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Review
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Biocatalysis for the Production of Pharmaceutical Intermediates: Statin Precursors

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Abstract

The application of enzymes in chemical synthesis, due to the recent advances, has a strong impact in multiple industries, especially the pharmaceutical industry. Namely, the use of enzymes shows remarkable advantages over classical chemical catalysis and therefore it is considered a 'green' solution. By using novel techniques, it is possible to tailor the enzymes to adapt them for a given process. Today, several pharmaceutical companies are successfully producing valuable precursors for the production of active pharmaceutical ingredients using enzymes, some of them being statin precursors. Statins are hypolipidemics, drugs used for the prevention of cardiovascular diseases and lowering cholesterol concentration in blood. Due to challenges in the chemical synthesis of statin intermediates, the production of statin intermediates using enzyme-catalysed reactions shows some notable advantages. Therefore, research and development laboratories, combined with reaction engineering techniques, have shifted their focus towards applying biochemical catalysis for the production of statin intermediates.

Keywords

Enzymes, biocatalysis, green chemistry, precursors, pharmaceutical industry, statins

1 Introduction

While catalysis refers to the application of chemical catalysts for the acceleration of chemical reactions, biocatalysis is the application of natural catalysts (enzymes) instead of chemical catalysts. Enzymes offer distinct advantages in the realm of industrial application, mainly due to their ability to perform highly stereoselective reactions while operating under mild reaction conditions, such as ambient temperatures, atmospheric pressure, and neutral pH values, making biocatalysis a viable choice for industrial application in organic synthesis. Moreover, the application of enzymes is aligned with the principles of Green Chemistry guiding the chemical industry in the last few decades.[1–6]

The application of enzymes for industrial purposes has a very storied history which has steadily grown from the days of Louis Pasteur (1857), who isolated a specific racemate with the help of fermentation by various microorganisms.[7] Since then, enzymes have been isolated, mass-produced, and re-engineered to fit almost any chemical reaction by the application of highly complex protein engineering techniques, like directed evolution[8–10] and *in silico* or *de novo* enzyme design.[11,12] While chemical catalysis with various metal catalysts is still prevalent in industrial organic synthesis,[13–15] it shows its own drawbacks in the form of high market price and limited availability of required precious metals, like rhodium and palladium,[16] along with multiple protection and de-protection steps that make the process economically unfeasible, and may result in the production of large amounts of dangerous or toxic waste.[17,18] Those drawbacks are especially noticeable in the pharmaceutical industry, which is known for its long and rigorous drug design and production times.[19] Thus, enzyme-catalysed processes represent a more feasible, economically beneficial, and environmentally safer alternative for the pharmaceutical industry. *Truppo*[20] has shown metrics of rapidly increasing publications and patents discussing "pharmaceutical biocatalysis" from 1985 onwards, to which we have added the period from 2015 to 2019, shown in Fig. 1, that can arguably be correlated to increased testing and usage of biocatalytic processes in the pharmaceutical industry.

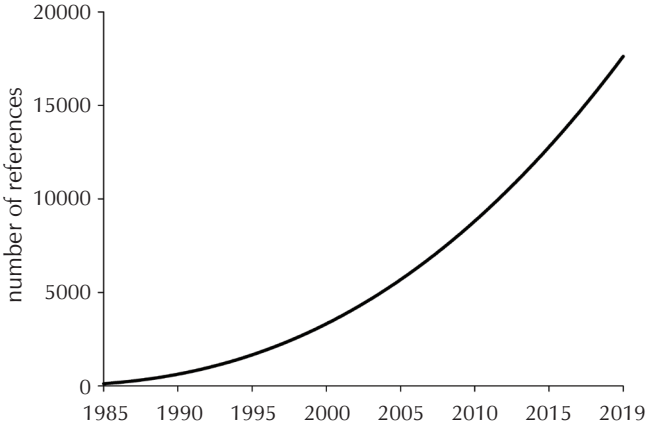

Fig. 1 – Number of publications and patents discussing "pharmaceutical biocatalysis" in the last 35 years. Metrics are taken from *Google Scholar*.

Slika 1 – Broj objavljenih publikacija i patenata na temu farmaceutske biokatalize u posljednjih 35 godina. Mjerni podatci preuzeti s *Google Znalca*.

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The main challenge in the pharmaceutical industry, regarding the production of highly functionalized building blocks, is the complexity of molecules and the number of chiral centres needed to be produced.[21,22] Chiral pharmaceutical compounds are usually manufactured in a single enantiomeric form,[22,23] since the opposite enantiomer may have adverse effects.[23] For the production of chiral drug intermediates, different routes are employed,[24] but the application and implementation of enzyme-based processes and their use in the pharmaceutical industry has shown great promise, since enzymes inherently satisfy the requirements expected for the drug production. Hence, several pharmaceutical companies are now outsourcing the synthetic stages to focus on discovery and formulation.[23]

2 Biocatalytic process development

Although running biochemical reactions is similar to running chemical reactions, it requires additional multidisciplinary skills, such as from biology and biochemistry, along with chemical and engineering sciences that are essential features of biochemical engineering.[25] The pharmaceutical processes are run on a smaller scale compared to chemical processes. For biocatalysis to be run on an industrial scale, compared to the academic research, the process requires significant substrate concentrations. With respect to biocatalyst usage, conversion, and optical purity, some general rules of thumb for a feasible enzyme-catalysed process can be roughly defined.[26–29] The main problem for industrial implementation of biocatalysis lies in the transfer of the biocatalytic process from laboratory to a larger scale, which can cause a loss in productivity and product quality,[28] but can be overcome by gaining insight into the reaction characteristics on a laboratory scale.[22,28] A methodology for development of a biocatalytic process is outlined in Fig. 2.

Naturally occurring enzymes, whether in isolated or whole cell form, often exhibit challenges regarding their application in relation with their stability, activity, and re-usability, especially when scaled-up from a laboratory to industrial scale.[21] There is a multitude of ways to minimize or even overcome those disadvantages. Biocatalyst engineering approach usually includes different types of enzyme immobilization and various protein engineering techniques. Of those two, the protein engineering technique currently seems to relish main focus largely due to its practical feasibility and speed of development, despite the increasingly growing possibility for combining the immobilization procedures with enzyme engineering techniques into one efficient solution.[30–33] In order to develop the optimal biocatalytic process, it is important to examine kinetic properties of enzymes in detail by integrating enzyme reaction kinetics and mass balance in the reactor.[34] The precondition for an effective scale-up of processes is the speed of development, which can be achieved by the use of miniaturized experimentation or mathematical modelling.[34] Mathematical modelling can be useful in finding the most cost-effective mode for the bioprocess on an industrial level.

3 Statins and statin precursors

A lucrative aspect[27,35–40] of the application of enzymes in the pharmaceutical industry is in the production of statin precursors. Namely, statins are hypolipidemics, a medication used for lowering LDL cholesterol (LDL-C) in blood, and for prevention of cardiovascular morbidity and mortality.[27,41–44] Besides lowering the LDL-C levels, and therefore reducing cardiovascular risk,[45] it has been reported that the use of statins has additional positive side effects.[46,47] Statins act as competitive inhibitors of HMG-CoA reductase, a key enzyme involved in cholesterol biosynthesis,[27,44,47–51] and therefore are one of the most prescribed drugs today.[27,52] Presently, there are a number of statins on the market, being either natural or synthetic products,[35] and all having in common the pharmacophore *syn*-3,5-dihydroxy carboxylate side-chain.[50] For the industrial production of statins, the chemical route is employed, which is a time-consuming, multi-step chemical process that can occur at very severe conditions.[28,35,53–57] Moreover, the two chiral centres present in the molecule are challenging to make by applying traditional chemical methods.[50,55] The increasing commercial demand for statins, and the need for simple preparation on the industrial scale, have led to immense efforts towards finding a more efficient and economical production of statin side chains.[28,29,38,51,58,59]

4 Biocatalysis as a response to the challenges arising from chemical synthesis of statin precursors

Driven by the requirements of high chemical and stereochemical purity, a variety of biocatalytic routes, involving different classes of enzymes and starting materials, have been developed for their enantioselective synthesis,[27,55] since enzymes inherently circumvent the challenges arising from the chemical synthesis.[60] Thus, some pharmaceutical (and biotechnological) companies, including Codexis,

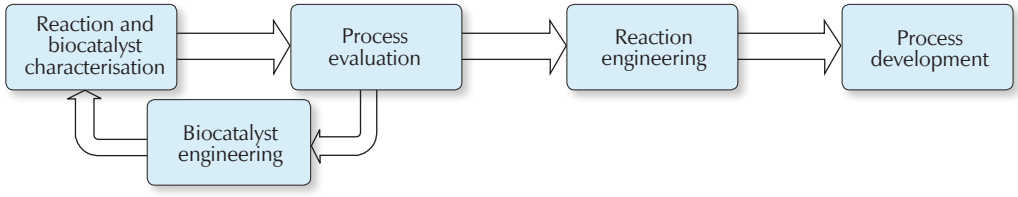

Fig. 2 – Methodology for the development of a biocatalytic process
Slika 2 – Metodologija razvoja biokatalitičkog procesa

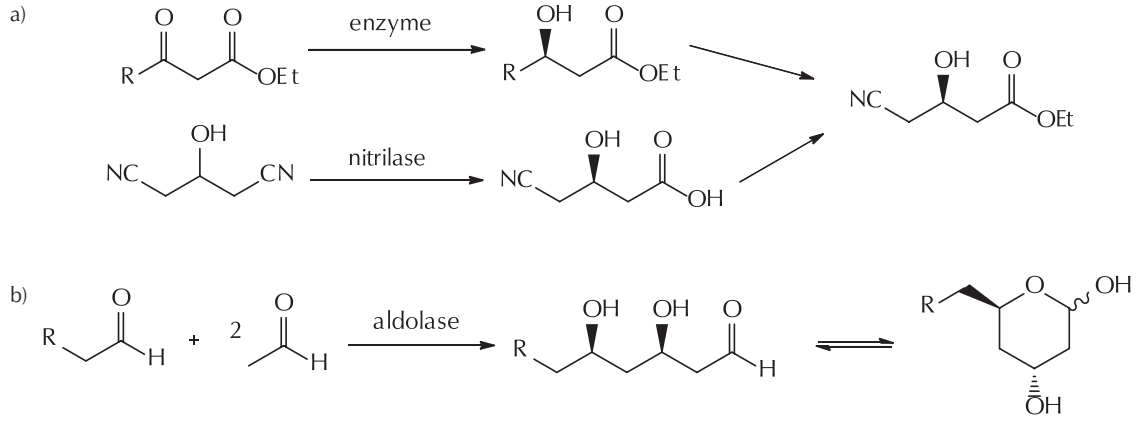

Fig. 3 – Biocatalytic strategies towards the production of statin side-chain intermediates. a) The first strategy results in the formation of ethyl (*R*)-4-cyano-3-hydroxybutyrate,[36,55] while b) the second strategy is based on an aldol reaction that results in the formation of a six-carbon intermediate.[54,55]

Slika 3 – Biokatalitički načini proizvodnje prekursora bočnog lanca statina. Prvi pristup (a) rezultira nastankom etil-(*R*)-4-cijano-3-hidroksibutanoat,[36,55] dok se drugi pristup (b) temelji na dvostrukoj aldolnoj adiciji kojom nastaje dihidroksi aldehid sa šest C-atoma.[54,55]

Pfizer, Bristol-Myers Squibb, Diversa Corporation, Dow Pharmaceutical Sciences, DSM Pharma Chemicals, Lek Pharmaceuticals, IMI TAMI (Institute for Research and Development Ltd.),[61] among others, have shifted their focus towards applying biochemical catalysis for the production of statin intermediates.

The most common biocatalytic methods towards the production of statin intermediates[27,29,38,39,50,62] can be broadly grouped into two strategies[36,55] (Fig. 3).

The first strategy (Fig. 3a), which encompasses most biocatalytic methods, involves the synthesis of the key chiral precursor, ethyl (*R*)-4-cyano-3-hydroxybutyrate, on which the second stereocentre is introduced at a later step. Most of the biocatalytic methods fall under this strategy,[55] although, according to some literature, the usage of nitrilases is classified as a separate third strategy.[37,38] The second strategy (Fig. 3b) involves the generation of both chiral centres in a single step using aldolases, which catalyse the aldol reaction that results with a more advanced six-carbon statin side-chain.[36,55] This strategy is often expanded by coupling aldolases with oxidoreductases and/or halohydrin dehalogenases, as possible enzyme combination,[27,36,55] to produce the key statin precursor.

Although different biocatalytic approaches have been developed, ever-growing attention is being shifted towards a simplified process containing fewer reaction steps with a high atom economy, in which the generation of both chiral centres is done in a single step using the deoxyribose-phosphate aldolase (DERA).[28,35,67,48,51,54,58,63–66] By using achiral substrates[28,48] the DERA enzyme catalyses the sequential aldol addition, which results in an enantiomerically pure lactol, a cyclized 2,4,6-trideoxyhexose,[27,68–72] which is a valuable chiral synthon[40,64,69] used for the production of statin intermediates.[40,64,67,68,70,73–75] Although the application of the DERA enzyme shows great potential in the production of statin intermediates, the enzyme demonstrates some major drawbacks for its practical application,[27,40,44,48,58,59] but due to the high interest in its application, the enzyme is being continuously re-engineered to have a better fit.[38,55,58,65,69,72] The DERA-based strategy is almost by default coupled with an oxidation step, in which the lactol is further oxidized into a lactone, such as cyclized 3,5-dihydroxyhexanoic acid, which is a more stable form of lactol[76] and thus presents a key product in the synthesis of statin intermediates.[27,36,38,50,54,72,73] The significance of those two reactions is evident also in the industry: DSM Pharma Chemicals[77,78] and Diversa Corporation[40] operate the DERA-catalysed aldol reaction followed by chemical oxidation of the produced lactol[27,39,40,77,78], while Lek Pharmaceuticals patented the DERA-catalysed reaction followed by oxidation catalysed by dehydrogenases (DHs), which results in a more economic process: reduced number of synthetic steps, improved and simplified reaction, *etc*.[59] By studying the available literature, it is evident that there are only a few known suitable ways for chemical oxidation of the enzymatically produced lactol,[40,51,54,65] whereas investigations on the enzymatic lactol oxidations remain rare.[38,51] This is especially interesting considering that the chemical oxidation of the DERA-produced lactols is done with the excess of harsh chemicals, while the biocatalytic oxidation uses oxygen as a co-substrate and produces water as a by-product, which classifies the latter approach in the area of green chemistry.[38] Based on the accessible literature, an interesting route towards statin side-chain intermediate production seems to be a multi-enzyme process consisting of DHs and halohydrin dehalogenases (HHDHs), where the HHDHs are used for their ability to replace the halide group with a strong nucleophile containing nitrogen,[36,62,79,80] such as nitrile[62] (Fig. 4).

A similar reaction of statin intermediate synthesis was patented by Codexis, who demonstrated a two-step three-enzyme catalysed reaction involving two DHs, where one is responsible for the substrate oxidation, and the other for

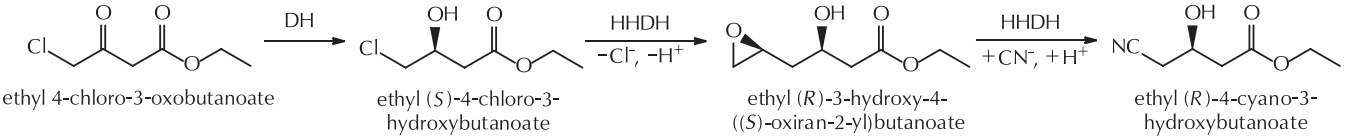

Fig. 4 – DH-catalysed oxidation of ethyl 4-chloro-3-oxobutanoate followed by HHDH-catalysed conversion of ethyl (*S*)-4-chloro-3-hydroxybutyrate into ethyl (*R*)-4-cyano-3-hydroxybutyrate.[36,62,79] The change in stereo-configuration from the substrate to the final product is caused by a switch in priority of the substituents at the chiral centre according to the Cahn-Ingold-Prelog (CIP) priority rules.[79]

Slika 4 – Reakcija oksidacije etil-4-kloro-3-oksobutanoata katalizirana enzimom DH nakon koje slijedi reakcija biotransformacije etil-(*S*)-4-kloro-3-hidroksibutanoata u etil-(*R*)-4-cijano-3-hidroksibutanoata katalizirana enzimom HHDH.[36,62,79] Promjena u RS-konfiguraciji uzrokovana je promjenom prostornog razmještaja atoma na kiralnome centru prema Cahn-Ingold-Prelogovim (CIP) pravilima prednosti.[79]

coenzyme regeneration, and an HHDH enzyme applied for the dehalogenation reaction and cyano-group addition.[81] They filed a new successive patent, in which they expanded the substrate scope and applied newly engineered enzymes.[82] Akin reactions were claimed by DSM Pharma Chemicals, who are using HHDH enzymes to synthesize epoxides, lactones, alcohols, and other intermediates in the preparation of active pharmaceutical ingredients (APIs), in particular statins.[83] In their patent, they also claim a completely chemoenzymatic pathway towards the production of the desired lactone by combining DERA and HHDH enzymes.

5 Multi-enzyme systems and reaction engineering for the production of statin precursors

The application of multi-enzyme (cascade) systems shows a promising direction to produce optically active chemicals. That way, the process results in higher yields, spends fewer chemicals, and saves time, especially when an unstable intermediate needs not be isolated. Moreover, the process conducted in this manner leads to a significant reduction in both waste and production costs on the industrial scale.[45] The main drawback, which can be overcome by applying mathematical modelling, is finding optimal process conditions. Therefore, when finding the most cost-effective mode for statin side-chain biosynthesis, especially for further industrial use, it is of great importance to understand the mechanisms and kinetics of the enzyme systems.[46] For this purpose, mathematical modelling, as reaction engineering tool, is used and has an important role in the study of enzymatic reactions.[46] Despite its undeniable importance for each of the synthetic steps towards the production of statin precursors, the available literature offers very little or virtually no kinetic data obtained based on kinetic models. The data concerning aldolase-catalysed reactions is scarce[53,84,85], and for now, only two research papers report mathematical modelling of the reaction towards statin side-chain production.[53,86] *Švarc et al.*[86] have reported a mathematical model (consisting of a formal kinetic model that includes the data-driven or the empirical model that has less kinetic constants) validated in multiple reactor configurations. Using the developed model, process simulations were done and optimal process design was proposed. *Ručigaj and Krajnc*[53] have reported a very complex model for the sequential aldol reaction with different substrates catalysed by crude DERA expressing culture lysate. Regarding reactions catalysed by NAD(P)-dependent DHs, for now, only two published research articles offer kinetic data on the enzymes used for oxidation of the DERA-produced lactols, but without further model development that would help in process optimization and scale-up. The first research was published in 2014,[51] in which the kinetic data of *E. coli* membrane-bound PQQ-dependent glucose dehydrogenase (GDH) was given. The authors employed a DERA-GDH coupled system using a whole-cell catalyst, but despite performing successful oxidation mediated by the PQQ-GDHs, the system resulted in a disadvantageous industrial application, due to the application of the PQQ as a cofactor and the low expression level of GDHs as membrane protein. This finding was followed by the research of *Xu* group in 2016[38], who carried out oxidation of the key lactol using a novel NADP-dependent aldehyde dehydrogenase (AlDH). Although they published values of kinetic parameters, they had not developed a mathematical model that would provide a basis for future application. In addition to the apparent lack of aldolase and DH kinetic data, the data related to HHDHs and the reaction of statin side-chain production remains unavailable.[35,87] It should be noted that, for the time being, catalytic data on the biocatalytic reaction of epoxide ring-opening in the presence of strong nucleophiles has yet to be estimated; thus, no mathematical models have been developed although these phenomena are of scientific interest.[27,87–89] Based on the previously mentioned research, it is obvious that an engineering aspect regarding development and optimization of multi-enzyme-catalysed reactions towards the biocatalytical production of statin intermediates is still lacking.

6 Conclusion

As we have shown in this review, biocatalysis is very much an active field of development in the pharmaceutical industry, and can still offer many new and beneficial economic and environmental advantages. Moreover, it could be said that statin precursor production using biocatalysis is still in its relatively early stages of development, and shows a lot of promise and untapped potential waiting to be explored and utilized.

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SAŽETAK

Biokataliza u proizvodnji farmaceutskih intermedijara: prekursori statina

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Napredak tehnologije usko je vezan uz primjenu enzima u kemijskoj sintezi. Biokataliza ima velik utjecaj na različite industrije a među njima se posebno ističe farmaceutska industrija. Primjena enzima pokazuje značajne prednosti u usporedbi s tehnikama klasične kemijske sinteze te se smatra i 'zelenim' rješenjem. Primjenjujući nove dostupne tehnike i tehnologije, enzime je moguće u potpunosti prilagoditi zahtjevima određenog procesa. Danas je poznato nekoliko farmaceutskih tvrtki koje uz pomoć enzima uspješno proizvode vrijedne intermedijare za proizvodnju aktivnih farmaceutskih spojeva, a u njih se ubrajaju i prekursori statina. Statini su hipolipidemici, lijekovi koji se upotrebljavaju za prevenciju kardiovaskularnih bolesti te za snižavanje koncentracije kolesterola u krvi. Metoda klasične kemijske sinteze intermedijara statina vrlo je zahtjevna te stoga njihova proizvodnja primjenom enzimski kataliziranih reakcija ima značajne prednosti. Iz tog razloga za proizvodnju intermedijara statina istraživanje i razvoj uz primjenu reakcijskog inženjerstva sve se više usmjeravaju prema primjeni biokatalize.

Ključne riječi
Enzimi, biokataliza, zelena kemija, intermedijari, farmaceutska industrija, statini

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