromobacter sp.*, *Acetobacter pasteuranium*, *Acetobacter turbidans, Bacillus megaterium*, *Klyvera citrophila*, *Pseudomonas melanogenum*, and *Xanthomonas citri and others*) as biocatalysts of semisynthetic betalactam antibiotic synthesis is described in literature [4, 9, 10].

The initial compound for the synthesis of the huge number of parenteral cephalosporins is Ceph C which is transformed to 7-ACA by two-stage enzymatic process with the use of D-amino acid oxidase, for example, from *Trigonopsis sp.* (BC_4) and glutaryl hydrolase, for example, from *Acetobacter sp.* or *Pseudomonas sp.* (BC_5) [11, 14]. Then the desacetyl-7-ACA is produced by 7-ACA chemical or enzymatic hydrolysis with acetyl esterase, for example, from *Trigonopsis sp.* as a biocatalyst (BC_7). The production of the desacetyl-7-ACA from Pen G as initial compound can be considered as an alternative to the above paraphrased approach. This biocatalytic technology includes the transformation of Ceph G to desacetyl-Ceph G with use of hydroxylase from *Streptomyces sp.* (BC_6).

Desacetyl-7-ACA is used mainly as intermediate compound for production of large number of derivatives by introducing substituents into C-3 position of cephem. The use of enzymatic methods for such derivative production instead of the chemical ones gives the indisputable advantages. The production of 7-amino-3-vinylcephalosporanic acid (7-AVCA—key amino acid for cefixim) by consecutive transformation of Ceph G with use of oxidases from *Rhizopus sp.* (BC_8) and *Pseudomonas sp.* (BC_9) and Pen G amidase from *E. Coli* (BC_{1-1}) is shown on the scheme as an example of the proposed approach.

The production of Ceph G from Pen G by enzymatic synthesis with use of expandase of betalactams from *Streptomyces sp.* (BC_{10}) [15] instead of traditional multistage chemical transformation is another perspective direction of pharmaceutical industry.

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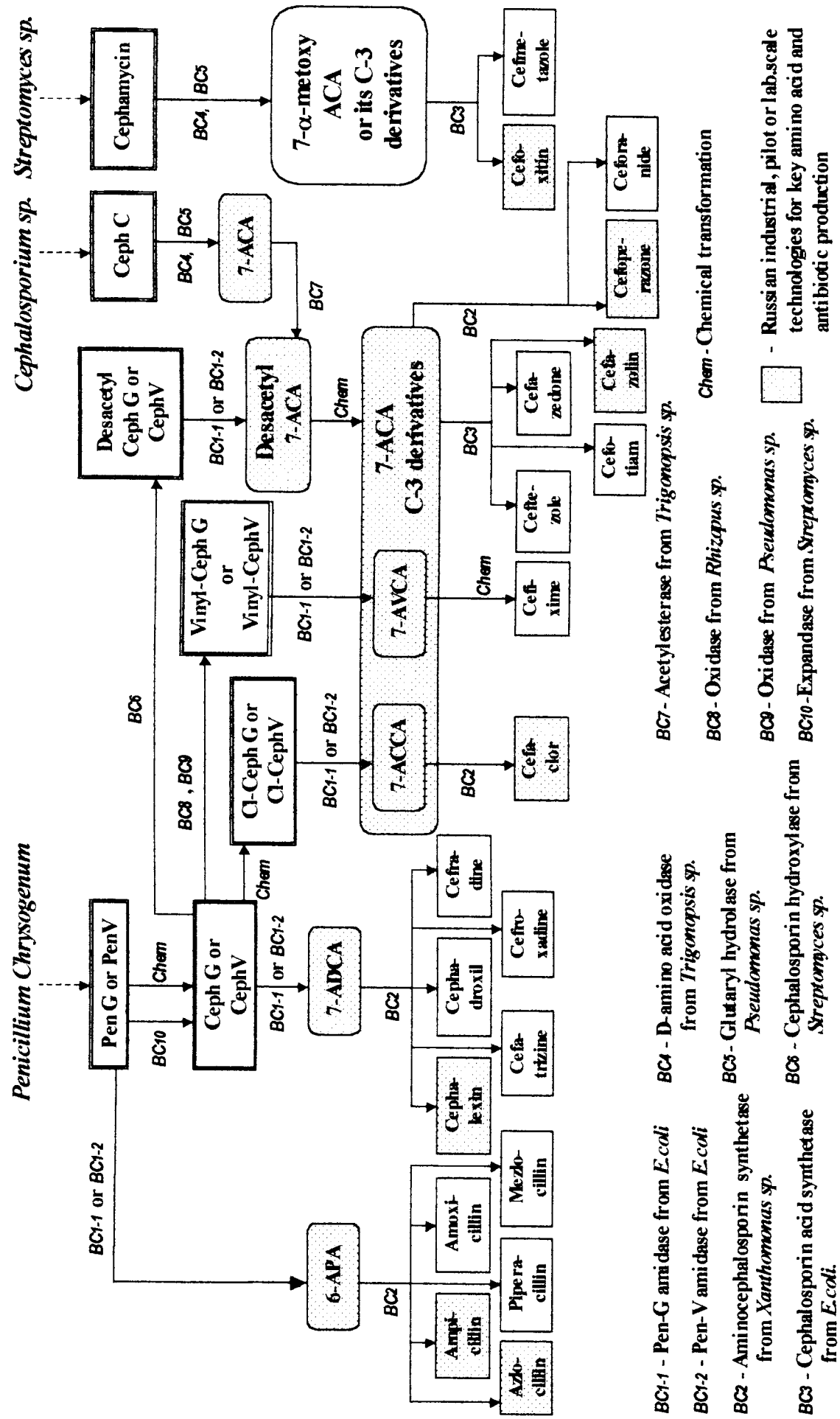

Fig. 1. Enzymatic Approach to Semisynthetic Betalactam Antibiotic Synthesis.

The complex solution of betalactam antibiotic enzymatic synthesis problem demands the deep physico-chemical approach to development of the technologies. The methodology of the studies, which are essential for the development of highly efficient processes of betalactam antibiotic synthesis and transformation, is developed [16, 17]. The methodology is based on: (1) the elucidation of the technology design and optimization with account of the interrelation of each stage efficiency parameters and the order of the stage variants by their preference for the large scale implementation; (2) the determination of the integral criteria of the entire technology efficiency on the base of particular and general criteria of the process stages; (3) the estimation of the deepness and the structure of physico-chemical investigations, which are essential for choose the optimal synthesis scheme, for designing technology and its optimization (Table 1).

The approaches to production of the biocatalysts on the base of immobilized enzymes, cell or enzyme-membrane complex, developed by us, include the next features [16–19]:

1. The estimation of the strength of required enzyme binding with the cell structures, responsible for enzyme localization, by the constant of binding (K_{bin}). The use of K_{bin} as a parameter of the optimization of the enzyme biosynthesis makes it possible to regulate purposefully this process to obtain the cell biomass, suitable for isolation of the free enzyme and its following immobilization or directly for the cell immobilization.

2. The isolation of enzyme from bacterial cells based on mild targeting effect of water immiscible organic solvents on the cell walls and the places of required enzyme localization, which provides the extraction of the preferably required enzyme. This method unlike the ordinary procedure of the total cell disintegration makes it possible to produce the enzyme preparations with high specific activity already on the stage of enzyme isolation [19].

3. The use of various methods of targeting modification of microbial cells, intended for immobilization, which makes it possible to increase mildly the cell wall permeability with preservation of the strength of binding of the final enzyme with the structures responsible for its localization [19]. The most preferential method of modification is that one, which provides the binding of additional quantities of required enzyme by cell structures responsible for its localization and the production of strong enzyme-membrane complex on the base of initial cells. For example, the dissociation constant of enzyme-membrane complex on the base of cells of *E.coli* and Pen G amidase from *E. coli*, which is deter-

Table 1

Complex of physico-chemical investigations, which are essential for choose of optimal synthesis scheme, for designing technology and its optimization

Technological stage	Physico-chemical characteristics	Efficiency parameter	The algorithm of the scientific research
Biotransformation (bt) and synthesis (syn)	Equilibrium (eq) parameters of enzymatic reactions ($K_{\text{eq}}, \Delta H$) Kinetic (τ) parameters of enzymatic reactions (V_m, K_M, E_a) Electrochemical properties of components (K_a) Stability of components (k_{in})	$\eta_{\text{eq}}^{\text{bt(syn)}} = f(K_{\text{eq}}, \Delta H, K_a, c_0, \text{pH}, t)$ $\eta_{\tau}^{\text{syn}} = f(V_m, K_M, E_a, c_0, \text{pH}, t)$	Investigation of reversibility of enzymatic reactions and characterization of main electrochemical properties of components. Statement of the possibility of direct synthesis of antibiotics. Estimation of efficiency parameters and design of technology for the direct synthesis. Analysis of the kinetic scheme of the processes in the frames of chosen model. Elucidation of the relationships between maximum values of the kinetically controlled yield and the operation conditions and kinetic parameters of the processes. Estimation of efficiency parameters and design of technology for synthesis. Development of a mathematical model of the process adequate to the kinetic model of enzymatic synthesis. Proceeding the optimizing calculations.
Precipitation (pr) of reaction mixture components	Electrochemical properties of components (K_a) Component solubility (s_i)	$\eta_{\text{pr}} = f\left(K_a, s_i^0, c_i, pH, t, \varepsilon\right)$ $\gamma_{\text{adm}} = f(K_a, s_i^0, c_i, c_{\text{adm}}, \text{pH}, t, \varepsilon)$	Study of component solubility in wide interval of pH, temperature values, dependence on dielectrical permeability (ε), etc. Estimation of efficiency parameters and design of technology of product isolation and component separation by direct precipitation. Optimization of precipitation technology.
Preliminary preparation (pp) of reaction mixture	Interphasic distribution (K_{d})	$\eta_{\text{pp}} = f\left(K_d^0, c_i, \text{pH}, t, \mu\right)$	Study of dependence of component interphasic distribution on pH, temperature, phase polarity (μ), etc. Estimation of efficiency parameters and design of technology which includes purification and concentration of reaction mixture by extraction, ionic exchange and chromatography.
Total (tot) technology		$\eta_{\text{tot}} = f\left(\eta_{\text{bt}}, \eta_{\text{pr}}, \eta_{\text{pp}}\right)$ $CC_{ii} = f(\eta_{\text{syn}}, \eta_{\text{pr}}, \eta_{\text{pr}}^i, \eta_{\text{pp}})$	Choose of efficiency criteria of entire technology (total yield of product (η) in case of biotransformation or consumption coefficients of the main raw material (CC) in case of synthesis). Estimation of efficiency criteria for various stage variants. Choose of optimal technological scheme.

Notes: c is concentration; γ is admixture (adm) content; K_{eq} is equilibrium constant; ΔH is enthalpy; K_a is constant of electrolyte dissociation; K_{d} is interphasic distribution constant; k_{in} is component inactivation constant; E_a is energy of activation; V_m is maximum velocity of enzymatic reaction; K_M is Michaelis constant.

Table 2

The Biocatalysts with Pen G Amidase Activity on the Base of Polyacrylamide Gel

No.	Biocatalyst	Activity, μmol/min · g wet	Half inactivation time, h
1	Immobilized enzyme	170	1000
2	Immobilized *E.coli* cells	70	2300
3	Immobilized enzyme-membrane complex	330	5000

mined in model experiments, is equal 1.2×10^{-4} M [19]. The 3–4-fold increase of enzymatic activity of such complex in comparison with the initial cell biomass is achieved owing to enriching the cells by the required enzyme by both the binding of its additional quantities by cell membranes and removing the ballast substances from the cells, as well resulted from increasing of accessibility of enzyme for substrates due to higher cell wall permeability. The enzyme stability in enzyme-membrane complex is very high and exceeds the both native enzyme and enzyme in the natural cell microenvironment (Table 2).

The production of mixed enzyme–membrane complexes, which are the 2nd generation biocatalysts, is possible on the base of immobilized cells and enzyme supplementing the cell activity in the two stage transformation of biosynthetic betalactams to their semisynthetic analogous. The use of BC on the base of, for example, immobilized cells of *E. coli* and synthetase from *Xanthomonas sp.* makes it possible to produce the aminopenicillins from Pen G or aminocephalosporins from Ceph G directly without key amino acids isolation.

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