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Biochemistry - Knowledge to Practical Applications - Examples

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ABSTRACT

Biochemistry is a branch of science trying to explain the different and constantly emerging, new aspects of life. It tries to elucidate our knowledge on those subjects from highly basic, yet very complicated level by dividing the complex problems into smaller and less difficult ones and working them out one by one. However one has to remember that there is no such thing in biochemistry as a "simple problem", because no matter how basic the encountered difficulty seems, it is always correlated with and dependent on another one. This is why those interactions are of great significance, because by working and acting together they make life as we see it. Cells in a living organism are good example of such interactions. Once they were said to be the building blocks of life, then scientists discovered and described their "insides" and a whole new chapter of protein, amino acid and nucleic acid biochemistry was born. However the greatest part of the knowledge broadening has to be that eventually people become capable of harnessing natural processes and making them work for their advantage. For instance amino acids, once only building blocks of life, now also employed in the food industry as flavor enhancements, another

good example are the enzymes that work as various kinds of therapeutic agents and are also applied as detergent additives. The lipid molecules are getting a lot of attention as well, due to their ability to work as fat substitutes or may be even more importantly as possible drug carriers. It is also very interesting to see how carbohydrates enable the development of different sectors of food industry as they constantly find applications as various types of sweetening syrups and ingredients. Nucleic acid molecules have to be mentioned as well, because indirectly thanks to Watson and Crick for describing the structure of DNA, the scientists are now able to apply that knowledge for example in the development of aptamer-based biosensing devices that are invaluable tools in the field of bioanalysis and biomedicine. Taking all into the account, this chapter is an attempt to introduce and interest the reader with the world of basic biochemical molecules and their current applications in life.

Keywords: Biochemistry; Amino acids; Proteins; Enzymes; Lipids; Carbohydrates; Nucleic acids

AMINO ACIDS AND PROTEINS

L- Amino Acids Production (Asp, Lys and Glu)- Food and Feeding Additives

Amino acids are the components of all peptides and proteins in the cells and represent basic building blocks of life. High content of nitrogen (~16%) distinguishes these compounds from fats and carbohydrates [1]. All natural amino acids may be classified into two categories: essential and nonessential. The nonessential amino acids can be synthesized in the body as opposed to essential ones which cannot be synthesized and they must be provided with food. Amino acids apart from the obvious role of building blocks can serve as nutrients, additives, and drugs in the food, feed and pharmaceutical industries. During the last decades the global use of some essential amino acids (e.g.*L*-lysine) significantly increased. The use of lysine as a feed additive optimizes the growth of farm animals and additionally improves the quality and quantity of meat. *L*-glutamic acid can serve an example of nonessential amino acid of high importance and application, it is used in food industry as the most known flavor-enhancing agent. The increasing year-on-year demand of different amino acids results in development of new, effective technologies. As opposed to traditional chemical methods the fermentation is an effective biotechnological tool that serves as the most cost effective process [2,3]. The utilization of microorganisms for food manufacturing was known for centuries, but without the knowledge about the nature of this process. The word "fermentation" was introduced by Pasteur in 19th century and since then a large number of processes using bacteria and fungi were developed [2,4,5]. With advanced knowledge of microbial physiology, molecular biology and genetic engineering, the fermentation has become one of the most efficient production methods that allowed obtaining different compounds. Many of the older methods of amino acids production such as an extraction from natural sources (e.g. hairs) and chemical synthesis, were replaced by biotechnological processes such as enzymatic catalysis and fermentation. Traditionally, amino acid-producing microorganisms were obtained by multistep

random mutagenesis and directed screening but such biocatalysts were genetically unstable and the process was time-consuming, because of the presence of large amount of unwanted mutants [6]. In the last few years targeted metabolic and genetic engineering were used to create the effective producers of amino acids and other products [7]. Generally, the biosynthetic pathway of amino acids synthesis is closely correlated with central metabolism. The best examples of the fermentative production of amino acids are *L*-lysine and *L*-glutamic acid (Figure 1).The *L*-glutamic acid is the most important amino acid due to its commercial application in the food industry. It is used as a carboxylate ion and salt (e.g. monosodium glutamate-MSG) and is known as glutamate and used as a flavor enhancer and has its own unique flavor called ''Umami''. The production of glutamic acid and its salts reaches 2.2 million tons per year [8]. Major producers of MSG are Ajinomoto, Miwon, Kyowa-Hakko and Cheil-Jedang. The biosynthesis of *L*-glutamic acid using *Corynebacterium glutamicum*- the most useful microorganism in fermentative production of amino acids, is quite simple and involves main glycolytic pathway. In this process glucose is converted through glycolysis and citric acid cycle to α- ketoglutarate which is finally transformed to *L*-glutamic acid. For efficient, industrial production of this amino acid, it is necessary to introduce some modifications of the microbial cell or/and cultivation conditions. The first is low content of biotin in medium. Deficiency of this vitamin implies the increasing of the permeability of cell membrane because of impaired fatty acids biosynthesis. This enables easy release of product into the medium. The effective removal of the product from the cell is crucial for production of large amount of the amino acid.

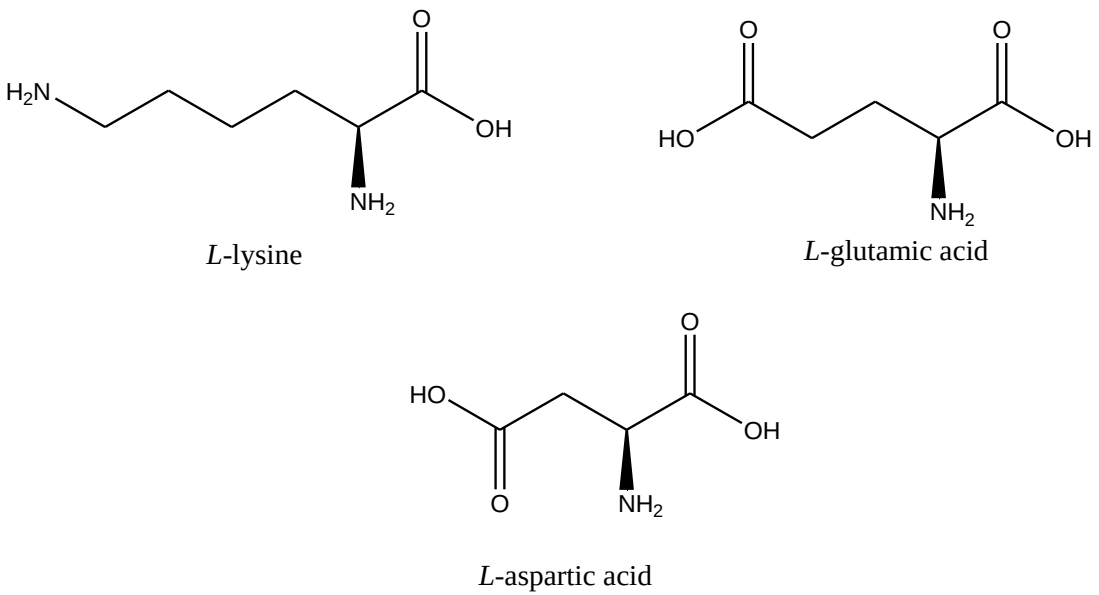

Figure 1: Examples of structure of amino acids produced on industrial scale.

The same effect - facilitated *L*-Glu releasing - can be achieved by using antibiotics (e.g. penicillin) or surfactants (e.g. Twen 40). Additionally, genetic modifications performed to increase the activity of the α-ketoglutarate dehydrogenase result in obtaining of microorganisms suitable for industrial applications. The use of raw materials as a production medium is essential for economy of the process. Especially the carbon source is a major part of variable production costs. Depending on the geographical location, the plant carbon sources like cane molasses, beet molasses, or starch hydrolysates from corn, potato or cassava are applied [9].

The application of *C. glutamicum* as an efficient producer of glutamic acid stimulates the development of other microbiologically-driven synthesis of the next amino acid: *L*-lysine. It is important because of the deficiency of this compound in the animal feed (based on corn, wheat or barley) and often in the human food. The world market for lysine was 1,697 kilo tons in 2011 and is expected to reach 2,518 kilo tons by 2018 (around $5.9 billion by 2018). The major producers of *L*-lysine are Ajinmoto (Japan, France, Italy, Brazil etc.), Archer Daniels Midlands (USA), and Chinese industries: Changchun Dacheng, Cheil Jedang, Global Biochem Technology and COFCO Biochemical. The industrial production of *L*-lysine is carried out in a similar manner to *L*-glutamate production and sugar cane or beet molasses are used as the substrates for its receiving. The *C. glutamicum* has two pathways for the biosynthesis of lysine [10,11] which give the flexibility in response to different environmental conditions. The aspartokinase (EC 2.7.2.4) plays the key role in the lysine biosynthesis, catalyzing the formation of aspartyl phosphate from aspartate and its activity is under thefeedback regulation by formed lysine and threonine [12]. The modifications of aspartokinase, dihydrodipicolinate synthase (DapA, EC 4.2.1.52) and the lysine exporter **(LysE)** were crucial for improvement of lysine production [13]. For the effectiveness of the process proper aeration and the molar ratio of oxygen to carbon dioxide are essential as well as supplementation with biotin and vitamin B1. Biotin is involved in the oxidation of glucose and in the synthesis of proteins as well as regulation of the cell permeability [14]. Furthermore, enzymes containing biotin provide aspartic acid - an intermediate in the synthesis of lysine [15]. Vitamin B1 is needed for oxidative decarboxylation of pyruvate and α-ketoglutarate and also for the increase of the formation of acetyl-CoA and oxaloacetic acid, what improves production of *L*-lysine. On the industrial scale submerged fermentation is a highly effective and economical method and *L*-lysine is produced as a crystalline product **(*L*-Lys x HCl)** and for feed industry as a granulate Biolys 60.

Some of the useful amino acids are produced using isolated enzymes rather than fermentation technique e.g. *L*-aspartic acid. As a dietary supplement, aspartate is combined with minerals (copper, iron, magnesium, manganese, potassium, and zinc) and it increases the absorption of the minerals. Some forms of this supplement are used to reduce brain damage caused by cirrhosis of the liver (hepatic encephalopathy) when administered intravenously by a healthcare professional. *L* -Aspartic acid is mainly prepared for the production of popular low-calorific sweetener aspartame. This amino acid is formed from the fumaric acid by the use of the aspartate ammonia-

lyase (aspartase) of *Escherichia coli* origin. This enzyme is quite unstable, but immobilization enhances its stability. An effective immobilized aspartate ammonia-lyase may be prepared by entrapping *Escherichia coli* cells in a κ–carageenan. The process is carried out in PBR (packed bad reactor) at pH 8.5, with the ammonium fumarate used as a substrate (Figure 2). This production method is an excellent example of lyases application in the industry [16].

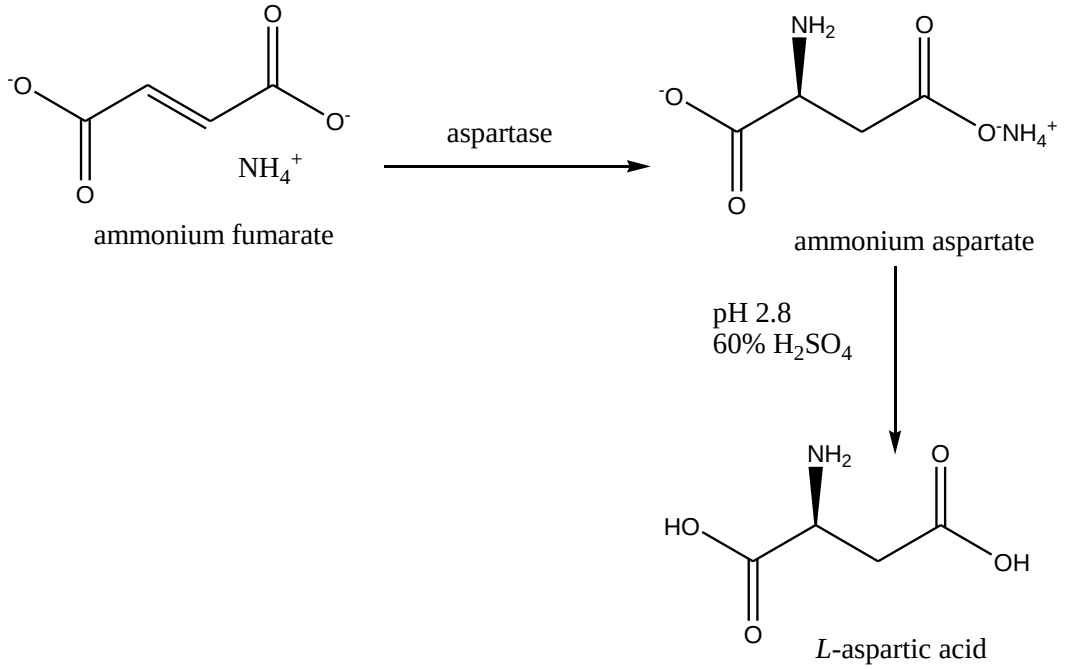

Figure 2: Scheme of aspartate synthesis.

Chiral Building Blocks

More than 700 amino acids were found in nature and nearly all are α-amino acids. Twenty of them are used by living cell for synthesis of proteins and are crucial building blocks of life [17]. Optically pure amino acids are used for the preparation of agrochemical target molecules [18] and as a chiral starting structures, auxiliaries and catalysts in organic synthesis [19]. Term "chiral building blocks" means that these optically pure components are incorporated into the structure of target molecule and supply the stereogenic center into the moiety, whereas chiral auxiliaries are removed from the final product and are used only to control the stereoselectivity of process. In turn, catalysts play only catalytic function. The incorporation of unnatural amino acid into the structure of peptide often implies obtaining the product which can influence the specific functions of the cell. Such strategy allowed to synthesize the biologically active compounds [20]. Optically pure *tert*-leucine is commonly used for this purpose, it has a sterically hindered side chain. Due

to its space-filling *tert*-butyl side chain, *tert*-leucine is especially important for the molecular conformational control of the whole moiety. In peptides this amino acid, usually replaces valine, leucine or isoleucine, keeping the same chirality but offering increased hydrophobicity and additional stability against enzymatic degradation. Both enantiomers of *tert*- leucine can be used in stereoselective synthesis as auxiliaries and chiral building blocks, however (*S*)-*tert*-leucine is more important in the synthesis of pharmaceutically active compounds (antitumor, antiviral, anti-inflammatory). Examples include the hepatis C antivirial protease inhibitors: telaprevir (Incivec, Vertex) [21], bocepravir (Victrelis) [22], or the Hiv protease inhibitor atazanavir (Reyataz) [23] and antitumor agents produced by Zeneca [19]. The use of *tert*-Leu and its derivatives in pharmaceutical applications as API (Active Pharmaceutical Ingredient) had been reduced for a long time by its limited availability. There are two main strategies of obtaining enantiomerically pure *tert*-leucine: resolution of racemic mixture of previously synthesized compounds or asymmetric synthesis. Apart from the chemical methods [24,25], biocatalytic processes were developed. Biocatalysis may be defined as the use of enzymes or whole cells of living organisms as biocatalysts for synthetic chemistry. Because of the significance of *S*-*tert*-leucine the different methods, both chemical and biocatalytical were developed. *S*-*tert*-leucine can be prepared by kinetic resolution using following enzymes: lipase [26], acylase [27], protease [28], amidase [29], but the maximum theoretical yield of the reaction is up to 50%. That is why asymmetric synthesis is usually preferred, because of possible and theoretical yield upto 100%. The first process of*S*-*tert*-leucine production was developed by Evonik Industries (in the past Degussa AG) in 1990s [30]. This method is based upon the reductive amination of trimethylpyruvic acid to (*S*)-*tert*-Leu, which is catalyzed by leucine dehydrogenase (LeuDH- E.C. 1.4.1.9) originally derived from *Bacillus cereus* (Figure 3). Applied enzyme under physiological conditions catalyzes the reductive amination of branched-chain α-keto acids to the α-amino acids. The enzymatic activity of dehydrogenase is dependent on effective coenzyme (NADH) regeneration system. In this case such system is supported by the addition of ammonium formate and formate dehydrogenase from *Candida boidinii* (FDH- E.C. 1.2.1.2). Ammonium formate serves both as a reductant for NAD^+ and as a donor of amine group in the main amination reaction (Figure 3). To preserve the cofactor from leaking across the membrane reactor, it is usually enlarged with polyethyleneglycol (PEG) [31]. To overcome the problem of high costs of above discussed method, whole-cell biocatalyst (*E.coli*) was genetically modified to produce both required enzymes (LeuDH and FDH) and was successfully applied for the synthesis of optically pure *L*-tert-leucine [32].

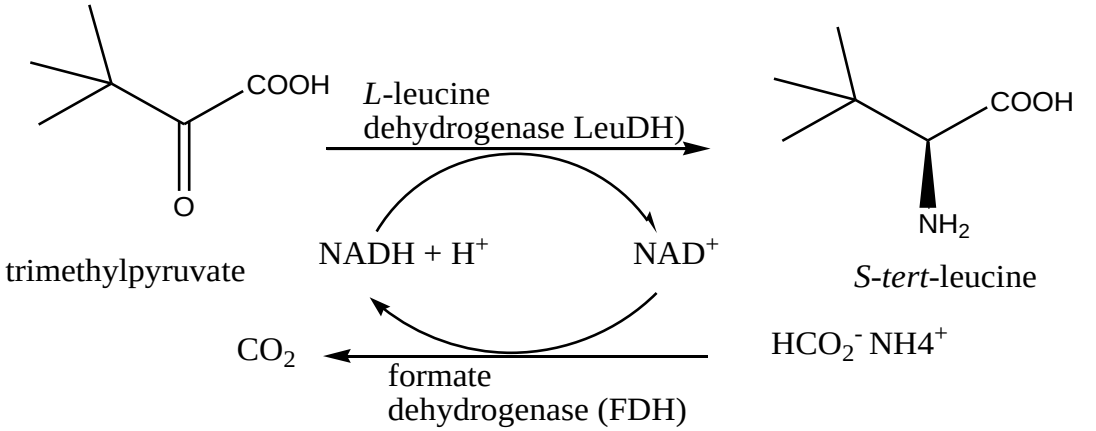

Figure 3: Reductive amination leading to enantiomerically pure (*S*)-*tert*-leucine.

Because of the enzymes stereospecificity (*R*)-*tert*-Leucine cannot be manufactured in the same way. Effective path of production of *R*-tert-leucine was developed by Evonik (Degussa). This method is based on the resolution of racemic hydantoin (*RS*-5-*tert*-butyl-hydantoin) with (*R*)-hydantoinase, what results in N-carbamoyl-*R*-tertiary leucine formation (Figure 4). The N-carbamoyl-*R*-*tert*- leucine is further converted to (*R*)-tertiary leucine either by another enzyme reaction with *R*- carbamoylase (Figure 4 route 2) or is decarbamoylized in a HCl-aqueous solution by the addition of a nitrite, such as sodium nitrite (route 1) [33]. Hydantoinase is produced by *Escherichia coli* and used in immobilized form. For fully enzymatic process it is possible to overexpress both of the enzymes: hydantoinase and carbamoylase.

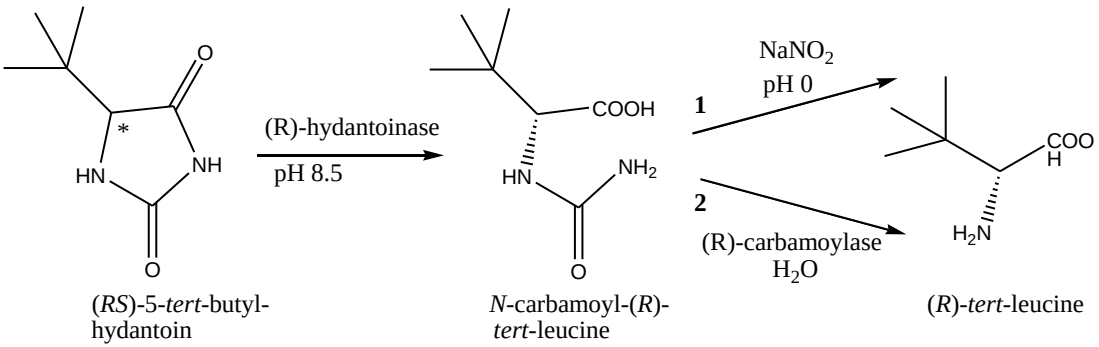

Figure 4: Synthesis of (*R*)-*tert*-leucine by Evonik (formerly Degussa).

Described kinetic resolution is limited by the maximum 50% yield of the reaction. There is a possibility of overcoming this problem by the creation of whole-cell biocatalyst with incorporated genes for the production of three enzymes: hydantoinase, carmamoylase and racemase. Then, kinetic resolution of racemic hydantoin gives one enantiomer-*R*-, which is converted to desired product *R*-*tert*-leucine, while the second one-*S*- remains unreacted. The biocatalyst with racemase activity is able to convert racemic mixture of substrate into enantiomerically pure *R*-*tert*-leucine

with theoretical yield of 100%. Direct application of *Escherichia coli,* which produces required enzymes, is an excellent resolution procedure for reaching the desired purpose [34].

Enzymes as Therapeutic Agents and Detergents Additives

Enzymes are highly selective catalytic proteins which control and regulate all biochemical processes in the organism. Several hundreds of different enzymes have been identified, and many of them have been characterized. Some of them have industrial applications as catalysts, therapeutic agents and food and detergents ingredients. *L*- Asparaginase is the first therapeutic enzyme with anticancer properties that has been deeply studied [35]. This enzyme (*L*-asparagine amido-hydrolase; EC 3.5.1.1), belongs to the amidohydrolases family, it catalyzes the breakdown of *L*-asparagine to ammonia and *L*-aspartic acid. *L*- asparagine is a crucial amino acid for the production of proteins necessary for the growth of both tumor and healthy cells. Normal cells have the ability to synthesize *L*-asparagine from aspartate using asparagine synthase. In turn, aspartate is a product of transaminase activity, which converts oxaloacetate to aspartate using glutamate as an amine group donor. Tumor cells cannot synthesize the asparagine, because of the inability to use the asparagine synthase, as a consequence they are dependent on the exogenous source of this amino acid. That is why delivery of *L*-asparaginase to cancer cells cleaved asparagine that was circulating in the blood vessels, what causes the tumor cells starvation and finally death [36] (Figure 5).

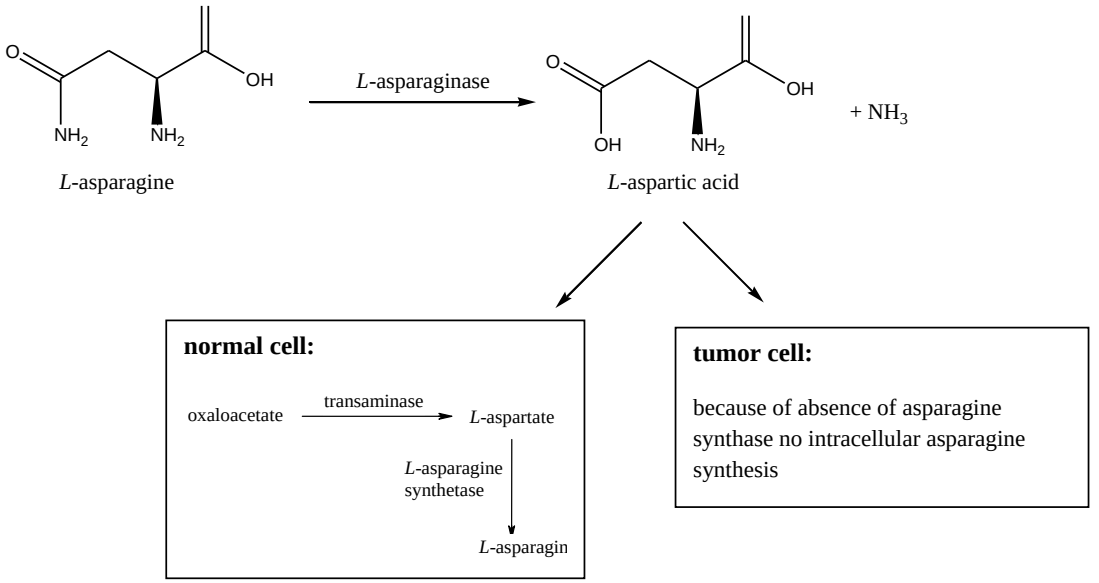

Figure 5: Mechanism of asparaginase action.

Also *L*-asparaginase is used in food industry for the production of dough based products, such as cookies, potato chips and French fries [37] to prevent acrylamide formation. *L*- asparaginase has been found in various organisms, including animals, plants, and microorganisms (bacteria,

fungi, algae and actinomycetes) but humans are not able to produce this enzyme. Microorganisms are a good source of *L*-asparaginase because they can be cultured easily and the production is quite economical [38]. Bacterial species *Escherichia coli*, *Corynebacterium glutamicum,Bacillus* sp and *Erwinia* sp. are the most common producers of *L*-asparaginase [39,40]. This protein, if provided by Gram-negative bacteria, can be categorized into two main types: type I and type II. Type I is active towards both *L*-glutamine and *L*-asparagine, while type II can be synthesized after induction, under anaerobic conditions and is specific only towards *L*-asparagine [41]. Fungi such as *Aspergillus*, *Mucor*, *Candida* and *Rhodotorula* sp. are another potential source of asparaginase[35]. Fungal enzyme is produced extracellularly and it is easy to purify. Additionally the response of human immunological systems against fungal enzyme, is lower than in the case of bacterial one [42]. *Actinomycetes* and algae are other sources of asparaginase [43-45]. Different methods for production of *L*-asparaginase are reported. Most common are solid state fermentation and submerged fermentation. The process conditions vary, they are mostly dependent on the applied microorganism [35]. However, only the asparaginases from *E. coli* and *Ervinia chrysanthemii* were approved to be used as a part of a multiagent chemotherapy to treat acute lymphoid leukemia (ALL) [46]. On the industrial scale the asparaginase is produced in rich medium, supplemented with amino acids, with limited oxygen access [47]. Currently, several commercially available asparaginases are derived from *E. chrysanthemi* (Erwinase) or from *E. coli* and they are marketed as native proteins (Asparaginase Medac, Kidrolase, Paronal, Leunase, Elspar) and as a PEGylated enzymes (Oncaspar) [48, 49].

The second group of hydrolases of strong importance for the biotechnology, are lipases (EC 3.1.1.3), which catalyze breakdown of esters of long fatty acids and glycerol over on oil-water interface. Lipases are also able to catalyze hydrolysis and esterification reactions of other non-physiological substrates. Lipases are produced by animals, plants and microorganisms. Enzymes of microbial origin are diversified taking into account their enzymatic properties and substrate specificity, they are commonly used for different industrial applications [50].The enzymes of microbial origin are used in food, detergents, textile, pharmaceutical, cosmetic, chemical biodiesel industries, [51,52]. Lipases and other hydrolases (proteases, amylases, celulases) are added to detergents (household dishwashers and industrial laundry) [53] to remove the fatty residues and to clean clogged drains [54]. The enzyme based detergents have better cleaning properties than synthetic formulations and they can be used in smaller quantity than synthetic ones. They can act under low washing temperatures and are easily biodegraded after using. Every year about 1000 tons of lipases are added to approximately 13 billion tons of detergents [53]. Such lipases are selected due to their low substrate specificity, stability under alkaline environment (pH 10-11) and under elevated temperature (30- 60°C), also stability at the presence of surfactants and other enzymes (proteases). Detergent enzymes must be effective, safe and cheap, that is why the most popular sources of this kind of factors are microbes. The detergent lipases are predominantly produced by species of *Acinetobacter* [55, 56], *Bacillus* [57, 58], *Burkholderia* [59], *Streptomyces*

[60], *Rhodococcus* [61], *Pseudomonas* [62], *Aspergillus* [63] and *Fusarium* [64]. Lipases are produced extracellularly and their production is strongly dependent on the medium composition and other cultivation conditions e.g. temperature, pH and dissolved oxygen. The most important factor for expression of lipases is the carbon source. It is also known, that the lipase production is induced by the presence of lipids (oils) or any other inducer such as triacylglycerols, fatty acids, Tween or glycerol [65]. Submerged fermentation (SMF) is often used for this enzyme production, because of its high effectiveness [57]. The other way is to use solid state fermentation (SSF) with the use of agroindustrial waste (wheat bran, sugar cane molasses, corn steep liquor etc.) as cheap substrates. The development of the application of commercial lipases for detergent purposes was rather slow, because of low fermentation yields and not enough stability of the obtained proteins under desired operation conditions. The possible way to overcome such difficulties was genetic engineering, which delivered improved lipase for applications under specific process conditions e.g. under low temperatures (Lipolase- Novozymes active below 20°C). Lipolase was originally isolated from *Humicola lanuginose* and expressed in *Aspergillus oryzae*, this protein remains active also during drying steps (in washing machines). The highest activity of Lipolase is observed when the moisture content of the textile is 20-30% [66].However, commercial bacterial detergent with high-temperature optima of working is produced by Genencor International with the use of genetically modified *Bacillus* strain with inserted genes of lipase production derived from *Pseudomonas mendocina* (Lumafast).

LIPIDS

Single - Cell Oil (SCO)

Oils and fats are products of great industrial importance. Obviously, the oleaginous plants serve as significant and predominant source of these compounds. Oils are extracted mainly from grains, seeds, and beans such as rapeseed (canola oil), soybean, sunflower, corn, peanut, cottonseed, palm fruit etc. Most of these oils are edible and produced for food market. However, it must not be forgotten, that this lipids are important compounds for other type of industry including biofuel production [67]. Vegetable oils are used in various industrial applications such as emulsifiers, lubricants, plasticizers, surfactants, plastics, solvents and resins [68]. World vegetable oil production has been increasing continuously in the past decades and passed 179 million metric tons in 2015/2016 [69].

In addition to plants, microorganism of various origin can also be considered as valuable source of oils [70]. A number of microorganisms including both bacteria [71], yeast [72] and molds [73] as well as microalgae [74] are able to produce and accumulate lipids inside their cells (sometimes in excess of 70% of their biomass) [75,76]. They are called oleaginous microorganisms, whereas oils of microbial origin produced *via* biotechnological fermentation are known as Single Cell Oils (SCOs). Although, it is evident, that microbial oils would never compete commercially with the major commodity plant oils [77], it occurs as a possibility to rationally manage some

industrial and municipal waste materials [78,79], that can be applied as carbon sources for lipid-accumulating microorganisms. As well as to produce SCOs of unique composition and properties such as contained polyunsaturated fatty acids with a carbon chain length and degree of unsaturation greater than those found in plants of great nutritional interest [80,81]. Furthermore, the number of genetic manipulation strategies and the accessibility of microorganisms to genetic and metabolic engineering can lead to obtain oils of controlled and desired chemical composition on one hand, but on the other hand it can make efficient manufacture process by facilitation of lipid extraction, what should have the direct impact on the commercialization opportunity [70].

Procaryotic microorganisms belonging to oleaginous microbes are simply defined as capable of accumulating lipids that amount to more than 20% - 25% of their dry biomass weight [82]. Lipid-accumulating bacterial species of SCO interest include mainly genera of *Rhodococus* and *Arthrobacter* [83], whereas cyanobacteria is represented by *Chlorella.* The definition of oleaginous microorganism, in the case of yeast and molds as well as some microalgae species, representing eukaryotic microbes, has been enhanced, because their specific metabolic capacities are associated with the presence of ATP-citrate lyase that is considered as crucial enzyme for lipid accumulation inside the eukaryotic cell [73].This lyase has been identified responsible for the synthesis of acetyl-CoA in oleaginous cells [78]. *Crypthecodinium* and *Schizochytrium* are eukaryotic microalgae with high lipid content. The group of oleaginous yeast contains primarily species belonging to *Cryptococcus, Lipomyces, Yarrowia* and *Rhodotorula* [79, 84] while the best known oleaginous fungi are typically found in genera *Mucor*, *Mortierella* or *Aspergillus* [85]. By all means, lipid content and profile differ between species and are dependent on cultivation conditions [86]. Lipids produced by microorganisms can be classified according to cellular function, as structural compounds – this group is represented by phospholipids and glycolipids – and energy storage lipids including glycerol triesters with non-polar fatty acids(triacylglycerides, TAGs). The latter compounds are the main constituents of vegetable oils, so microbial oils containing such neutral lipids are also of special interest as the alternative for them. Compared to yeast, fungi and microalgae known for TAGs accumulation, synthesis of these compounds by bacterial cells is rare and limited to actinomycetes group [83].

Oleaginous cell factories are highly affected by the type of carbon source and conditions applied [87]. They are able to utilize various carbons sources (both hydrophilic as well as hydrophobic ones) for metabolic purposes as lipid production and accumulation, including glucose, xylose, glycerol, starch, cellulose hydrolysates, and industrial and municipal organic wastes [77]. Biochemistry of lipid accumulation in oleaginous microorganisms has attracted much attention and is still extensively examined [88-91]. Two different pathways of lipids accumulation are reported, dependently on the character of carbon source used for fermentation process. Lipid production as a result of fermentation of sugars and related substrates used as carbon source is termed *de novo* production (Kennedy pathway [92]), whereas microbial synthesis of oils from hydrophobic substrates (fatty acids, alkanes etc.) takes place according to *ex-novo* pathway [84].

De novo accumulation of cellular lipids is an anabolic biochemical process in which, by virtue of quasi-inverted oxidation reaction series, acetyl-CoA issued by the intermediate cellular metabolism, generates cellular fatty acids. Fatty acids are then esterified with glycerol generating structural and reserve (mainly TAGs) lipids [88]. TAG storage occurs at the end of the exponential growth stage and in periods of metabolic stress. In all cases, accumulation of lipids takes place under conditions of limitations caused by a nutrient other than carbon. When cells run out of a key nutrient, usually nitrogen, excess carbon substrate continues to be assimilated by the cells and converted into storage fat. The lack of phosphorus, sulfur, and zinc has also been shown to trigger this process [93]. The use of fats or hydrophobic materials as the carbon source for microbial growth, causes lipid accumulation being a growth-coupled process (*ex-novo* pathway). It includes the degradation of substrates followed by the incorporation of hydrophobic substrates inside the cell [78]. The incorporated fatty acids are either dissimilated for growth needs or become a substrate for synthesis of fatty acid profiles which did not exist previously in the substrate. Principal biochemical differences exist between *de novo* and *ex novo* lipid biosynthesis; in the latter case, lipid accumulation occurs simultaneously with cell growth, being entirely independent from nitrogen exhaustion from the culture medium [88,91].

Large scale processes of various SCO production are still under optimization [86, 94], but some examples of industrially relevant technology are demonstrated [87]. Currently, SCO rich in specific long-chain PUFAs (omega-3 and omega-6) are produced commercially [90]. These oils are manufactured due to high nutritional value and used in infant formula milk and as health supplements.

Lipid Nanoparticles

Currently, pharmaceutical and cosmetic sectors of industry gave a great importance to the use of effective delivery technologies [95, 96]. Very often, only developing of powerful active compound in combination with suitable drug carrier system can provide desired results *in vivo*. Nanocarriers (NCs) are a group of nano-sized vehicles devised to deliver biologically active molecules to the desired destination [97].

Solid lipid nanoparticles (SLNPs) and nanostructured lipid carriers (NLCs) are among the most promising bioactive carrier systems. Active compounds can be incorporated into carriers structure, what ensures the protection of the molecule against chemical degradation as well as facilitates the modulation of compound release. Because various routes of administration of lipid nanoparticles were demonstrated including dermal [98], oral [99], parenteral [100] and ocular [101] one, the range of their application is very wide. These products have been developed in order to reduce toxic side effects of the incorporated, highly potent drugs and increase the efficacy of the treatment. Lipid nanoparticles constitute an alternative carrier system to liposomes, emulsions and polymeric nanoparticles, with improved quality [102]. SLNPs were developed at the beginning of the 1990s, whereas NLCs regarded as the second generation of lipid

nanoparticles, were presented at the end of the 1999 and the beginning of the 2000 [98]. These particles have an average size of 40 to 1000 nm and a spherical morphology [103]. SLNPs are derived from o/w emulsion, in which solid lipid is incorporated instead of liquid lipid (oil). This fact is crucial in the view of active compound (drug) release – the rate of movement in solid lipid is lower than in oily phase [104]. Lipid components of SLNs are solid at both body and ambient temperature and include triglycerides (e.g. tristearin, tricaprin, tripalmitin), partial glycerides (e.g.glyceryl monostearate), fatty acids (e.g. stearic acid, palmitic acid), steroids (e.g. cholesterol) and waxes (e.g. cetyl palmitate) [104,105]. All compounds are pure and of physiological origin, what should resolve the problem of toxicity. Aside from solid lipid, water and emulsifiers are components that are used for SLNs formulations. All classes of emulsifiers (with respect to charge and molecular weight) have been used to stabilize the lipid dispersion, in concentrations of about 0.5 to 5%, but the choice of proper compound depends on the route of particles administration. Additionally, it has been found, that the application of mixtures of various emulsifiers is favorable because it might prevent particle agglomeration [104]. It should be underlined, that the proper selection of both ingredients: lipids and surfactants, are extremely important because it can affect physicochemical properties and quality of SLNs such as particle size and active compound loading as well as the tendency to agglomeration.

Several different techniques have been reported for the production of SLNs including high pressure homogenization, solvent emulsification/evaporation, supercritical fluid extraction of emulsions (SFEE), ultrasonication or high speed homogenization and spray drying [102]. Depending on the method of preparation, SLNs have various diameters and can be used as carriers for both hydrophilic and hydrophobic molecules. Commonly used method for preparation of SLNs is high pressure homogenization that can be performed using either the hot or the cold homogenization technique. An important advantage of this approach is the feasibility of process scaling up. For both techniques, the active compound is dissolved, solubilized or dispersed in the melted lipid [105]. In the hot homogenization protocol, the active compound containing lipid melt is dispersed in hot surfactant solution of the same temperature by high-speed stirring. The obtained pre-emulsion is then passed through a high pressure homogenizer [106]. In the cold homogenization method, the active compound containing lipid melt is cooled and after solidification, the lipidic mass is ground to yield lipid microparticles. The lipid microparticles are dispersed in cold surfactant solution by stirring, yielding a macro-suspension. This suspension is passed through a high-pressure homogenizer, the microparticles are broken down to solid lipid nanoparticles [98].Hot homogenization is the most frequently applied technique leading to obtain smaller particles and in general even temperature sensitive compounds can be processed because the exposure time to elevated temperatures is relatively short. The cold homogenization technique is recommended for extremely temperature sensitive compounds and hydrophilic compounds, which might partition from the liquid lipid phase to the water phase during the hot homogenization [106]. SLNs except of many advantages including biocompatibility, molecule

protection and good release profile, environmentally friendly methods of production, feasibility of scaling up and low cost, they have some limitations. The common ones are unpredictable gelation tendency and inherent low incorporation rates resulting from the crystalline structure of the solid lipid [107]. To overcome some potential limitation associated with SLNs, novel lipid-based formulations were created. These are called NLCs - nanostructured lipid carriers and exhibit improved properties such increased loading and long term stability of active compound [97,108,109]. NLCs consist of an unstructured solid lipid core made of a mixture of blended solid and liquid lipids and an aqueous phase containing a surfactant or a mixture of surfactants. Typically, solid lipids are mixed with liquid lipids in a ratio of 70:30 up to a ratio of 99.9:0.1, whereas the surfactant content ranges 1.5%-5%(w/v) [98]. Production of NLCs is carried out based on the same techniques as in case of SLNs.

SLN and NLC are complex systems with great potential. They have remarkably wide range of application and have shown to greatly control the skin penetration of several actives, delivery of food and drugs, cosmetics and other applications [110-112]. Up to now, commercial application of these products concerned the application of NLCs in the cosmetic formula. Cutanova Nanorepair Q10 cream was the first NLC containing cosmetical product introduced to the market in October 2005 [113].

Fat Substitutes

Lipases are versatile biotechnological tools applied in both laboratory scale as well as in large industrial scale to produce wide range of compounds of academic or industrial interest [114]. They are enzymes that catalyze hydrolysis of triacylgycerides into free fatty acids and glycerol at the lipid water interface but exhibiting also the capability to carry out the reverse processes in the environment of organic solvents. Enzymes of microbial origin are predominantly used and their applicability is closely related to the wide substrate tolerance and peculiar precision of their action expressed in chemo-, region- and stereoselectivity of lipases. The main area of lipase application concerns food modification, detergent formulation, cosmetic, pharmaceutical, leather, textile, and paper industries, biodiesel and biopolymer production, or pretreatment of lipid-rich wastewaters [115].

Lipases are also used to modify lipids and synthesize compounds of desired structure and nutritional benefits [116]. These lipids, represented by acylglycerols, such as triacylglycerols which are the most common types of food lipids, diacylglycerols, and monoacylglycerols as well as phospholipids, are called structured lipids **(SLs)** [117]. Structural changes including modification of fatty acids positions and/or the composition of fatty acids in lipid molecule cause the alteration of functional properties, expanding the range of SL application. Lipases catalyze modification of lipid substrates either by transesterification or interesterification reactions [118], what allows to rearrange the molecule structure according to the output assumptions. Transesterification involves an exchange of acyl moieties between a triacylglycerol and a fatty acid (acidolysis),

an alcohol (alcoholysis), or glycerol (glycerolysis), and interesterification involves exchange of acyl moieties between two triacylglycerols. Among SLs, cocoa butter substitutes and human milk fat substitutes constitute the examples of special commercial interest. In the production process, lipases of fungal origin, with 1,3-positional specificity are applied [118]. Cocoa butter **(CB)** extracted from the cocoa bean, is the basic and essential component of chocolate. It mainly consists of symmetric monounsaturated triacylglycerols (97%). The remaining 3% are minor components, such as free fatty acids, mono- and diacylglycerols, phospholipids [119]. Fatty acids composition of CB includes palmitic (20 to 26%), stearic (29% to 38%), oleic (O, 29% to 38%)) acid and lower amount of linoleic (2% to 4%) and arachidic acid (± 1%) [120]. Very high cost of this raw material and its limited availability, have forced industry to look for some cheaper alternatives.

The development of lipase mediated technology allows the production of CBS from low-cost vegetable oils such as palm, sunflower, rapeseed or olive oil containing TAGs, in which sn-2 position is mainly occupied by oleic acid. The first patents of Unilever [121] and Fuji Oil [122,123] for the lipase catalyzed synthesis of CBS is from palm oil midfraction and stearic acid. Palm oil midfraction consists of 1,3-dipalmitoyl-2-mono-oleine **(POP)**. Interesterification with tristearin or acidolysis with stearic acid in presence of 1,3 specific lipase byields 1(3) palmitoyl-3(1) stearoyl-2-mono-oleine **(POSt)** and 1,3-distearoyl-2-monoleine **(StOSt)** [118]. As a result a cocoa butter-like fat can be produced, in which the fatty acid composition resembles closely that of cocoa butter.

Lipase mediated biotransformation provides a useful way to improve the properties of dietary fat. An example of this is the production of human milk fat substitutes **(HMFS)** [124]. Typical human milk fat is mainly composed of palmitic and oleic acids, which constitute about 25% w/w and 40% w/w of the total fatty acids, respectively. Palmitic acid is predominantly esterified at the *sn*-2 position (55% w/w), whereas the *sn*-1,3 position is mainly occupied by unsaturated fatty acids, such as oleic acid (45% w/w) [125]. Thus, 1,3-dioleoyl-2-palmitoyl-glycerol **(OPO)** is one of the major TAG species present in human milk fat [126]. Betapolis a brand name for a newly developed human milk fat substitute from Loders Croklaan (Unilever). This is a product based on tripalmitin-rich oils such as palm stearin, which more closely mimic the specific structure of HMF as well as closely matching HMF fatty acid composition. Betapol is produced by modern enzyme technology which involves position-targeted reactions catalyzed by sn-1,3 specific lipases [127,128].

CARBOHYDRATES

Non-lactose Food

The knowledge about the saccharides structures, isomerism and their biochemical transformations inside the living cells allowed to diagnose a number of dysfunctions implying from the disorder of sugar related metabolic pathways. One of the examples is the lack of the

enzymes involved in the hydrolysis of glycoside bonds linking the saccharides monomers. Among others, the deficiency or lack of lactase, which belongs to the family of enzymes known as β-galactosidases, is a good model object for further considerations. This enzyme is normally produced by the cells named enterocytes, which are the integral part of the brush border - small intestinal walls, the barrier, which is crossed by the digested food. This is particularly important for mammals, whose development and growth depends on the milk digestion, especially during infancy, because lactase is produced to hydrolyse the lactose, also called as milk sugar, responsible for its sweetness. Lactose is a disaccharide, built from the galactose and glucose, which are linked by β-1,4-galctoside bond, which is decomposed by lactase according to scheme below (Figure 6):

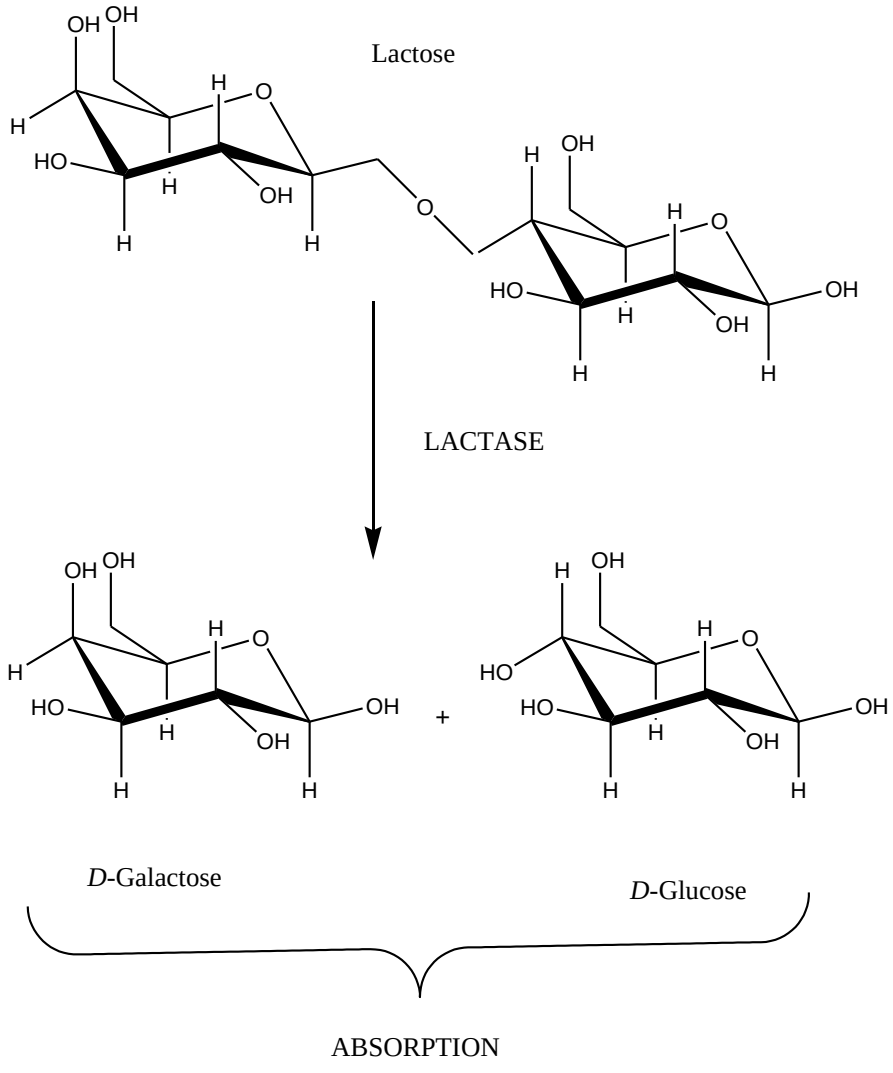

Figure 6: Decomposition of glycoside bond by lactase.

This reaction enables to absorb the monosaccharides products from the small intestinal lumen, otherwise lactose remains undigested and can serve as a nutrition for the bacterial strains, living inside the digestive track. As a consequence products of bacterial lactose fermentation are released into the intestinal lumen, what causes many symptoms: disturbing pain, flatulence, diarrhea and weight loss in children – called an intestinal distress - lactose intolerance [129-132]. The level of lactase production, in human population is particularly abundant during infancy and then genetically encoded and irreversible reduction of its activity is observed, what results in the malabsorption also named as lactose intolerance. The inability to cleave the glyosidic bond in lactose is observed in neonates and adults as a result of inherited deficiency of the enzyme (primary lactose malabsorption) or intestine disease (secondary hypolactasia) [133]. There are at least few ways to overcome this problems starting from the general, reasonable food limitation *via* decreasing the amount of the lactose intake with the meal to the complete lactose elimination from the diet by choosing the lactose free food. The modification of the dairy components in diet always takes a risks of the decreasing of the level of calcium and vitamin D below the necessary one. Also the other possibility exists - the supplementation by the pharmaceutical preparations - capsules consisting of the bacterial strains, which are able to produce lactase and are safe for the human health or consisting purified enzyme - lactase of microbial origin. The production of the lactose-free food is a very important branch of food industry. The knowledge about the biochemistry of the enzymes action (including lactose) and pathways of sugars breakdown and production in living organisms allowed elaborating procedures of industrial meaning in the human nutrition. Thus, the production of lactose-free milk, in the most cases is based upon the fermentative methods with the use of microorganisms, which are able to hydrolyse glyosidic bond. Such milk can serve as a raw material for further production of lactose-free dairy. Microorganisms applied for this milk production are a source of the lactase, which should be thermostable, because it should remain active under the milk pasteurization (65°C, 30 min) conditions and also using microbial cells in food production or modifications – the problems of contamination should be overcome [134]. As an example: *β*-glucosidase of prokaryotic origin from *Pyrococcus furiosus* (extremophilic - temperature resistant species of Archea) is applied for the milk lactose hydrolysis under pasteurization conditions, enzyme remains active in the presence of the calcium ions and glucose, which concentration increases with the lactose hydrolysis progress [134]. After the reaction is completed – the lactose free milk is produced, the cleaved sugar is a source of glucose and galactose, which are digestible components. The lactose intolerance symptoms are also overcome by the application of the pharmaceutical preparations, which are usually capsules loaded with the lactase. This time there is no necessity to use the thermostable enzymes, because these drugs are subjected to operate in the digestive tract, under mild temperature. That is why, in most cases, in pharmaceutical industry fungal lactase is applied. As an example “Ensymm” - German lactase supplier receives the *β*-galactosidase from the fungus strain *Aspergillus oryzae* for further applications, also for drugs formations.

HFCS - High Fructose Corn Syrups

Another large scale application of biochemical laws is the production of high fructose corn syrups (mixtures of dextrose - *α-D*-glucose and fructose), which are used commonly as sweeteners in food industry. HFCS are valuable products because of their industrial parameters such as: good solubility – remain liquid under the processing conditions, hygroscopic properties and low price, which is a derivative of the cheap raw material - corn starch. HFCS are present in: baking and cooking ingredients, beverages, breads, breakfast cereals, candy bars, cookies, cakes, cough syrups, dairy, ice creams, meats, salads dressings, snacks, almost every category of food is supplemented by this sweetener. The consumption of this product constantly increases with time passing, because there was no evidence of it adverse influence on the human health. Currently, scientists recommend greater caution in this matter, since the literature data have considered the coincidence between the HFCS consumption and the metabolic disorders (obesity, diabetes) and also cardiovascular diseases [135]. This sweetener production is based upon the knowledge about the mechanisms of the enzymatic reactions involved in sugars conversion and was developed in 1957 in the USA [136]. Industrial production also known as a corn isomerisation is performed according to Figure 7.

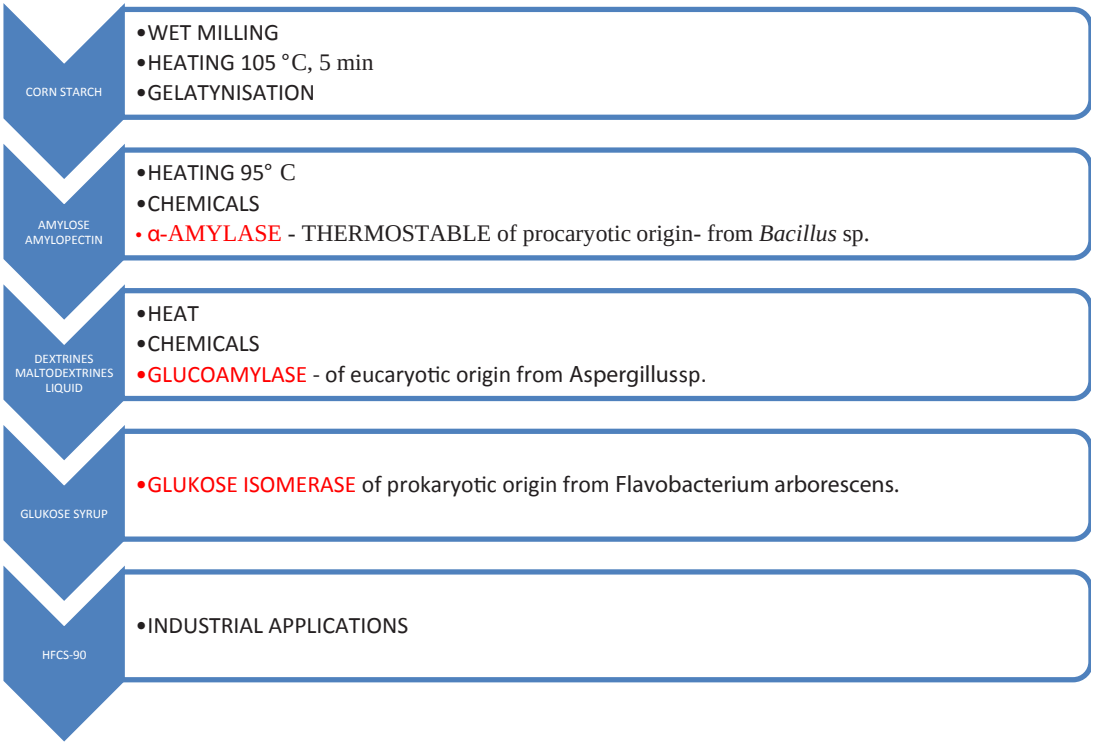

Figure 7: The main steps of the industrial production of HFCS.

As seen on the graphic above, three different enzymes are involved in the HFCS production, two of them:*α*-amylase and glucose isomerase are bacterial and the third one - glucoamylase is of fungal origin. Corn starch – glucose polymer, which is obtained as a product of milling and heating, is converted into the monomers *via* subsequent enzymatic reactions performed by the *α*-amylase and glucoamylase, according to Figure 8:

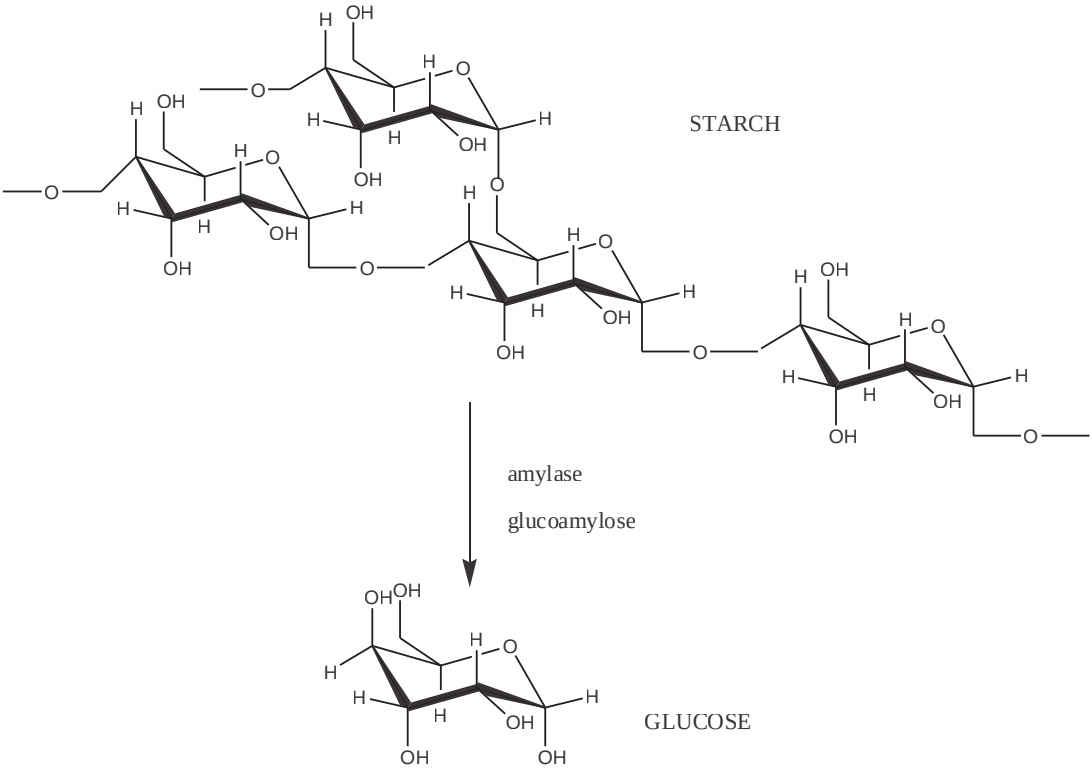

Figure 8: Enzymatic starch hydrolysis.

Glucose - product of starch hydrolysis is a substrate for the crucial reaction in the HFCS formation - for the glucose to fructose isomerisation, which is biocatalyzed by the glucose isomerase. This enzyme is one of the most important proteins in the food industry, taking into account the global volume of the sweeteners production e.g. in European Union current output is capped at 700000 tones and is predicted to reach the level of about 2 million tons by the 2023 year (Foodnews, 08.06.2016, by GOODPACK, Lorena Ruibal) [137]. As a consequence the production of the glucose isomerase also constantly increases. This enzyme is produced and distributed among others by Novo-Nordisk as Sweetzyme®, by Miles Kali-Chemie as Optisweet® or by Finnsugar as Spezyme®. Isomerization of the glucose is performed according to Figure 9. The process conditions allowed to obtain the HFCS, which consists of 90% of fructose and can be mixed with glucose to obtain products of low fructose concentration (42% - the lowest useful concentration) for further applications. The setting of the conditions for such complicated process as HFCS

production, which involvs three different enzymes was possible thanks to the deep investigations of sugar metabolism and to the understanding of the outstanding meaning of the enzymes in their conversions.

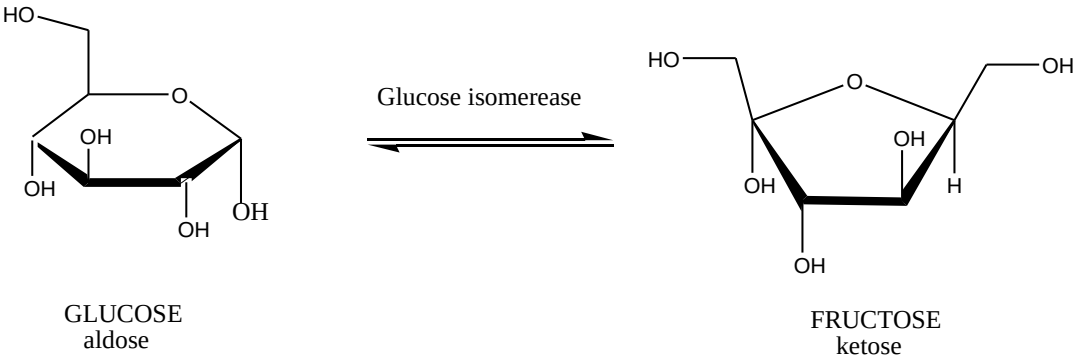

Figure 9: Enzymatic glucose isomerization.

Oligosaccharides as Food Ingredients

- The application of oligosaccharides ranges from the food *via* pharmaceutical to agriculture industries [138]. As food ingredients they are divided into two groups: digestive and non-digestive carbohydrates. This last compound act as sweeteners, dietary fibre, weight controlling agents and also are considered as supplies promoting the growth of particular intestinal bacterial strains to improve the human health. They are also applied as delivery structures for active molecules in pharmaceutical and cosmetics industries. Among the non-digestive carbohydrates – functional oligosaccharides are of outstanding meaning because of the consumer benefits. These structures are named as prebiotics and were defined in 1994 as "a non- digestible food ingredients that beneficially affects the host by selectively stimulating the growth/or activity of one or limited number of bacteria in the colon and thus improving host health" [139]. Also they belong to the colonic food that passes through the digestive tract to its bottom part without the structure changing. Carbohydrate to be classified as prebiotic must meet restrictive criteria [140]: it cannot be hydrolysed in the upper part of digestive system

- it must cause the growth and/or activation of one or limited number of the colonic bacteria by being a selective substrate
- it must be able to change the microflora of the colon to healthier composition
- it must induce beneficial luminal or systemic effect to the health of the host by delivery of the health promoting metabolites, produced by the stimulated colonic bacteria

Among others, fructoligosaccharides **(FOS)** are prebiotics, which meet all required criteria [139, 140], they are also known as a functional food. Organic chemistry classified the

oligosaccharides as carbohydrates that consist of 2 to 9 monomers, whereas polysaccharides are built up from more than 9 monosaccharides [141] but usually the term oligosaccharide means the carbohydrate built up from the 2 to 20-25 monomers. These structures are water-soluble and are of lower sweetness than sucrose. FOS (also named as oligofructose) are obtained by the enzymatic sucrose conversion, which is catalysed by *osyl*-transferases (glucosyltransferase, fructosyltranserases) or by the acidic or enzymatic (with innulinase) hydrolysis of sugar inulin (obtained from e.g. chicory) [142]. Considering the structure of fructoligosaccharides they belong to the two groups. First one consists only of the β-*D*-fructose units, which are bonded by the *α*-1-2-glycoside bonds and the second group includes the *D*-terminal glucose molecule also linked by the *α*-1-2 bond, what is illustrated below (Figure 10):

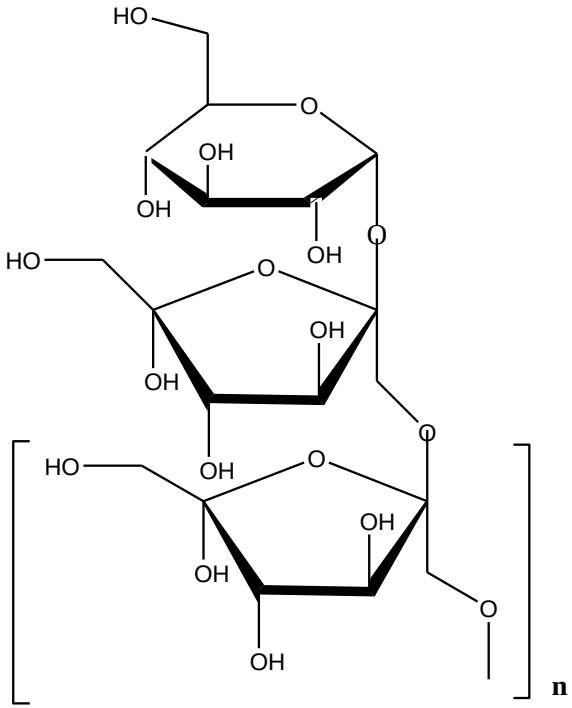

Figure 10: FOS - structure.

Enzymatic FOS synthesis can by performed with use of fructosyltransferase (FTase, E.C.2.4.1.9) or *β*-fructofuranosidase (FFase, E.C. 3.2.1.26) [146]. For commercial purposes this enzymes can be produced by following fungi: *Aspergillus* sp., *Fusarium* sp., and others [143-145]. Fructosyltransferase catalyses the transfer of fructosyl group from one moiety of sucrose to the other one, promoting the extension of the structure and releasing the glucose. Operating parameters for such synthesis are as follows: up to 850g/L of sucrose concentration (substrate), temperature 50-60°C, what assures the proper substrate viscosity and improves its

processing [147] allowing to obtain about 50-60% of conversion degree. Such effective methods of oligosaccharides synthesis are the answers to the still increasing need for functional food production. At the beginning of 21st century the US market of such food was valued at 27 billion $ and still growing. Discussed paragraph also illustrates the outstanding meaning of understanding the rules of biochemistry and physiology and the benefits, which implies from this knowledge.

NUCLEIC ACIDS

Biosensors - Aptamer Application

Aptamers as biomolecules have been firstly reported in the 1990 by three groups simultaneously and independently of one another [148-150]. They are oligonucleotides comprised of single-stranded DNA or RNA molecules that can bind to their target of interest (metal ions, small molecules, proteins or cells) with highly specific affinity, by folding into secondary and tertiary structures [152]. Due to their target-binding properties aptamers are often compared with their biological equivalent – antibodies, over which they exhibit a significant number of superior features, such as small size, ability to sustain reversible denaturation, easy and controllable modification, slow degradation kinetics, nontoxicity and lack of immunogenicity [153]. Aptamers are therefore excellent molecules for the researchers in the field of broadly defined bioanalysis and biomedicine (Figure 11).

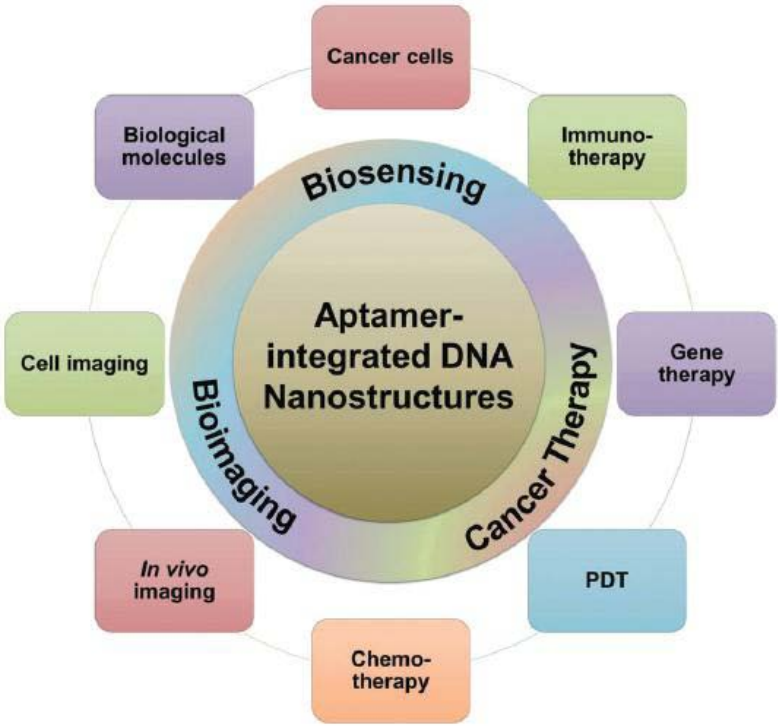

Figure 11: Scientific and research trends that may exploit the superior qualities of aptamer molecules binding their targets [153].

Aptamers are synthesized through the systematic evolution of ligands by exponential enrichment **(SELEX)** method of which the schematic diagram is depicted in Figure 12. Generally this process involves the incubation of random synthetic oligonucleotides obtained from the library with the target of interest. Subsequently the probes are subjected to washing, during which all of the unbound DNA strands are discarded and the bound sequences are eluted and amplified with polymerase chain reaction **(PCR)**. This process is repeated until the specific binding intensity of the sequences towards the target is obtained 154,155]. There have been many variants of SELEX method developed throughout the years:

- Primer-free SELEX [156];
- Toggle SELEX [157];
- Tailored SELEX [158]
- Cell SELEX [159];
- Genomic SELEX [160];

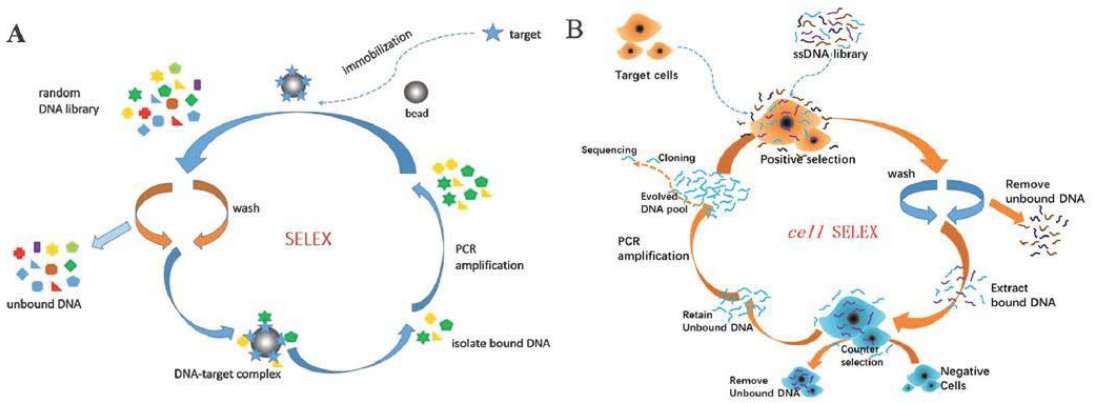

Figure 12: Schematic of SELEX method with regard to: A) Proteins, B) Cells [153].

Over the years biosensors have become a very powerful tool in modern biomedicine thanks to their ability to provide information about pivotal steps and monitoring of the biological and biochemical processes. Typical biosensor is composed of two main parts: a transducer (electrochemical, optical, thermal etc.) and a biological recognition element (enzyme, antibody, receptor etc.) [152]. Owing to their excellent properties aptamers are often integrated as the biological elements into the biosensor structure. Apart from quantum dot-based detection and mass sensitive detection biosensors can be divided into four main classes depending on the type of the detection method. First of them is the electrochemical sensor group (Figure 13A.) in most of which the aptamers have to be immobilized on the electrodes, they are often used to detect various kinds of macromolecules for example interferon **(IFN-γ)** [161] orthrombin [162], but also smaller compounds such as aminoglycosidic antibiotics [163]. Second class of biosensors is based on the

chemiluminescence detection of the target molecule (Figure 13B). Those sensors do not need any kind of external light source to produce the light signal, additionally they are simple in structure, cheap and highly sensitive. Chemiluminescence biosensors can also be used to detect the level of various proteins, such as α-fetoprotein **(AFP)** or coronavirus nucleocapsid protein [164] and smaller targets like Pb^{2+} [165], adenosine [166] or cocaine [167]. Third class is the fluorescent detection based biosensors (Figure 13C) that use one of the most sensitive methods to detect molecular interactions. This group of sensors is also simply built, since aptamers can be readily modified and labeled with the fluorescent tag. Fluorescent biosensors can offer subpicomolar sensitivity in detection of small compounds, such as cocaine [168], but can also be used to detect cancerous cells [165] and give insight into the cell metabolism [169]. The last class of biosensors is the group based on colorimetric detection (Figure 13D). Although they are less sensitive the colorimetric sensors do not need any inconvenient, specialized equipment for the detection of the emitted signal, in contrast to fluorescent biosensors for example. They simply rely on the properties of the metallic nanoparticles, such as very high extinction coefficient, which will cause a visible color change upon metal aggregation. In this approach scientists often use mixtures of polyelectrolytes with target molecules and aptamers that combined lead to the aggregation of metallic nanoparticles and change of solution color in the end [170].

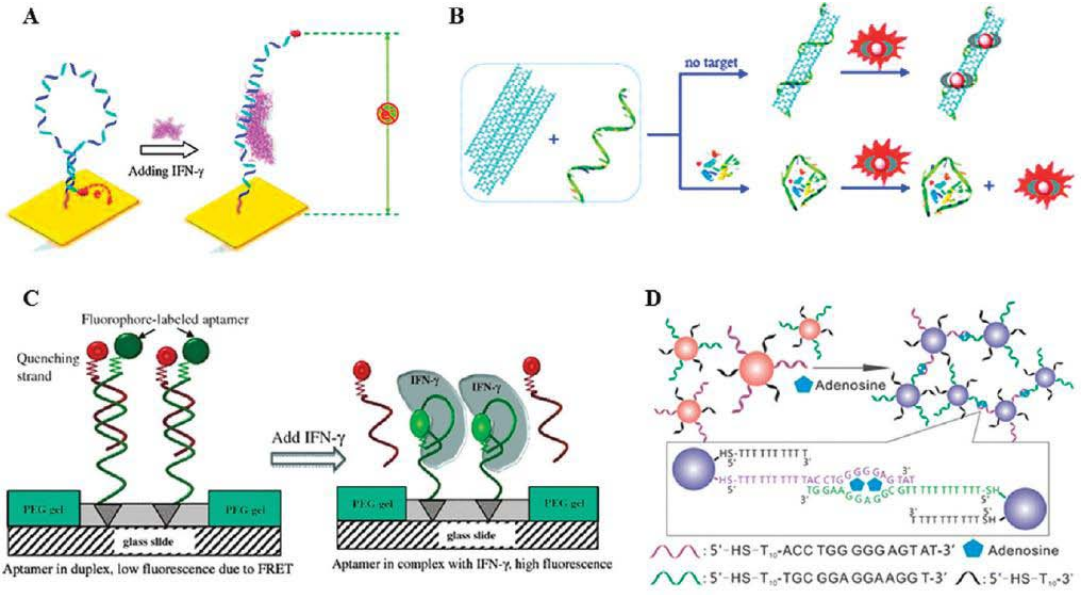

Figure 13: Examples of different classes of biosensors according to the method of detection: A) Electrochemical, B) Luminescent, C) Fluorescent and D) Colorimetric [152].

Nucleotides - Food Additives

Nucleotides are one of the most important groups of the biological compounds, they are involved in a very broad range of metabolic, catalytic and regulatory pathways in living organisms.

They are also a basic structural unit of DNA and RNA, so they take part in fundamental biochemical processes, like DNA replication, transcription, translation and many more [171]. Nucleotides contain couple of main structural components, namely heterocyclic nitrogenous base that can be either purine or pyrimidine. Pyrimidines consist of hexagonal cyclic ring with 2 nitrogen atoms, whereas purines have additional imidazole ring (Figure 14A). Aside from bases nucleotides also consist of sugar (ribose and deoxyribose in case of RNA and DNA respectively) and a phosphate moiety (Figure 14B).

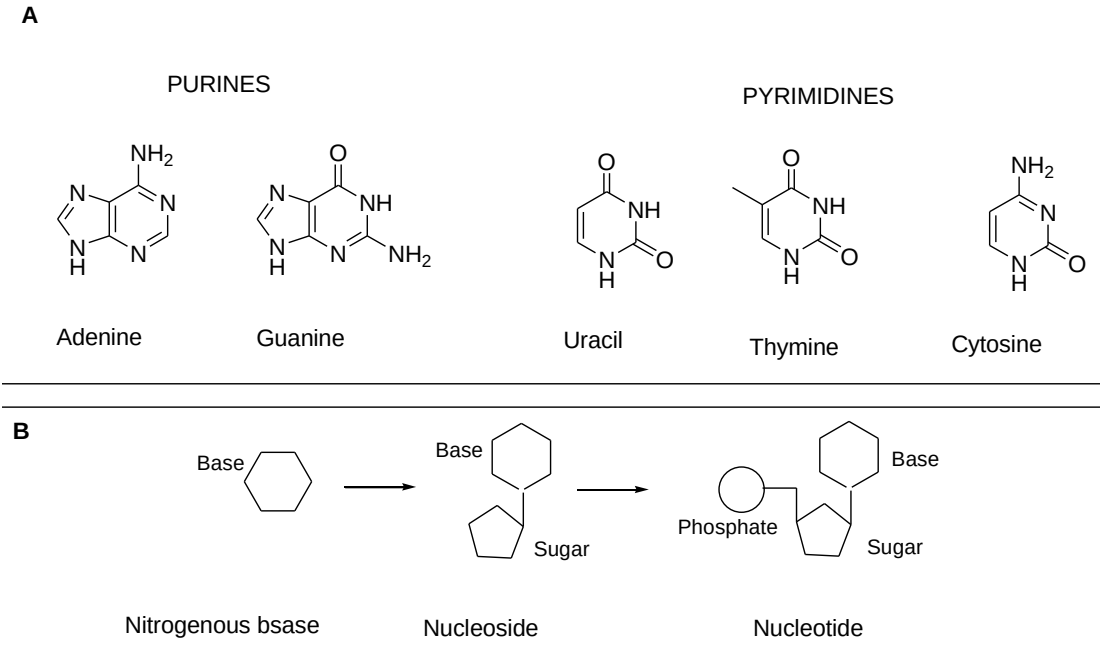

Figure 14: Structure of nucleotide components: A) Nitrogenous bases, B) Basic structure of a nucleotide [172].

Vast majority of the vital nucleotides is formed through the reactions of endogenous synthesis. However they can also be acquired in the form of nucleoproteins (proteins bound to nucleic acid) that are naturally present in food of animal and vegetable origin [173]. Concentration of nucleotides in food is cell density dependent, hence solid food, like meat and seeds is more abundant in RNA and DNA then for example milk [174]. Over the years nucleotides also found an application as synthetically or naturally synthesized food additives for flavor enhancement (disodium inosinate, monosodium glutamate or disodium guanylate). There have been many clinical trials concerning dietary nucleotides and the influence they have on immune and gastrointestinal systems of animals and humans, some of the results are presented in Table 1 and 2 [172].

Table 1: The outcome of animal studies with nucleotides and the effect they have on immune and gastrointestinal system [172].

Study	Tissue or System	Outcome
Dietary adenine, uracil, or RNA and sepsis	Immune	Improved survival in uracil and RNA groups Increased phagocytic activity by all test groups
Dietary adenine, uracil, or RNA and candidiasis	Immune	Longer survival time in all test groups Reduced recovery of *Candida albicans* in kidney and spleen
Dietary nucleotides and dextran sulfate sodium and distal colitis	Large intestine	Nucleotide group had increased inflammation and delayed healing
Dietary nucleosides fed to old mice after food deprivation	Gastrointestinal	Nucleoside-supplemented group restored jejunum and ileum faster upon refeeding

Table 2: The outcome of infant studies with nucleotides and the effect they have on immune and gastrointestinal system [172].

Study	Tissue or System	Outcome
Term infants breastfed and fed formula with and without nucleotides for 2 months	Immune	Natural killer cell percent cytotoxicity higher in breastfed and nucleotide-supplemented groups
Infants fed formula with and without nucleotides for 12 months; third group had breast milk for 2 months and then formula	Immune	Nucleotide group had increased antibody to *Haemophilus influenzae* and diphtheria vaccine No difference in immune response to tetanus and oral polio vaccine Breastfed group had greater antibody response to oral polio vaccine
Infants fed formula with and without nucleotides for 12 months; third group had breast milk for 2 months and then formula	Immune	Nucleotide group had improved humoral immune responses vs nucleotide-free group Nucleotide group had shifts in T cell populations indicative of improved immune cell maturation
Infants fed formula with and without nucleotides for 12 weeks	Gastrointestinal and immune	Nucleotide group had less diarrhea Nucleotide group had 13% higher incidence of upper respiratory tract infections
Term infants fed formula with and without nucleotides for 5 weeks	Circulation and gastrointestinal	Nucleotide group had increased superior mesenteric artery blood flow 90 minutes postprandial

The current state of knowledge suggests that dietary supplementation of nucleotides is advisable when organism is influenced by strong physiological stress that might impair its immune, hepatic or cell growth function. However the doses and the concentration vary upon certain conditions, such as rapid growth, injury or infections etc. Dietary nucleotides are therefore necessary for the organism to maintain sustainable physiological functioning that supports its homeostasis [172].

DNA Vaccines

DNA vaccines are generally known to be molecules of simple structure that involves short, circular fragment of DNA containing gene encoding the antigen and a promotor/terminator sequence to enable the protein expression in mammalian cells [175]. The first reports about DNA vaccines dates back to 1992, when Tang and Johnston used a gene gun to insert DNA into mice skin as an attempt to deliver the human growth factor into the organism. The aim of this gene therapy was to generate antibody response against specific transgene products [176]. The research that followed was focused on the use of DNA vectors in such a way, that they would induce both humoral and cellular immune responses against pathogens and tumours antigens *in vivo*. Scientists also discovered that naked DNA plasmids can generate immune responses against influenza virus antigens in mice [177, 178].

DNA vaccines mechanism of action is still not fully explored, its idealized pathway model is summarized in Figure 15. The optimized gene sequence is inserted directly into intradermal layer of skin or into muscles. With the use of host's proteins and enzymatic machinery the DNA plasmid enters the nucleus of transfected cells and the antigen gene expression starts. Once expressed the antigenic proteins may trigger the immune response of class I major histocompatibility complexes (MHC) and class II antigen presenting cells (APC). Subsequently the APCs travel through lymph nodes, where native T cells are monitoring their antigen-MHC complexes, this action may lead to the initiation of immune response by activation and expansion of T cells or alternatively to activation of B cells that triggers the antibody expression cascades. This way DNA vaccines generate both humoral and cellular response [179].

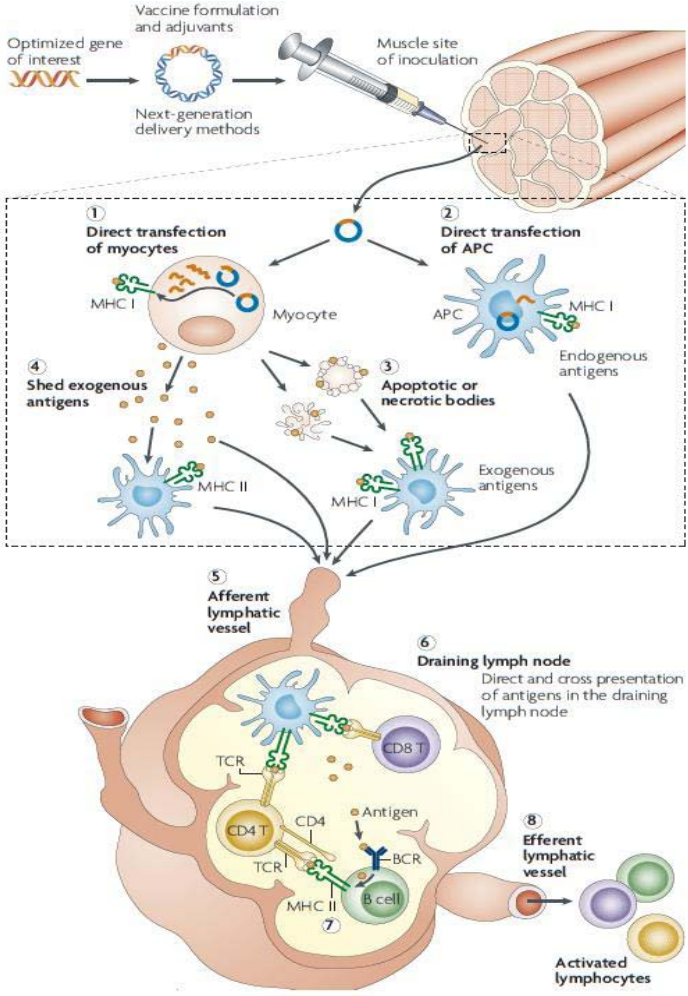

Figure 15: Idealized model of action of DNA vaccines [179].

DNA vaccines present a series of advantages in comparison to relative antigen-specific approaches (Figure 16). Above all DNA plasmids are far more stable, cheap and easy to manufacture [180].

1. Adaptive immunity—can induce robust CTL responses, helper T cells and antibodies.
2. No MHC restriction—epitopes applicable to any MHC haplotype can be presented from a DNA plasmid encoding an entire antigen.
3. Safety—DNA vaccines have demonstrated safety in multiple early clinical trials. Moreover, they preclude handling of virulent pathogens or pathogenic proteins from other vaccine approaches that could potentially subdue immune responses, mask critical epitopes, or cause infection or transformation. No immune response against the vector is induced.
4. Adjuvant effect—double-stranded DNA and hypomethylated CpG motifs of plasmid DNA can stimulate innate immune receptors to cause cytokine release.
5. Adaptability—can encode altered proteins or epitopes to enhance immune responses. Can be coupled with various adjuvants in protein or DNA form.
6. Stability—plasmid DNA is a stable moiety and does not require unusual storage and transport conditions.
7. Economy—can be easily and cost-effectively manufactured.

CTL = cytotoxic T-lymphocyte.

Figure 16: The advantages of DNA vaccines [180].

The early clinical studies of DNA vaccines revealed that those compounds are well tolerated and safe when used in human organism. Throughout the years a large number of scientific research trends emerged, pursuing the goal of the development of various kinds of DNA vaccines against cancers, HIV-1, influenza, malaria, hepatitis B and many more [181-187].

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