Overview on Reactions with Multi-enzyme Systems

Z. Findrik* **and Đ. Vasić-Rački**
Faculty of Chemical Engineering and Technology, University of Zagreb,
Marulićev trg 19, HR-10000 Zagreb, Croatia

Review
Received: April 6, 2009
Accepted: September 18, 2009

Dedicated to the memory of Professor Dr. Valentin Koloini

Production of special chemicals and pharmaceuticals includes multi-step procedures that are still carried out in the traditional way: by isolating the intermediary product of each step and using it as a substrate for the next step, which is money and time consuming. These are also procedures that require many chemicals, energy and labour. Over the last couple of decades scientists have been working on new, integrated processes that require fewer resources and are more close to nature, as they produce less waste. These kinds of processes are discussed in this paper. The advantages of enzyme catalyzed reactions are well documented and numerous. Enzyme reactions are carried out at mild reaction conditions. The enzymes are enantioselective and stereoselective, which is important particularly for the pharmaceutical industry. By combining the action of different enzymes we can imitate the processes in the living cells and produce the desired compounds. Enzyme catalyzed reactions can also be combined with chemical reactions in one-pot chemo-enzymatic synthesis. The progress in the development of these reactions will be presented.

Key words:
Multi-enzyme systems, kinetic model, enzyme reaction, fine chemicals

Introduction

The importance of today's chemical and pharmaceutical industry is unquestionable. However, the fact that these are considered one of the world's largest pollutants resulted in their bad public image.[1] Since environmental protection is one of the most important issues of the 21st century, the chemical industry has to adapt to new rules and laws. It is important to develop new processes that will generate less waste, use less energy, resources and water, which will also positively affect the environment, and lower the process costs as well.

The progress of chemical synthesis is very rapid. New compounds are being synthesized every day, and it is no longer a question of what we can synthesize, but how we do it.[1] The classical approach of synthesizing compounds usually involves multi-step reactions that are carried out in separate stages. Each intermediate product is isolated and purified from the reaction solution and serves as a substrate for the next step.[2] Such synthesis results in low yields and expensive products, as it lasts a long time and uses too many chemicals for separation steps. It is clear that there is a need for new kinds of processes which will resolve these issues. These processes, i.e. reactions will be addressed in this paper and they are often referred to as cascade,[3,4,5,6,7] domino[1,4,8] or multi-step reactions.[2,9,10] They occur in multiple steps without product recovery, and can be catalytic or non-catalytic. Enzyme-catalyzed reactions which in a way imitate the chemical processes in the living cells are also a type of cascade. There are a few known definitions; more or less similar. One of the first definitions came from Hemker who defined cascades as a sequential array of enzymatic reactions in which the product of one reaction serves as the substrate in the next.[3] Similar cascade definition was elaborated by Mayer *et al*. who considered domino or cascade reactions as transformations of reactants into products in several inseparable steps, which usually go through highly reactive intermediates.[4] If such multi-step reaction is enzyme catalyzed, it results in high enantio- and regioselectivity.[11] A different definition comes from Bruggink *et al*. who defined cascades as catalytic reactions that happen in multiple steps without product recovery after each step – similar as in the living cells.[5] Tieze's definition of domino reactions as transformations that occur in multiple steps (one after another)[1] is quite general. However, Tieze considers the term *cascade* wrong for multi-step processes as it does not describe their real meaning.[1] Unlike Bruggink *et al*. who defined them as a catalytic reaction, Tieze's and Mayer's definitions of cascades are not that narrow. No matter which definition we embrace, in this paper dif-

* Corresponding author: e-mail: zfindrik@fkit.hr,
phone: +385 1 4597 157; fax: +385 1 4597 133

ferent multi-step reactions are discussed. Special attention is addressed to their advantages, complexity, the problem of their optimization, kinetic modelling etc. Enzyme-catalyzed reactions are discussed mostly, since their superiority over the chemical reactions is well recognized. Numerous industrial processes have been established where biocatalysts are employed in the form of whole microorganisms or purified enzymes.[12] Some chemo-enzymatic reactions are discussed as well, since these also represent industrially interesting processes.[2]

There are many advantages in the use of multi-enzyme systems in the biosynthesis: the enantioselectivity, stereoselectivity, high yields and lower expenses for downstream processing, the equilibrium shifting by removing one of the products from the reaction mixture etc.

Reactions with two enzyme systems

Several examples of reactions with two-enzyme systems are mentioned here. The first one, crucial for the industrial application of oxidoreductases is coenzyme regeneration.[13] The results so far clearly demonstrate that cofactor regeneration is no longer an economical issue. It is known that some cofactors (pyridoxal phosphate, biotin) are tightly bound to the enzymes and are essentially self-regenerating, while others, which are not bound to the enzyme structure, should be regenerated *in situ* to avoid high coenzyme expense.[14] There are many methods developed for that purpose and described in the literature.[13] The most common and the most used method is coenzyme regeneration by the additional enzyme.

Degussa's industrial synthesis of L-*tert*-leucine is presented in Fig. 1. In this reaction L-leucine dehydrogenase was used for reductive amination of trimethylpyruvate to L-*tert*-leucine, and cofactor regeneration was carried out by using formate dehydrogenase from *Candida boidinii*. Due to the highly effective coenzyme regeneration system, a low concentration of coenzyme is necessary.[15] By using the enzyme reaction engineering principles, proper enzyme concentrations could be estimated to achieve the maximum conversion and volume productivity, with the minimum amount of biocatalyst and coenzyme.[16,17] The process was developed[16] in the continuously operated enzyme membrane reactor and in the two-stage cascade of identical enzyme membrane reactors with the conversion of over 93 %. It needs to be emphasized that kinetic modeling plays an important role in the process development and it played its role in the development of this process. The advantage of cofactor regeneration is not only in the reduction of cofactor cost, but

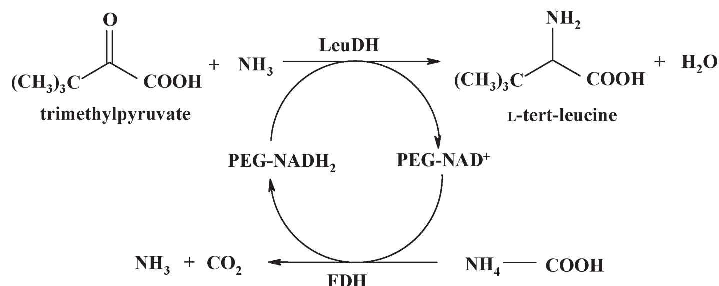


Fig. 1 – *Synthesis of L-tert-leucine by reductive amination of trimethylpyruvate with cofactor regeneration*

also in shifting the reaction equilibrium and driving the reaction to completion. In this manner, the product isolation is simplified, and accumulation of inhibitory cofactor by-products is prevented.[14] There are other industrial processes where dehydrogenases and coenzyme regeneration systems are used.[18] Beside formate dehydrogenase, other enzymes like glucose dehydrogenase or lactate dehydrogenase can be used. Even though NADH regeneration is no longer an issue, new methods are investigated for NAD regeneration which is required if oxidation is carried out by using oxidoreductase. In these cases, formate dehydrogenase cannot be used, and substrates and products for other enzymes available (lactate dehydrogenase, glucose dehydrogenase etc.) can be inhibitors for the catalytic enzyme of the main reaction. Therefore, there are still new methods being developed and investigated. New coenzyme regeneration enzymes that are investigated and isolated still await industrial application. NADH oxidase is one of the newer promising enzymes for NAD regeneration,[19,20,21] but much work is still required on its stabilization.

Beside coenzyme regeneration systems, there are other two-enzyme systems that need to be mentioned. Nakajima and co-workers studied the production of enantiomerically pure amino acids and found that chemically and enzymatically produced L-amino acids, in particular L-methionine, often contain a small amount of the D-enantiomer.[22] Therefore, D-amino acid oxidase was used to remove the unwanted enantiomer from the process.[22,23] Catalase was the second enzyme in the reaction required for the subsequent removal of hydrogen peroxide. The same process can be done with L-amino acid oxidase if D-amino acids are produced.[24] The system is presented by the reaction scheme in Fig. 2. Nakajima *et al*. have developed a process in an immobilized enzyme membrane (slurry) reactor which operated in the continuous mode. The enzyme kinetics were not investigated. However, the process was successful and operated for 14 days.[22] The oxygen was continuously supplied as a second substrate. Since this reaction sys-

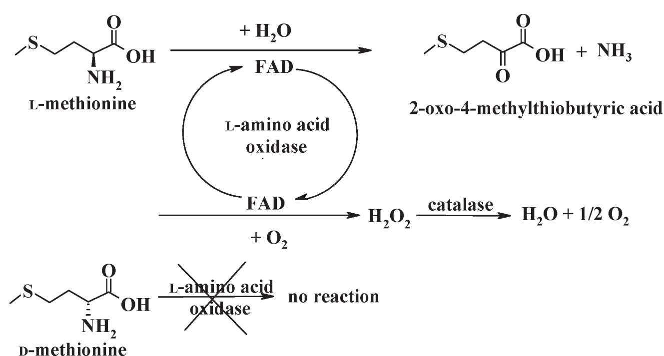


Fig. 2 – *Oxidative deamination of* L*-methionine catalyzed by* L*-amino acid oxidase from Crotalus adamanteus. Hydrogen peroxide removal by the addition of catalase.*

tem was used for the removal of low enantiomer concentration (1 mmol dm^{-3} D-methionine), it is important, from the kinetics point of view, that the used D-amino acid oxidase has low value of Michaelis constant. This means that the substrate has high affinity towards the enzyme, and it exhibits high activity even with such low substrate concentrations. Otherwise, too much enzyme would be consumed. Additionally, amino acid oxidases are competitively inhibited by α-keto acids,[23,24] which would make a continuously operated enzyme membrane reactor a bad reactor choice because it works in stationary conditions at constantly high product concentration (high conversion). Since Nakajima *et al*. used this reactor mode for the removal of low concentration of D-enantiomer, only a small amount of α-keto acid was produced, which did not significantly affect the enzyme activity.[22–24] This reaction system is an excellent example for the enzyme's enantioselectivity which can rarely be found in a chemical reaction. The continuously operated reactors are industrially very attractive since they deliver a product of constant quality, require less manpower, and are easy to operate.[12]

There are examples of synergistic action of two enzymes in sugar production as well. Glucoamylase and glucose isomerase are enzymes required for the production of high fructose syrup. This is a two-stage process in the industry. However, co-immobilization of these two enzymes has several advantages.[25] The proximity of the two enzymes also reduces the diffusion time of the substrate to the second enzyme.[26] Such systems save money on equipment and energy as compared to the present process. The fructose output is much higher in the co-immobilized system, than that of free enzymes.[25]

Our group is also working on a similar two-enzyme system – starch hydrolysis by α-amylase and glucoamylase, which is an important industrial process[27] for the production of glucose syrup. The process is usually carried out in two stages: the first stage, liquefaction is catalyzed by α-amylase and is carried out at approximately 80 °C, while the other, saccharification catalyzed by glucoamylase, is carried out at 60 °C.[28] We have carried out starch hydrolysis with synergistic action of α-amylase and glucoamylase to produce glucose in a one-pot process. Data will be published soon. This process was carried out at a temperature of 65 °C in order to save energy. In addition, both enzymes were found to be more stable at these conditions.

Systems with two enzymes are the most frequently investigated among all multi-enzyme systems and results are frequently reported in the literature. Many efforts are being made to investigate three or more enzyme systems. This is discussed in the next section.

Reactions with three-enzyme systems

Many multi-enzyme reactions run simultaneously side by side in a microbial cell, which makes them highly complex and efficient molecular machines. Enzymes responsible for catalyzing these reactions enable biological processes in all living cells[29] with great efficiency and specificity. By investigating these processes *in vitro* the overall knowledge about metabolism functioning can be increased and used for our own benefit in the production of many chemicals. Enzyme reaction engineering can help in this area in order to make the appropriate processes economically feasible.[16,30,31] Considering that different enzymes require different pH and temperature for their action, the process involving more enzymes becomes more complex. It is therefore necessary to find the optimal process conditions that ensure the environment for enzyme activity. Relevant parameters must be considered: enzyme kinetics, pH and temperature dependence on the enzyme activity, reaction thermodynamics etc. Although the choice of conditions is quite clear with a simple reaction involving just one enzyme, in the case of two or three enzymes, parallel or consecutive reactions coupled via common reactants, dependencies rapidly arise and can no longer be easily grasped.[12] That is why it is practical to develop a mathematical model of the process that enables process optimization and can give insight into process dependencies which can be quite complicated. Model simulations decrease the number of unsuccessful experiments and provide the conditions required to achieve the desired conversion or productivity, without the necessity of excessive experiments. If complex multi-enzyme systems function in nature, there is no particular reason for them not to function *in vitro*.

Three-enzyme systems are covered by many authors.[32,33,34,35,36,37] However, not many of them

study these processes in detail nor do they optimize or model them. Probably one of the first investigations carried out on this three-enzyme system subject was the work done by Srere *et al.*,[33] who investigated an immobilized three-enzyme system consisting of malate dehydrogenase, citrate synthase and lactate dehydrogenase. They tried to mimic the rate of oxaloacetate production and utilization in mitochondria.[34]

Yun and co-workers[35] have investigated a highly complex three-enzyme system in which (R)-1-phenylethanol and (R)-α-methylbenzylamine were produced from a racemic mixture of α-methylbenzylamine. They combined ω-transaminase, alcohol dehydrogenase and glucose dehydrogenase. By using such a system they produced ketone *in situ* from the corresponding amine compound by the action of enantiomeric ω-transaminase in the first reaction, and transformed the ketone into alcohol by the action of alcohol dehydrogenase in the second reaction. Glucose dehydrogenase was used for coenzyme regeneration and to shift the reaction equilibrium towards the wanted product. In this manner, the authors did not have to use organic solvents to dissolve ketone as a substrate for ADH, since its solubility in water is quite low, unlike amines whose water solubility is good. Moreover, the addition of organic solvent often decreases the enzyme activity and stability.[38] A kinetic model of this reaction system was not developed. However, enzyme inhibitions were detected and taken into account. Acetophenone inhibition of ω-transaminase was overcome by the use of three enzymes, since the produced acetophenone is consumed in a subsequent reaction catalyzed by ADH. It is obligatory to examine the presence of substrate of product inhibition of each enzyme in such a complex system, because if inhibition occurs, the reaction might not proceed efficiently,[35] or it might not proceed at all. The reaction was carried out in the batch reactor. 100 mmol dm^{-3} of racemic α-methylbenzylamine was converted to 48 mmol dm^{-3} (R)-1-α-methylbenzylamine and 49 mmol dm^{-3} of (R)-1-phenylethanol, which was very successful.

Three-enzyme systems are used in biosensors for biotechnological process monitoring, such as in the production of alcoholic and non-alcoholic drinks and sweets.[39] Soldatkin and co-workers[36] have developed a first conductometric biosensor for sucrose determination using three-enzyme membrane immobilized onto conductometric transducer, and used as a sensitive element of the biosensor.[36] Since sucrose is a component of food and beverages, its concentration is an important factor for the quality assessment.[36] The enzymes used are invertase, mutarotase and glucose oxidase. In this reaction system sucrose is gradually decomposed by invertase, mutarotase and glucose oxidase to hydrogen peroxide and D-gluconolactone. D-gluconolactone is spontaneously hydrolyzed to gluconic acid which dissociates to the acid residuum and a proton, and the conductivity of the solution is being changed which can be registered by the conductometric transducer.[36] This is a good example of how enzyme cascade reactions can have other applications in addition to biochemical synthesis.

Ishii *et al.*[40] worked on the three-enzyme system presented in Fig. 3. The studied cascade reaction is a part of the glycolysis cycle and it includes the biotransformation of D-glucose to fructose-1,6-diphosphate with glucose-6-phosphate and fructose-6-phosphate as intermediate products. These reactions are catalyzed by the following enzymes: glucokinase, phosphoglucoisomerase and phosphofructokinase. Ishii *et al.* purified three glycolytic enzymes from the *Escherichia coli* and determined their individual kinetic parameters.[40] They also collected the experimental data on metabolites concentration *vs* time under the identical conditions and carried out the dynamic simulation to analyze the results and explain the behaviour of an *in vivo* system. This kind of investigation provided significant insights into the regulation mechanism of metabolic systems.[40]

Schmidt et al.[37] have done research on the production of L-phenylalanine from the racemic mixture of D,L-phenyllactate. They used three enzymes for this synthesis as shown in the reaction scheme

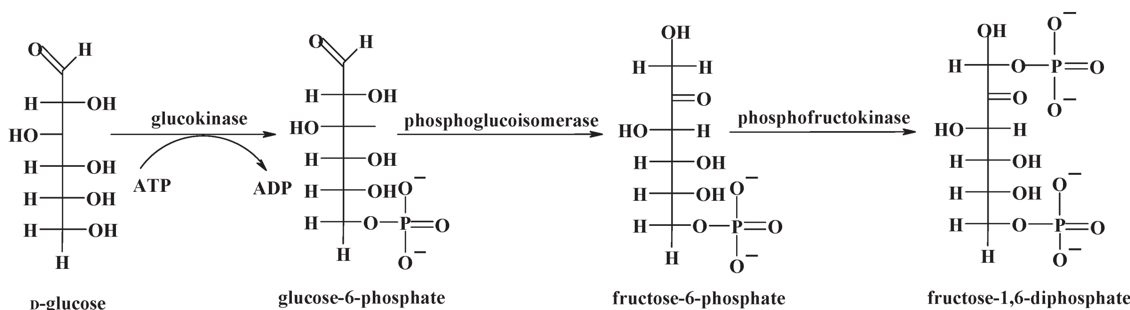
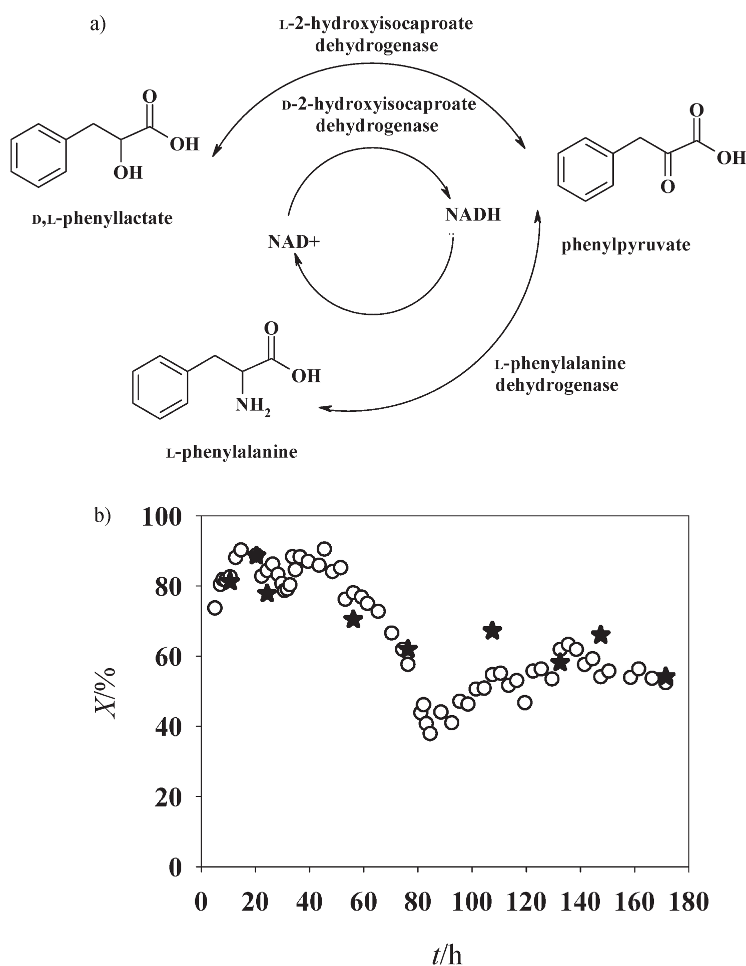


Fig. 3 – *Biotransformation of D-glucose to fructose-1,6-diphosphate in the three-enzyme system*

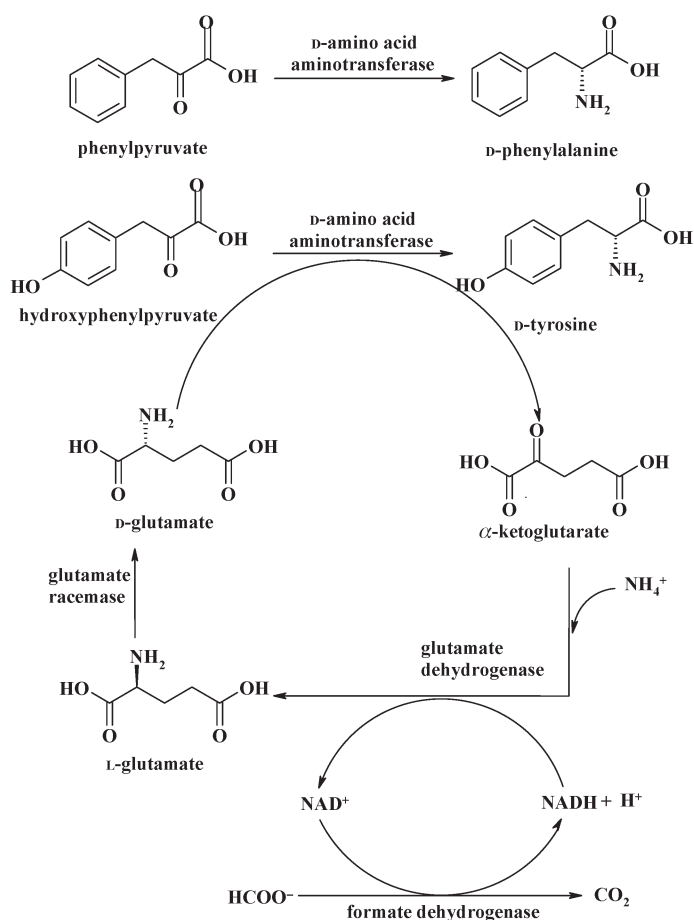
in Fig. 4a. In the first step, racemate is dehydrogenated to phenylpyruvate by two enzymes – D- and L-hydroxyisocaproate dehydrogenase. In the second step, phenylpyruvate is reductively aminated to L-phenylalanine by L-phenylalanine dehydrogenase.[37] Both steps are coenzyme dependent, and since the first step requires NAD and the second NADH, the coenzyme is regenerated in the presented system and requires no additional enzymes for regeneration.[37] The advantage of this research is detailed kinetic analysis and the reactor model which enabled the calculation of the optimum enzyme ratio, coenzyme concentration as well as the phenylpyruvate concentration. This work shows the importance of the mathematical model and process optimization to increase the process productivity and to minimize the influence of different inhibitions that might occur in such complex reaction systems. The limitations of this system were explained by the mathematical model. Phenylpyruvate and NADH are competing between L-phenylalanine dehydrogenase forward and D-hydroxyisocaproate dehydrogenase reverse reaction. The D-phenyllactate inhibits L-phenylalanine dehydrogenase significantly. Due to high Michaelis constant value for PEG-NAD of D-hydroxyisocaproate dehydrogenase, high amount of coenzyme is necessary (0.7 mmol dm^{-3}). The reaction was carried out in the continuously operated enzyme membrane reactor at the residence time of 3 h with a mean conversion of 43 %. The substrate concentration was 50 mmol dm^{-3} D,L-phenyllactate. Experimental results are presented in Fig. 4b.



F i g . 4 – *Biotransformation of D,L-phenyllactate into L-phenylalanine in three-enzyme system. a) Reaction scheme, b) Enzymatic production of L-phenylalanine from the racemic mixture of D,L-phenyllactate (0.1 mol dm^{-3} Tris-HCl, pH 8.5, 25 °C, continuously operated enzyme membrane reactor, $V_{reactor}$ = 10 cm^3, τ = 3 h, $c_{D,L\text{-}phenyllactate}$ = 50 mmol dm^{-3}, c_{NADH} = 0.7 mmol dm^{-3}) (empty circles – measured conversion, black stars – conversion calculated according to the mathematical model).*[37]

Reactions with four-enzyme systems

Bae and co-workers reported the production of aromatic D-amino acids in the multi-enzyme system consisting of the following enzymes: glutamate racemase, D-amino acid aminotransferase, glutamate dehydrogenase and formate dehydrogenase.[41] The system was demonstrated on D-phenylalanine and D-tyrosine production. The reaction scheme is presented in Fig. 5. Each aromatic D-amino acid was produced from the corresponding α-keto acid

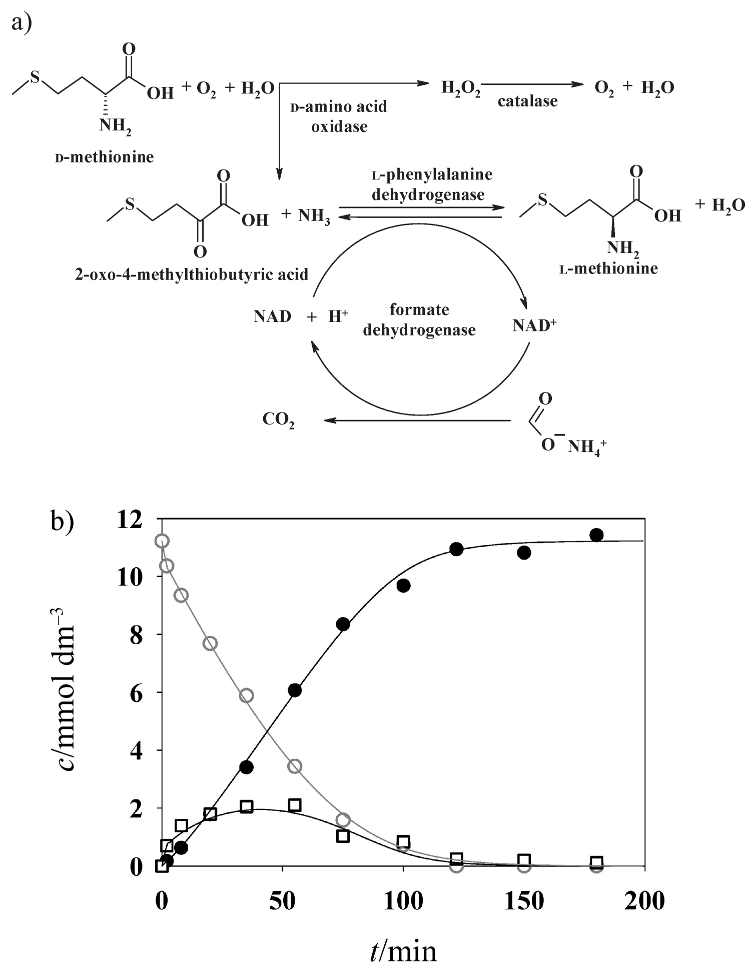


F i g . 5 – *Biosynthesis of aromatic D-amino acids in the four-enzyme system*

by D-amino acid aminotransferase reaction and the consumption of D-glutamate. D-glutamate is continuously regenerated by the coupled reactions of glutamate dehydrogenase and glutamate racemase from α-ketoglutarate, NADH and ammonia. NADH is regenerated by formate dehydrogenase.[41] The experiments were carried out in the batch reactor with a successive feeding of phenylpyruvate. Even though a mathematical model was not developed and the reaction kinetics analyzed, the influence of phenylpyruvate, ammonium formate and NAD on the enzyme activity was examined. Concentrations of phenylpyruvate over 50 mmol dm^{-3} decreased the production of D-phenylalanine. Only low amounts of NAD and formate were necessary for the production of D-amino acids.[41] This example shows an effective method for D-amino acid production without unwanted by-products. A similar four-enzyme system was studied by Galkin *et al.*[42] Their system included the presence of D-amino acid aminotransferase, alanine racemase, L-alanine dehydrogenase and formate dehydrogenase.

Findrik and Vasić-Rački[43] have developed a mathematical model for the biotransformation of D-methionine to L-methionine in the four-enzyme system presented in Fig. 6a. The aim of the corresponding system was to obtain optically pure amino acid from the corresponding racemate without the necessity to separate the α-keto acid which simplifies the procedure. The enzymes used were D-amino acid oxidase, L-phenylalanine dehydrogenase, formate dehydrogenase and catalase. D-methionine was oxidized to 2-oxo-4-methylthiobutyric acid by D-amino acid oxidase. The hydrogen peroxide formed in this reaction was removed by the action of catalase. 2-oxo-4-methylthiobutyric acid was reduced to L-methionine by L-phenylalanine dehydrogenase. Since this enzyme requires coenzyme NADH for the reaction, formate dehydrogenase was used for coenzyme regeneration. Each reaction step was studied in detail and enzyme kinetics was determined. The kinetic modelling enabled the optimization of the initial conditions of the experiment which ensured high conversion of the substrate. 100 % conversion of D-enantiomer to L-enantiomer was accomplished as presented in Fig. 6b.[43] The mathematical model and estimated kinetic parameters enabled different simulations which revealed that high formate concentration should be used due to high K_m value of formate. The first example of such a reaction system was published by Nakajima *et al.* who studied this system on different amino acids as substrates, but the system was not studied in detail.[44]

Schoevaart and co-workers[45] synthesized carbohydrates from glycerol in four enzymatic steps presented in Fig. 7. The first step was the phosphory-



F i g . 6 – *Biotransformation of D-methionine into L-methionine in the four-enzyme system. a) Reaction scheme, b) Conversion of D-methionine to L-methionine in coupled enzyme system (30 °C, 0.2 mol dm^{-3} phosphate buffer, pH 8.0, γ_{DAAO} = 0.0057 mg cm^{-3}, $\gamma_{L\text{-}PheDH}$ = 0.0038 mg cm^{-3}, γ_{FDH} = 1.30 mg cm^{-3}, $\gamma_{catalase}$ = 0.2 mg cm^{-3}, $c_{D\text{-}methionine}$ = 11.22 mmol dm^{-3}, c_{NAD^+} = 0.362 mmol dm^{-3}, c_{NH_4F} = 1000 mmol dm^{-3}). (empty grey circles – D-methionine concentration, empty squares – 2-oxo-4-methylthiobutyric acid concentration, black circles – L-methionine concentration, line – mathematical model).*[43]

lation of glycerol by reaction with pyrophosphate in the presence of phytase. Racemic glycerol-3-phosphate was synthesized. The L-enantiomer of the latter underwent selective aerobic oxidation to dihydroxyacetone phosphate in the presence of glycerol-phosphate oxidase and catalase. Subsequently, fructose-1,6-biphosphate aldolase catalyzed the aldol reaction of dihydroxyacetone phosphate with butanal. Finally, dephosphorylation of the aldol adduct was mediated by phytase resulting with 5-deoxy-5-ethyl-D-xylulose in 57 % yield from L-glycerol-3-phosphate.[7] This alliance of four different enzymes in one-pot cascade of four enzymatic transformations provides an attractive procedure for performing aldol reactions with DHAP aldolases starting from cheap, readily available glycerol and pyrophosphate. In this manner, differ-

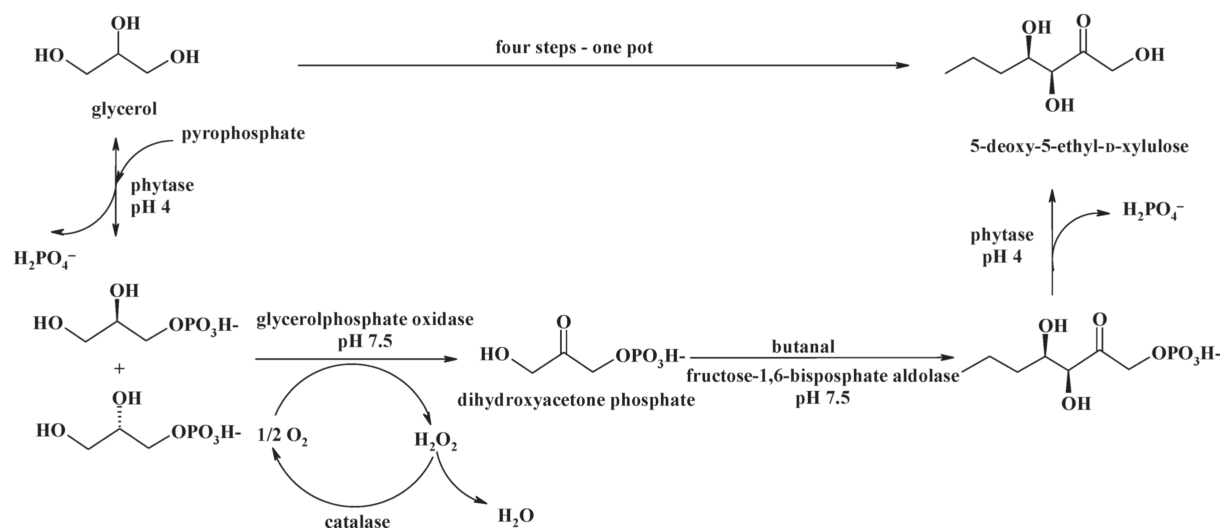


Fig. 7 – *Biotransformation of glycerol into non-natural carbohydrates in the four-enzyme system*

ent carbohydrates can be synthesized from glycerol.[7] A specialty of this system is a pH switch, i.e. pH is changed during the reaction from 4 to 7.5, to suppress the phytase activity, and again to 4 to activate phytase when necessary. This enables total control over phytase activity. Enzyme catalyzed aldol addition is a powerful tool for building carbohydrates derivatives.[45]

Other multi-enzyme systems

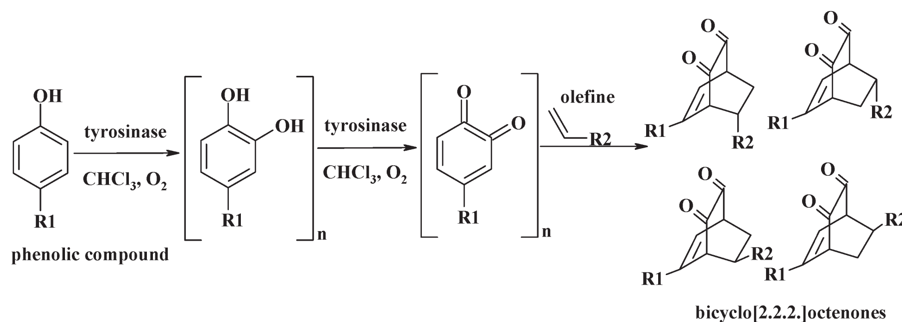
As it can be seen from the previous sections, there are many reported multi-enzyme systems. As the number of enzymes in the process increases, the number of reports about them decreases. These systems are mostly covered from the aspect of biology, biochemistry or organic chemistry, and as a possibility of synthesis. However, from the aspect of biochemical engineering, these systems are unknown and need to be studied in more detail. For example, a group of scientists have studied a one-pot synthesis of riboflavin isotopomers.[46] The overall reaction comprised six enzyme-catalyzed reaction steps for the synthesis of the vitamin and two auxiliary enzymes for in-situ recycling of cofactors. The enzymes used in this pathway are hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, 6-phosphogluconate dehydrogenase, 3,4-dihydroxy-2-butanone 4-phosphate synthase, 6,7-dimethyl-8-ribityllumazine synthase and riboflavin synthase. The overall yields[46] of riboflavin based on isotope-labelled glucose were 35–50 %.

Roessner and Scott have written a review on genetically engineered synthesis of natural products.[47,48] In their report, they covered many examples of one-pot multi-enzyme systems which include carbohydrate synthesis and catabolism, synthesis of porphyrins and corrins, and synthesis of bacterial polyketides. Among those systems the most impressive result is the one-pot 12-enzyme synthesis of hydrogenobyrinic acid, an advanced cobalt-free corrin precursor of cobalmin, accomplished by Roessner and co-workers. This synthesis consisted of 17 steps, and the overall yield was 20 %. Even though the overall yield is not very high, the results are impressive if the number of steps is considered. Such syntheses demonstrate the power of a heterologous multienzyme system as an aid in resolving the functions of uncharacterized enzymes in a multi-step pathway with unstable intermediates.[47]

Reactions with chemoenzymatic systems

The first successful combination of chemoenzymatic cascade was reported by Müller and co-workers.[8] The reaction scheme in Fig. 8 presents the transformation of phenols into bicycle[2.2.2]octenones in three steps. The first step is hydroxylation of phenols to catehols. This intermediate is immediately oxidized to form coloured *ortho*-quinones in the second step. Both of these reactions are catalyzed by tyrosinase and in the presence of oxygen. As *ortho*-quinones are highly reactive, they subsequently undergo Diels-Adler reaction in the presence of dienophiles forming the bicycle cycloaddition products[8] (bicyclic α-hydroxy ketones) in yields up to 94 %. These compounds can be useful intermediates for further transformations. The reaction was carried out in the batch reactor; kinetics investigations were not done.

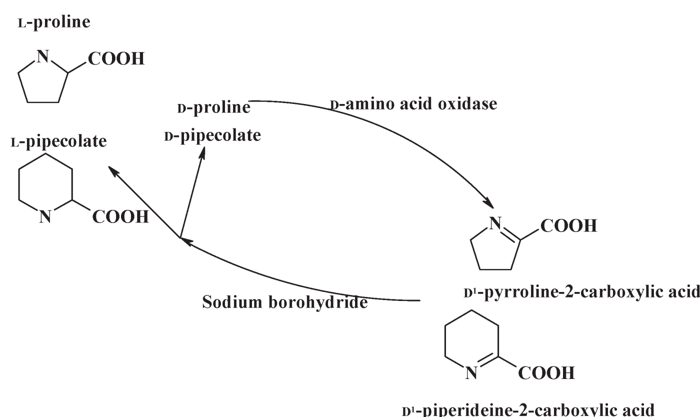
The second example of chemoenzymatic synthesis presented here was reported by Yun and co-workers.[35] They synthesized the derivatives of CMP-sialic acid in a one-pot two-enzyme system.



F i g . 8 – *Enzyme-initiated chemo-enzymatic synthesis of bicycle[2.2.2]octenones*

ManNAc was generated *in situ* from GlcNAc in the reversible chemical epimerization, and then reacted with pyruvate and CTP in the one-pot enzyme reaction, containing aldolase and CMP-sialic acid synthetase to produce activated Neu5Ac. The yields in this system[35] were low – up to 15 %. Neu5Ac is an important pharmaceutical compound which is used as a starting reagent for the production of pharmaceuticals.[49]

Soda and co-workers reported the first one-pot chemo-enzymatic conversion of racemic compounds (amino acids) to their enantiomers (L-amino acids) through an enantiospecific oxidation of the starting material and non-enantiospecific reduction of the achiral intermediate.[50] The reaction scheme is presented in Fig. 9. D-amino acids are enantiospecifically oxidized to form an achiral heterocyclic compound that is simultaneously reduced to form D,L-amino acid – one-pot coupling of these reactions results in enantiomerization with more than 98 % yield. Such a method combining enzyme oxidation and chemical reduction is applicable for other substrates and compounds, as described in the paper of Soda *et al*. The advantage of this method over possible enzyme reaction is the fact that different enzymes have different pH optima, and sometimes it is not advantageous to carry out the enzymatic process.



F i g . 9 – *Chemo-enzymatic synthesis of L-amino acids from their racemates by a coupled reaction of D-amino acid oxidase and sodium borohydride*

The application of chemo-enzymatic catalysis has great prospect in the chemical industry, as well as in the pharmaceutical industry, where waste generation can be higher than 100 kg kg^{-1} product.[6,51] Other examples of chemo-enzymatic syntheses can be found in the literature.[5,52,53,54]

Integration of catalysis and biocatalysis will progress in the future, and will broaden the scope of biocatalysts applications.[55]

Conclusion

Although much work is being done on the development of multi-step catalysis, there is still much to do. This area represents a vast field of interest due to attractive yields in these processes. Since more catalytic steps imply more complex dependencies between the process variables, it is necessary to use mathematical models and simulations as a tool for process analysis. These can also be used for the improvement and optimization of the process.

ACKNOWLEDGEMENTS

This work was supported by the Croatian Ministry of Science, Education and Sports (Contract Grant Number 125-1252086-2793).

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