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Modern Trends in Biocatalytic Synthesis of Chiral Compounds

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Abstract—Characteristic features and modern trends in the biocatalytic synthesis of chiral compounds have been discussed. New processes of biocatalytic synthesis use at least two enzymes. Whole cells of recombinant strains are utilized as biocatalysts, and the complete set of target enzymes can be expressed in a single cell.

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Synthesis of chiral compounds is one of the most rapidly progressing fields of modern chemistry. The current importance of this field is due to the fact that many drugs contain chiral centers. As a rule, a positive therapeutic effect is due to only one optical isomer. The other isomer can cause serious side effects (as was the case with thalidomide) or rapid habituation (as antiasthmatic drugs based on β2-receptor agonists). In this context, since 1999, the US Food and Drug Administration requires that the optical purity of drugs containing a chiral center (centers) should be at least 99%.

The Nobel Prize in chemistry in 2001 was awarded for the development of chemical syntheses and chiral compounds. However, classical organic syntheses of these compounds have a number of significant drawbacks, which hinder their wide use. The most serious are as follows:

(1) the absence of a high enantioselectivity (the reaction usually produces a racemic mixture; in the majority of the other cases, the prevalence of one isomer is insignificant);

(2) the absence of high regioselectivity (in the presence of two groups with identical structures and reactivities (e.g., two keto groups), in most cases, a selective transformation of one group is impossible without the same reaction occurring at the other);

(3) a low overall selectivity of the process (in classical organic chemistry, as a rule, several alternative reactions can occur concurrently; refinement from byproducts increases the cost of production of the desired product);

(4) a low yield of the desired product (because of the synthesis of the second isomer and byproduct formation, the yield of the desired isomer only infrequently exceeds 60–70%);

(5) strong deviations of the process parameters from the standard temperature and pressure (as a rule, many reactions in classical organic chemistry are carried out at elevated pressures and temperatures and require the use of organic solvents).

The above factors necessitate additional safety measures and greater expenditures for refinement and the elimination (recycling) of organic solvents. The use of enzymes as biocatalysts for synthesis of chiral compounds can easily eliminate the weaknesses of classical organic chemistry. Enzymes have extremely high regio- and enantioselectivity; they catalyze reactions without byproduct formation, and the processes are carried out in aqueous solutions at 25–35°C. Target products are obtained in more than 85–90% yields (very frequently, 95–99%) and have an optical purity of 99% or higher. As a result, the refinement cost is only a small part of the overall cost of the process.

All biocatalytic processes of chiral synthesis can be categorized into two groups. One group comprises enzymatic reactions based on the selective chemical transformation of one optical isomer in the racemic mixture. This approach was one of the first in use. It is employed when ordinary chemical synthesis is a sufficiently cheap and simple route to racemic mixtures compared to the preparation of precursor compounds for chiral-center-generating processes (see below). Chemical transformation of one isomer can either conserve the chiral center or not. The first variant is exemplified by reactions with hydrolases; hydrolases can specifically catalyze the formation or hydrolysis of esters or amides of one isomer. The second variant is exemplified by reactions with oxidases (amine oxidases, D- or L-amino acid oxidases). Evidently, the yield of the desired isomer from the racemic mixture is at most 50%. With D- or L-amino acid oxidases, byproduct α-ketoacids are also valuable compounds; their preparation by classical organic synthesis is not a trivial problem.

The other group of processes is intended to prepare chiral compounds from achiral precursors. Evidently, in this case, the maximal yield of the desired isomer amounts to 100%.

The three major types of reactions used in this type of process are described below.

Reduction of various groups with the use of dehydrogenases and oxidoreductases. Dehydrogenases are enzymes with the highest stereoselectivity. For example, the well-known alcohol dehydrogenase from baker's yeast makes only one stereochemical error (synthesis of the second (undesired) optical isomer) per seven billion catalytic cycles. Although lactate dehydrogenase is inferior to alcohol dehydrogenase in its stereoselectivity (one stereochemical error per seven million catalytic cycles), it helps to prepare samples with optical purity several orders of magnitude higher than the current norm of 99%.

A weakness of reactions with the participation of dehydrogenases and reductases is the need to use the reduced species of the coenzyme nicotinamide adenine dinucleotide or nicotinamide adenine nucleotide phosphate (NADH and NADPH, respectively). These compounds are very expensive. The production cost of 1 kg of NADH and NADPH is about US$5000 and US$100000, respectively. Therefore, a second enzyme is introduced into the system to reduce the cost; this enzyme catalyzes the reduction of the oxidized coenzyme species formed in the main reaction ($NAD(P)^+$) back to NADH and NADPH. The enzymes used for NAD(P)H regeneration should have a broad pH optimum of activity and use cheap substrates; the reactions catalyzed by them should be irreversible to ensure a thermodynamic impact on the equilibrium of the main reaction and a conversion in the desired process of 100%. In addition, the substrate and the product of the second reaction must not inhibit the major enzyme and must be easily removed during the purification of the final desired product. In principle, the major enzyme (e.g., isopropanol/alcohol dehydrogenase) can also catalyze the second reaction; numerous experiments, however, showed that it is more reasonable to use a special enzyme. Formate dehydrogenase and glucose dehydrogenase, two widely used enzymes, showed the best results. Formate dehydrogenase exceeds glucose dehydrogenase in almost all parameters. We should especially note that the energy content of formate is several times that for glucose; the formate oxidation product (CO_2) is easily removed from the reaction mixture, whereas additional efforts are needed to remove the glucose oxidation product. A drawback of formate dehydrogenase is its lower specific activity compared to glucose dehydrogenase (about one-tenth). Each enzyme, however, has found its own use.

Of the processes of the second group, reactions catalyzed by dehydrogenases and reductases are most widely used for synthesis of chiral compounds. Currently, the efficiency of NAD(P)H regeneration reaches one million cycles; as a result, the cost of the coenzyme use accounts for less than 1–2% in the overall cost of the target product. The main problem here is the stability of coenzymes (especially of $NADP^+$ and NADPH) during the reaction. Fresh portions of coenzyme should be added to compensate for those degraded. Degussa in the middle 1990s implemented a process for *tert*-L-leucine synthesis using leucine dehydrogenase as the major enzyme and formate dehydrogenase as the enzyme for NADH regeneration.

The reaction is carried out in a cascade of two flow-through membrane reactors; polyethylene-immobilized NAD^+ is used for the retention of coenzymes in the reaction zone. Currently, this process is used for enzymatic chiral synthesis on the largest scale (producing tens of thousands of tons per year).

Oxidation reactions. These reactions are catalyzed by various hydroxylases (e.g., cytochromes P450) and monooxygenases. Stereochemical oxidative hydroxylation reactions are abundant in nature. They produce various steroids. Cyclomonooxygenases catalyze the reactions in which the cycle increases by one atom as a result of oxygen incorporation. This reaction is known in organic chemistry as the Bayer–Villiger reaction. The centenary of its discovery was celebrated in 2006. Processes involving hydroxylases and oxygenases also require the reduced coenzyme species NAD(P)H, and all said above regarding coenzyme regeneration fully applies to them. Most (more than 98%) reactions with enzymes of this group require the use of NADPH.

Native formate hydrogenases are distinguished by high NAD^+ specificities and very low (or null) activities with $NADP^+$. This retards the development of these processes with hydroxylases and monoxygenases as compared to those using NAD^+-specific dehydrogenases and reductases. Activities in this field started with the design of mutant $NADP^+$-specific formate dehydrogenases from *Pseudomonas* sp. 101.

Stereochemical condensation of cyanide ion with ketones and aldehydes. This reaction is catalyzed by special enzymes, nitrilases. Although this reaction has come into use only recently, it has received much attention because of the simplicity of its implementation (with the provision of an enzyme with the required substrate specificity and stereospecificity) and because it does not need other substrates.

Characterizing the modern trends in biocatalytic synthesis of chiral compounds, we can note the following:

(1) New biocatalysts are searched, and their catalytic properties and stability are improved. To some extent, the high substrate, regio-, and stereospecificity is a drawback of enzymes; a highly specific biocatalyst for one process cannot be used for another. Genetic engineering and bioinformatics are now widely used to find new enzymes. Sequencing of a large number of genomes and development of bioinformatic approaches to annotation of new enzymes enable researchers to search known genomes for enzymes with the desired catalytic activity in a targeted way theoretically (we may say, "with the tip of a pen"). Natural genomes are even more diverse, especially in uncultured microorganisms from deep-sea sources, volcanos, salt lakes,

and other sources. In this case, metagenome screening is used, which does not require the isolation of pure cultures. Sources range from samples of Arctic and Antarctic seawaters (the well-known lipase from *Candida antarctica* was discovered in this way) to bovine rumen or the intestines of simple worms.

The next stage is to refine the catalytic properties and stability of the enzyme found. The methods widely used for this purpose include targeted search (rational design) and random search (directed evolution, experimental evolution, genomics, and other methods). Intentionally designed highly productive recombinant strains are used to prepare biocatalysts.

(2) A complex approach using several enzymes is implemented. Recall that the yield of the desired optical isomer and racemic mixture cannot exceed 50%. For large-scale production (tens of tons or higher), this amount of waste inevitably requires the development of special utilization processes; the overall cost of the product will increase. Instead, it would be more pertinent to develop additional processes for converting wastes into the desired product. Two approaches are now used. If the product of the enzymatic reaction is the target compound, the other optical isomer is treated with the specific enzyme racemase, and the desired product is produced by the major reaction from the resulting racemic mixture. If the undesired isomer is removed from the racemic mixture, the second enzymatic reaction can convert it to the target product.

(3) Whole cells, and not separated ones, are used as biocatalysts. The synthesis and purification of an enzyme are rather expensive processes. Therefore, the use of whole cells of recombinant strains (producing desired enzymes) as biocatalysts has been progressively increasing during the last decade. In this case, the absence of enzyme purification stages decreases the process cost. When dehydrogenases, reductases, hydroxylases, and oxygenases are used for biotransformation, additional coenzymes are not needed. The amounts of coenzymes contained in the cell are sufficient. In addition, coenzyme biosynthesis by the cell itself compensates for coenzyme lost by degradation. With the use of whole cells, glucose dehydrogenase is widely used for the regeneration of NADH and, especially, NADPH; the product of glucose oxidation is then used by the cell to maintain its life. Formate dehydrogenase is also widely used for NADH regeneration in cells because of the occurrence of an additional reaction for the production of NADH, independently of the existing metabolic routes, for utilization in both biotransformation and in the cell life cycle without additional metabolites.

The use of recombinant strains—biocatalyst producers—has one advantage over the native precursor strains. In this case, it is possible to express the entire required set of enzymes in the desired proportion in one cell. Unnecessary metabolic enzymes can be excluded from recombinant strains by genetic engineering. As a result, intermediate metabolites are intentionally utilized only in the synthesis of the desired product, whereas byproduct formation and nonspecific utilization of the desired compound are inhibited. An example is the use of *Rodococcus* strains for preparing acrylamide from acrylonitrile. The final product is a 40% aqueous solution of acrylamide, which can be used (after a minimum purification or, in most cases, as synthesized) for the designated purpose.

For recombinant strains (producers), methods have been developed for preparing high-density cultures with yields of up to 70–100 g primary cells per liter of the medium, which is unachievable for native strains. Low-cost synthetic media are used for recombinant strains, which makes it possible to reach high cell yields with high precision. For native strains, if the supplier of these components is changed, variations in the components of the medium require the optimization of growth processes, which increases costs. The production cost of biocatalysts based on recombinant strains is at least one to two orders of magnitude lower than that for native strains. As a result, the reuse of the biocatalyst becomes unnecessary. Most companies (Degussa, DSM, Dow Chemical, and others) now follow this route.

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