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BIOCATALYSTS – PRODUCTION AND APPLICATION

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ABSTRACT

A short review of the stages of enzymology development is presented in the paper. The processes for producing enzymes with different purity are presented schematically. The possibilities for their application in different arias of industry are considered. Particular examples with the enzymes pectinesterase, cyclodextrin glucanotransferase, lipase, etc. are presented.

Keywords: biocatalysts, production, application, cyclodextrin glucanotransferase, lipase

Two centuries from the discovery of the first enzyme diastase (amylase) in 1814 by the russian scientist Konstantin Sigimundovich Kirhoff will be fulfilled in several years [11]. His discovery was a result from the research on the problem with the sugar insufficiency in Russia in the beginning of the nineteenth century, which occurred as a result of the slaves rising in the sugar plantations and Napoleon blockade. The progress in the research in the biocatalysis processes raised the questions if the biocatalysis reactions are a variety of the chemical reactions, if the chemical rules are valid, if the chemical methods are suitable for investigation of the biochemical reactions.

The answering of these questions has taken more than a century and was connected with plenty of methodological difficulties. The history of the enzymology can be divided into the following stages:

- Production of the first enzyme preparations (1814-1860);
- Beginning of the simultaneous investigation on the biocatalysts and the biochemical processes (1860-1890);
- Research on the specificity of the enzyme action (1894-1920) [8];
- Isolation, purification; and determination of the enzymes composition and their action mechanism (1920-) [23,29];

- Investigation on cofactors, development of the concept for the enzyme two components nature and for the cofactoring functions vitamins (1920-1940);
- Application of the enzymes in different spheres of industry (1910-) [1,26];
- Preparation and application of immobilized enzymes (1914-) [10,12,28,30,].

The discoverer of the enzymes Konstantin Kirhoff could hardly imagine that he put the beginning of a whole field of science, which would allow processes intensifications, their performance at extremely mild conditions, environment protection, production of ecology clean and high quality products, performance of high precise analysis, etc.

When preparing this review, I have established that biocatalysis was a subject of a great deal of investigations. Over 40000 titles appeared only in the period 2001-2006. Summarizing this scientific wealthy is a rather complex task. Without having any claims for comprehensiveness and thoroughness**,** I will try to present a part of the achievements in this area in combination with some of the researches in the department, where I work, focusing on the biocatalysis application. Prior to the discussion of the theme, I will accentuate on the production of enzyme preparations [4,13].

The scheme on Figure 1 gives a brief summary of the production of different types of enzyme preparations. The first stage is the choice of an appropriate enzyme source. There are three possibilities: plant, animal, and microbial sources. The first two sources are limited, since the raw material is insufficient (for the animal enzymes), and the content of the enzyme in the plant materials is rather low, which necessitates processing of a great amount of raw material at low yield. Basic source for enzyme production is microorganisms, which possess the following advantages:

- A great number of enzymes with various properties;
- Possibilities for application of genetic methods, which leads to yield increase of the enzyme produced [25];
- High growth rate of the cells;
- Secretion of the enzymes in the culture liquid, which enables easy isolation.

Depending on the application, enzymes preparations with different degree of purity can be produced, which affects their final price. The most simple scheme is the production of crude enzymes (variant I). More complicated is the scheme for production of purified enzymes (variant II and III), and the most complicated method is for the homogenic ones (variant IV) [9].

The possibilities for enzymes applications are comprehensive (Figure 2). The broadest application is in the food and flavor industry. The most widely used sweet substance in every day life is sugar, which is produced from sugar beet and sugar-cane. However, corn could be also used for raw material. “Liquid sugar” with 60 % higher effectiveness in comparison to the traditional sources could be produced from corn starch by the means of several enzymes: α-amylase, glucoamylase, glucoseisomerase, pulullanase, etc [18]. The world production of this product is about 30-35 % in comparison to the total amount of sugar consumed [19,24].

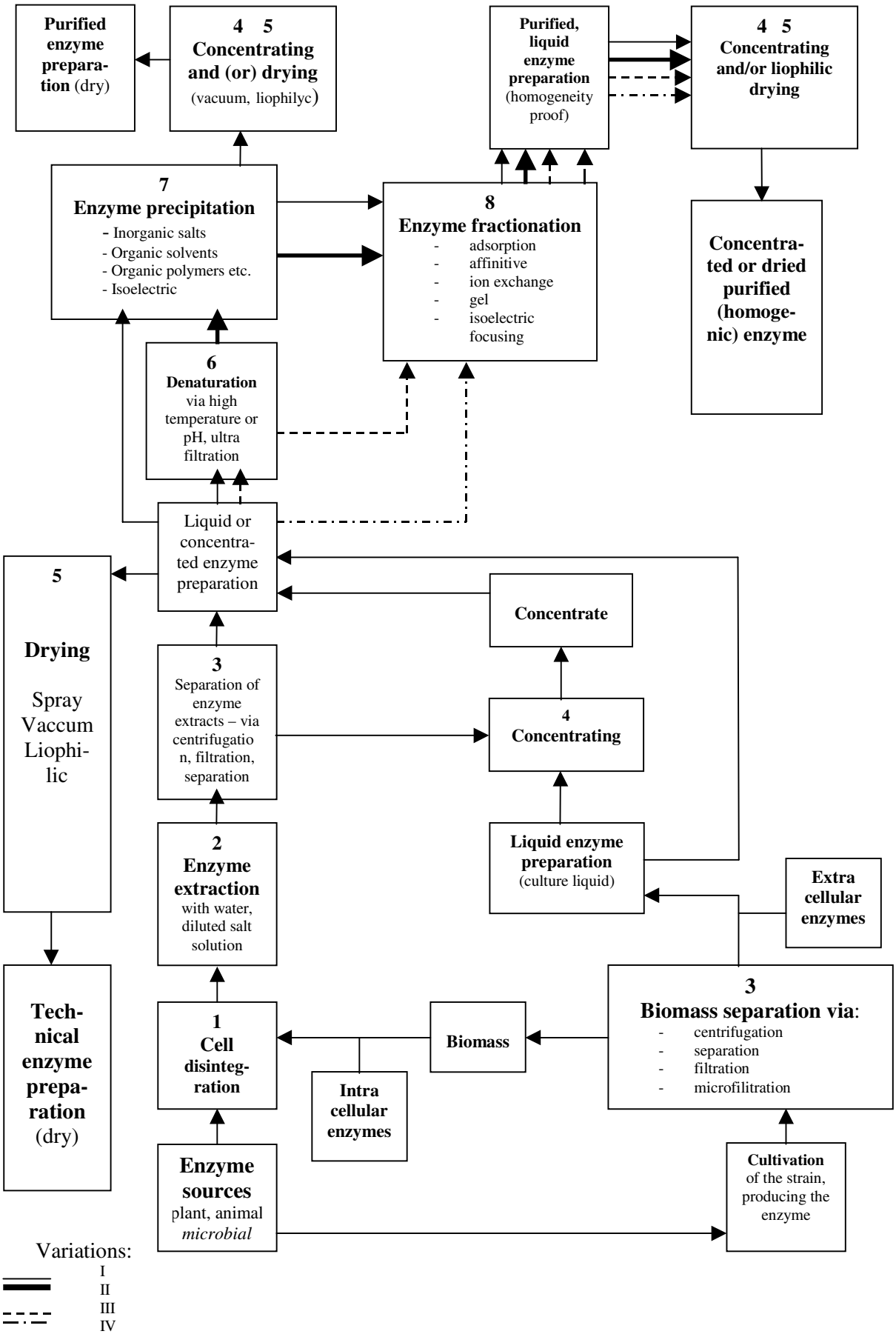

Figure 1. *Scheme for isolation and purification of enzyme preparation*

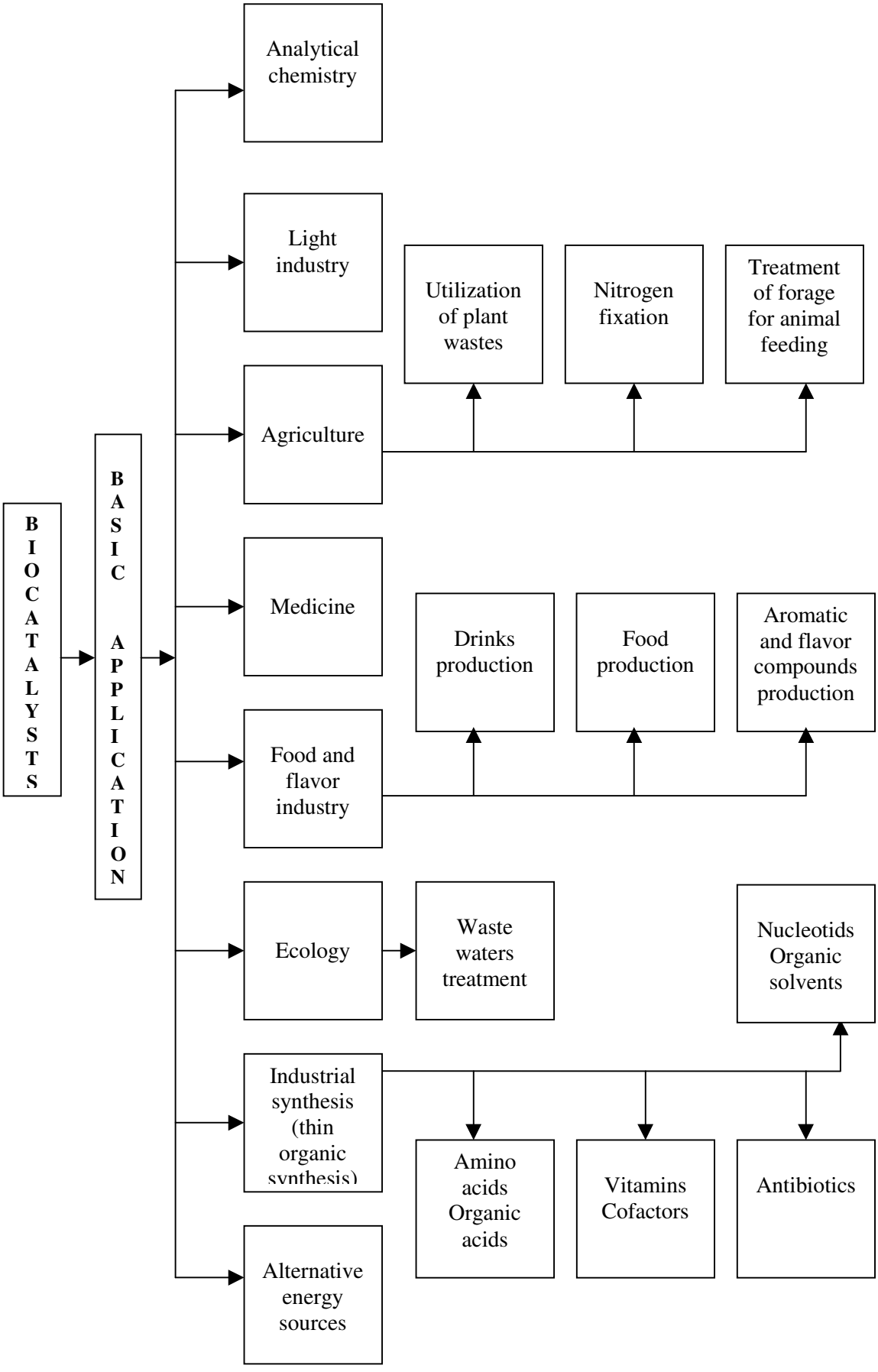

Figure 2. *Enzyme applications*

The liquid sugar is preferred for many food products. In our country this production is performed by Amylum-Razgrad. The glucose-fructose syrup is only a part of their products. Liquid and dry glucose, high inverted syrups, suitable for brewery and alcoholic industry, are also produced by the means of enzyme hydrolysis. The advantages of these technologies are as following: environment friendly, complete utilization of the raw material, high conversion degree of starch (over 95 %). For these reasons amylases comprise about 30 % of the commercially produced enzymes [24]. Strains have been selected, which produce thermostable α-amylases, useful at starch claysterization (100-110°C) and liquefaction (80-90°C) [2,5,6,18,20,21,27].

The taste of the dairy products (different cheese) is well known for everybody. Their production is an example of fine biocatalysis action. It is enough only one peptide bond from the stable casein molecule from the milk to be degraded in order to unstabilize it. The milk protein begins to precipitate, the precipitate formed aggregates gradually, and the valuable milk products are formed as a result of additional biochemical processes. The tool for this transformation is the enzyme rennin. In order to answer the increased demand of the dairy products, it was necessary to find a substitute of the naturally produced enzyme from young animals. Such microbial enzymes have been already produced and their properties are very close to the natural enzyme, which guaranties a high quality of the dairy products.

Bread is a food product, which we use every day. The production of high quality bread requires a great skill of the bakers. Nowadays, science is helping them. Different additives, for the bread industry have been made. They allow improvement of the bread consistence, and aroma. These additives constitute of enzymes such as α-amylase, xylanase, and glucoamylase [3,7]. Their action enables formation of greater amount fermentable sugars, higher content of CO_2, which is hold back in the dough, leading to increased bread volume, and improved texture. Together, the Meyer reaction is accelerated, and the content of the aroma substances is increased.

The examples could be proceeded, but I will focus on the ones, which are a subject of research in the department of Biochemistry and molecular biology, UFT.

The first one is connected with the enzyme pectinesterase. It hidrolyses the ester bonds of methanol in the pectin molecule, reducing the esterification degree. As a result moderate or low esterificated pectins are formed. They have altered properties and are valuable additives for pectin containing products, improving their nutrient and biological characteristic. The enzyme pectinesterase is not produced pure. It is in a complex with other pectolytic enzymes, which degrade pectin molecule. The separation of this complex is almost impossible procedure, even using chromatographic methods. We have succeeded to work out an easy method, connected with the change of pH of the enzyme solution, resulting in an isolation of a pure pectinesterase [14]. This allowed us to perform a controllable biochemical process for deesterification of pectin at mild conditions and to produce pectins with desirable esterification degree [15,16,17]. The production of this enzyme is performed in the enzyme factory in Botevgrad. Unfortunately pectin production in our country is at standstill in recent years, and the use of the enzyme is stopped.

The other example is connected with the enzyme cyclodextrin glucanotransferase, which is produced only by microorganisms, mainly bacteria. Extremely valuable compounds – cyclodextrins (CD) are formed by the means of the enzyme. They are starch derivatives, cycling molecules consisting of 6, 7 or 8 glucose units, called respectively α-, β-, and γ-CD (Figure 3).

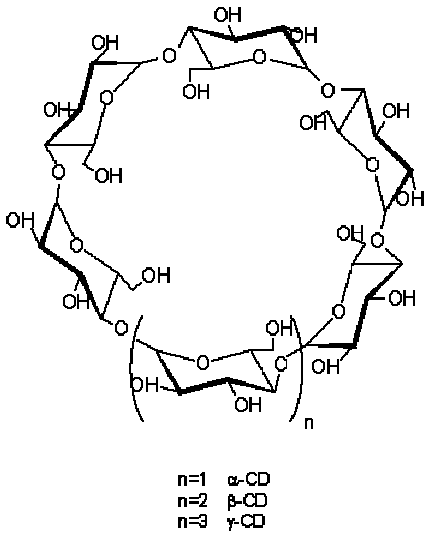

Figure 3. *Cyclodextrin view*

In their molecule a cavity is formed, in which different compounds – vitamins, aroma substances, lipids, drug formulations, pesticides, microelements can be situated. As a result complexes are formed, in which the guest molecules are with increased stability towards hydrolysis, oxidation, dehidratation, evaporation, thermal treatment, etc. The complexes gain valuable specific properties, which enable the nutrition and biological properties of food products to be preserved for a long time, the pharmaceutical and pharmacological characteristics of drugs to be improved by prolonged action of the active compound. For these reasons CD are applied in food and flavor industry, medicine, biotechnology, cosmetics, agriculture, etc.

One of our projects is about biosynthesis and characterization of this enzyme. The optimal nutrient medium composition and cultivation conditions for maximal enzyme amount have been established. Some of its properties were also determined (Figure 4).

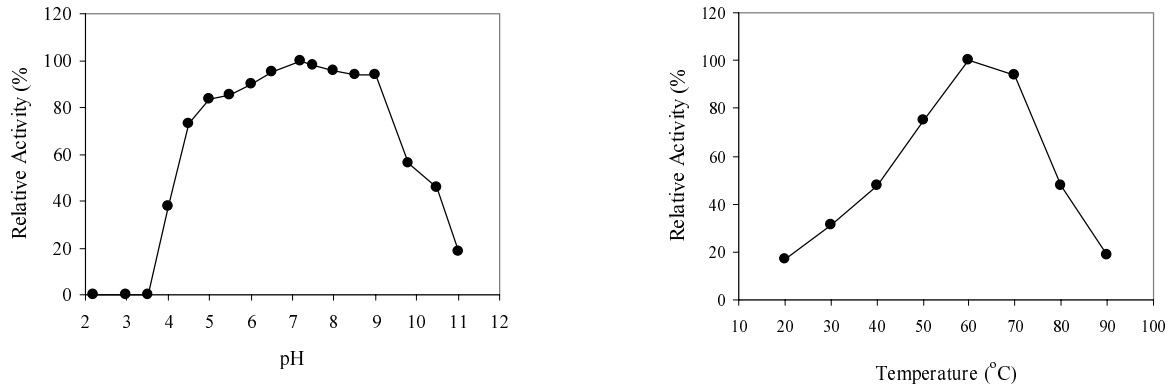

Figure 4. *Effect of pH and temperature on purified CGTase activity (pH 7.5)*

The possibilities for CD production with the isolated enzyme have been investigated in another research. Interesting results have been registered, which presume high starch conversion degree in CD (Table 1).

Table 1. *Effect of reaction time on CD yield*

№	Time, h	CGTase 1 U/g		CGTase 2 U/g			CGTase 4 U/g		
		YIELD*, %							
		With shaking	Without shaking	With shaking	Without shaking		With shaking	Without shaking	
		With toluene		With toluene		Without toluene	With toluene		Without toluene
1	1	14.75	12.62	18.20	20.70	16.84	18.68	28.41	27.32
2	2	21.93	21.26	22.27	28.55	24.85	26.60	42.77	33.96
3	3	25.32	23.62	27.65	35.77	26.07	43.45	54.97	35.72
4	4	37.00	33.70	40.30	44.60	29.79	53.63	56.90	35.96
5	5	40.62	34.72	46.71	51.20	31.29	57.90	59.47	38.60
6	8	42.68	40.28	51.94	56.90	31.43	63.41	65.01	39.07
7	24	48.80	49.28	56.31	59.90	33.82	69.50	71.91	40.97
8	24**	41.62	46.85	45.75	50.43	29.56	51.05	53.92	30.02

5 % starch solution; 40°C; pH – 6.0; * Reaction mixture is used for analysis; ** Analysis of isolated CD

The investigations in the last decade have shown that enzymes and whole cells can be successfully used in the organic synthesis. The enzymes are most often applied in hydrolysis and isomerisation reactions, and the whole cells are used in synthesis reactions, in which the cofactors should be regenerated. This is easier in vivo than in vitro.

One of enzymes advantages is their extreme specificity, selectivity. It enables their use as valuable catalysts in the production of biologically active compounds with chyral centers or position isomers. They allow simple or complicated transformations to be performed without the use of blocking and deblocking steps, characteristical for the organic synthesis. Another advantage of the enzymes is that by-products are not formed.

Some of the products produced enzymaticaly at several well known chemical companies are presented in Table 2 [22].

The use of the enzymes in organic synthesis has become possible as a result of the explanation of the enzymes action mechanism. As an example I will consider the enzyme lipase. It degrades the ester bonds between the fatty acids and alcohols or polyalcohols. The group of lipases consists of a great number of enzymes, which catalyses different ester bonds, i. e. they do not have a high specificity. The active site of lipases contains the amino acids serine, histidine, aspartic or glutamic acid. The hydrolysis of the ester bonds involves formation of acid-enzyme complex (Figure 5). The biocatalysis process begins with nucleophilic attack of serine OH group towards the carbon atom of COOH group of the ester bond. The complex is degraded via nucleophilic attack of water, the fatty acid is released and the enzyme is restored.

Table 2. *Biocatalytic systems at several chemical companies*

Product	Substrate	Reaction	Enzyme	Scale, ton/year	Yield	Source
Amides, alcohols, acids						
Enantiopure alcohols	Racemic alcohols	Resolution	Lipases	Thousand	Excellent	BASF
R-amide; S-amine	Racemic amines	Resolution	Lipases	Hundreds	Excellent	BASF
R-mandelic acid	Racemic mandelonitrile	Hydrolysis	Nitrilases	Several	Excellent	BASF
Amino acids, penicillins						
Non-proteinogenic L-amino acids	Racemic aminoacid amides	Kinetic resolution	Amidases	Several hundred		DSM
L-Aspartic acid	Fumaric acid	Addition of ammonia	Aspartic acid liase	Thousand		DSM
Aspartame	L-Aspartic acid phenylalanine Methyl ester	Selective coupling	Thermo-lisine	Thousand		DSM
6-APA	Penicillin G/V	Hydrolysis	Penicillin acylase	Thousand		DSM
Semisynthetic penicillins	6-APA	Selective coupling	Acylases	Several hundred		DSM
N-heterocyclic compounds						
6-Hydroxy nicotinic acid	Niacin	Addition of water	Niacin xydroxylase	Few	65 g/l	Lonza
6-Hydroxypirazine carboxylic acid	2-Cyanopyrazine	Addition of water	Nitrilase/ xydroxylase	Development product	40 g/l	Lonza
6-Hydroxy-5-nicotine	5-Nicotine	Addition of water	xydroxylase	Development product	30 g/l	Lonza

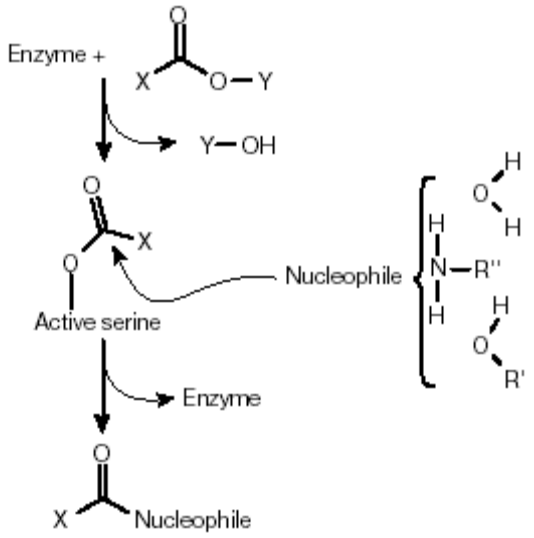

Figure 5. *Reaction mechanism of lipase biocatalysis*

As lipases are also active in organic solvents, water can be substituted by another nucleophile, alcohols for example. As a result preesterification or transesterification proceeds. Only the one isomer (enantiomer) from racemic mixtures can be acylated, which leads to selective transformation. Suitable acidic donors are

vynile esters, anhydrides or diketones. The reaction is non convertible and the unused alcohol and the ester produced are easily separated. This approach is used for production of pure enantiomeric alcohols. Amines could be also nucleophiles, which allows racemic amines to be separated. Characteristic for this approach is the high yield, selectivity and the minimal enzyme amount needed.

These are only a small part of the enzyme processes applied in the production of organic compounds, including biologically active substances. Their amounts are in the range of hundreds tons.

REFERENCE

1. Boyce, C. 1984. Developing a new industrial enzyme application: A strategy. J Oil Chem Soc 61, 1750-1753.
2. Canganella, F., Andrade, C., Antranikian, G., 1994. Characterization of amylolyticand pullulytic enzymes from thermophilic archaea and from a new Ferividobacterium species. Appl. Microb. Biotechnol. 42, 239–245.
3. Courtin, C.M. and Delcour, J.A. 2002. Arabinoxylans and endoxylanases in wheat flour bread-making, J.Cer. Sci., 35, 225-243.
4. Cowan, D. 1996. Industrial enzyme technology. Trends Biotechnol. 14 (6), 177-178.
5. Crab, W., Mitchinson, C. 1997. Enzymes involved in the processing of starch to sugars. Trends Biotechnol. 15, 349-352.
6. Demirijan, D., Moris-Varas, F., Cassidy, C. 2001. Enzymes from extremophiles. Curr. Opin. Chem. Bull. 5, 144–151.
7. Durton, A. Georis, J., Genot, B., Dauvrin, T., Collins, T.,Hoyox, A., and Feller, G. Use family 8 enzymes with xylanolytic activity in baking. Patent number WO 2004023879 A1, 2004.
8. Fischer, E. 1909. Untersuchungen uber Kohlenhydrate und Ferments, Bd.I. 1884-1909, Berlin.
9. Gessesse, A. 1998. Purification and properties of two thermostable alkaline xylanases from an alkaliphilic Bacillus sp. Appl. Environ. Microbiol. 64, 3533–3535.
10. Groboillot, A. 1994. Immobilization of cells for application in the food industry. Crit. Rev. Biotechnol. 14, 75–107.
11. Kirchhoff, G.S.C. 1815. Uber die Zuckerbildung beim Malzen des Getreides und beim Bebruhem seines Mehls mit kochendem Wasser. Schweigger.-J.Chem.Phys., 14, 389-398.
12. Krastanov, A. 2000. Removal of phenols from mixtures by co-immobilized laccase/tyrosinase and Polyclar adsorption, J. Ind. Microbiol. Biotech., 24, 1-6.
13. Leuschner, C., Antranikan, G. 1995. Heat stable enzymes from extremely thermophilicand hyperthermophilicmic microorganisms. World J. Microbiol. Biotechnol. 11, 95–114.
14. Ljutzkanov, N., Pishtiyski, I. Method for production of enzyme preparations of pectinesterase. Avtorsko svidetelstvo N 29659/2.06.1981.

15. Ljutzkanov, N., Pishtiyski, I., Koleva, L., Tziporkov, N. Method for deesterification of pectin. Avtorsko svidetelstvo N 38239/01.06.1984.
16. Ljutzkanov, N., Pistijsky I., Influence of pH on deesterification of pectin by microbial pectinesterase, Travaux scientifiques, HIFFI, XXXIII, I, 1986.
17. Pistijsky, I., Ljutzkanov, N., Koleva, L. 1986. Influence of some factors on pectin deesterification by means of microbial pectinesterase, Travaux scientifiques, HIFFI, XXXIII, I.
18. Poonam, N., Dalel, S. 1995. Enzyme and microbial systems involved in starch processing. Enzyme Microb. Technol. 17, 770–778.
19. Rakshit, S.K. 1998. Utilization of starch industry wastes. In: Martin, A. (Ed.), Bioconversion of Waste Materials to Industrial Products, second ed. Blackie Academic and Professional, London.
20. Rudiger, A., Jorgensen, P., Antranikan, G. 1995. Isolation and characterization of a heat stable pullulanase from the hyperthermophilic archeon Pyrococcus woesei after cloning and expression of its gene in Escherichia coli. Appl. Environm. Microbiol. 61, 567–575.
21. Sarikaya, E., Higassa, T., Adachi, M., Mikami, B. 2000. Comparison of degradation abilities of α- and β-amylases on raw starch granules. Proc. Biochem. 35, 711–715.
22. Schmid, A., Dordick, J.S., Hauer, B., Kieners, A., Wubbolts, M., Witholt, B. 2001. Industrial biocatalysis today and tomorrow, Nature, vol.409, 258-268.
23. Vakhlu, J., Kour, A. 2006. Yeast lipases: enzyme purification, biochemical properties and gene cloning, Electronic J Biotechnol. 9 (1), January 15.
24. Van der Maarel, M., Van der Veen, B., Uitdehaag, H., Leemhuis, H., Dijkhuizen, L. 2002. Properties and applications of starchconverting enzymes of the α-amylase family. J. Biotechnol. 94, 137–155.
25. Van der Veen, B., Uitdehaag, J., Penninga, D., Van Alebeek, G., Smith, L., Dijkstra, B., Dijkhuizen, L. 2000. Rational design of cyclodextrin glycosyltransferase from Bacillus circulans strain 251 to increase α-cyclodextrin production, J. Mol. Biol. 296, 1027-1038.
26. Vellard, M. 2003. The enzymes as drug: application of enzymes as pharmaceuticals. Curr Opin Biotechnol 14, 1-7.
27. Viara, N., Elena, P., Elka, I., 1993. Purification and characterization of a thermostable alpha-amylase from *Bacillus licheniformis*. J. Biotechnol. 28, 277–289.
28. Wang, P., Sergeeva, M.V., Lim, L., Dordick, I.S. Biocataliticplasticsasa active and stable materials for biotransformations. Nature Biotechnol. 15, 789-793.
29. Willstatter, R., Bamann, E. Uber die Proteasen der Magenschleihaut. I. Uber Enzyme der Leukozyten. Z physiol.Chem., 180, 127, 1928-1929.
30. Yang, Z. et al. 1995. Activity and stability enzymes incorporated into acrylic polymers. I.Am.Chem.Soc.117, 4843-4850.