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A Review on Extremozymes Biocatalysis: A Green Industrial Approach for Biomaterials Production

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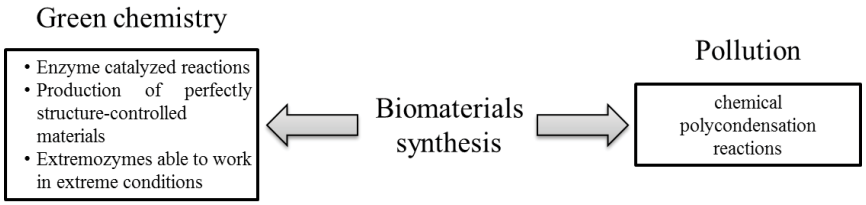
Abstract

In the last few years, the industrial attention on biomaterials production has focused on designing and developing of methods and processes to minimize the use and the generation of polluting products. In this context of green chemistry, the enzymatic catalysis could be the right way to obtain high level of polymers industrial production without the use of hazardous reagent and pollution. This review focuses on the enzymatic approach to polymer synthesis, and in particular on enzymes from extremophiles. This class of enzymes is industrial attracting showing good resistance to solvents, temperature, pH, and, in general, extreme reaction conditions. Moreover, in this manuscript are reported also the future perspectives of enzyme molecular engineering to obtain new species with more industrial interesting features.



Keywords: Enzyme catalysis; Polysaccharides synthesis; Extremophiles; Green chemistry; Polyesters production

Introduction

A biomaterial is any non-drug material that can be used to treat, enhance or replace any tissue, organ or function in an organism. Most biomaterials for environmental and medical applications are based on common polymers, such as polypropylene, polycarbonates, polyurethanes, polysulphones, poly(ethylene terephthalate) and poly(ethylene ether ketone), and mainly aliphatic polyesters. In all these cases, the polymer synthesis occurs via chemical polycondensation reactions catalyzed by a wide range of molecules such as acetates of manganese, zinc, calcium, cobalt and magnesium, antimony oxide, and titanium oxides [1]. Such reactions not only request elevated temperatures (180-280°C) but also cause undesirable side reactions and are not specific for complex polymers production.

During the past few decades, the demand for an industrial synthetic process safer, environmental "green" and more efficient for production of biomaterials is greatly increased.

The use of the enzymes as catalysts allows to obtain a green synthetic process: enzymes are recyclable eco-friendly non-toxic materials, so the complete process of polymerization is considered as a "green chemistry", reducing toxic waste and side products [2,3]. Moreover, compared to chemical synthesis, biocatalysis allows to bypass the problem of the reaction specificity, leading a production of perfectly structure-controlled material taking advantage of enzymes propriety e.g. enantioselectivity, chemo selectivity, regioselectivity, stereoselectivity, and choroselectivity [4,5]. The import of these characteristics into *in vitro* polymerization reactions involves in a perfect control of polymer structure (also for the creation of a new structure), saving energy, time and costs.

Enzymes are commonly classified, via a system of Enzyme Commission (EC) numbers, into six division, based on the chemical reactions they catalyze: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Table 1)[6].

Enzymatic catalysis has now been applied not only to polymer synthesis but also to polymer functionalization and polymer hydrolysis [7-9]. For these reactions, only hydrolases, oxidoreductases, and transferases are being used extensively in polymers and biomaterials as shown in Table 2.

Bio-catalytic Applications of Extremophiles

Many industrial reactions that produce chemicals and pharmaceuticals are often carried out under extreme conditions such as high temperature and pressure, non-neutral pH, and in non-aqueous environments. In this context, enzymes capable to work in unusual conditions are very interesting.

Extremophiles are microorganisms that grow in environments

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considered too hostile to support life. The extreme conditions may be high or low temperature, high or low pH, high salinity, high metal concentrations, very low nutrient content, very low water activity, high radiation, high pressure and low oxygen tension (Table 3) [10]. Although several extremophiles belong to the Bacteria domain, the majority of these microorganisms are members of Archea.

Enzymes from these microorganisms, sometimes called "extremozymes", perform the same enzymatic functions as their non-extreme counterparts, but they can catalyze such reactions in conditions which inhibit or denature the less extreme forms. Extremozymes are expected to be a powerful tool in industrial biotransformation processes according to their characteristics of extreme stability also under conditions usually incompatible with biological materials [11-14].

Although the extreme growth conditions of these microorganisms could be a great inconvenient, the cloning techniques allow to overexpress extremozymes in mesophiles, producing a great improvement in industrial use [15]. The majority of proteins produced in mesophilic hosts are quite similar to the wild type enzymes: they maintain the thermostability, the correct fold and the capacity of working in extreme conditions e.g. in presence of organic solvents and chemical denaturants [16,17]. Other advantages are the resistance to the host proteases and the facility of the purification (e.g. they can be purified by using thermal denaturation of the mesophilic host proteins). The degree of enzyme purity obtained is generally adequate for most industrial uses.

Each group of the extremophiles has unique features, which can be harnessed to provide enzymes with a wide range of applications [18].

Within all extremozymes the thermophilic ones are the most interesting in industrial and/or biotechnological applications due to their excellent stability in high temperature conditions and their resistance to organic solvents and chemical denaturants.

Running biotechnological processes at elevated temperature has many advantages: high temperature enhance the bioavailability and solubility of organic compounds, decreasing, at the same time, the viscosity, leading higher reaction rates and minimizing undesired by-products [19,20]. Furthermore, another great advantage of performing biological processes at high temperatures is the reduction of the risk of contamination.

To date, many thermophilic and/or hyperthermophilic enzymes are used in industrial applications, such as polysaccharide degrading enzymes (e.g. cellulases, amylases, pullulanases, xylanases, mannanase, pectinases and chitinases), and also proteases, lipases, esterases or glycosidases.

Most of industrial applications of extremozymes are involved in the surfactants and detergent field, in cosmetics and mostly in bioremediation of solids and waste waters.

The most famous thermophilic enzyme is the DNA polymerase, isolated from hyperthermophiles, which is currently used in molecular biology to perform the polymerase chain reaction (PCR).

Examples of industrial applications involving thermophilic cellulases or amylases are the bio-stoning of jeans, the pretreatment of plant biomass or the composting materials.

Typically lipases are used for fat hydrolysis, esterification, and organic biosynthesis.

Instead the ones from thermophiles are mostly used in waste water treatments, while the ones from psychrophiles and alkaliphiles are added to detergent formulation as polymer-degrading agents. This review is focused on the extremozyme uses in biomaterials production.

Polymer Synthesis with Enzymes Catalysis

Polysaccharides production

Chemical synthesis of polysaccharides is made difficult by the presence of a large number of stereo- and regioisomers: the enzyme-catalyzed synthesis, instead, allows the control of both the regioselectivity and the stereochemistry of bond formation.

Usually two classes of enzymes are involved in this kind of reaction: glycosyl transferases and glycosyl hydrolases. The first one catalyzes the transfer of monosaccharide moieties from a glycosyl donor to specific acceptor molecule, and is *in vivo* responsible for the synthesis of most cell-surface glycoconjugates [21-23]. The acceptor molecule of a glycosyl transferase can be a monosaccharide homologous to the

EC Number	Enzyme class	Catalyzed reactions
1	Oxido-reductases	Catalyze redox-reactions by electron transfer.
2	Transferases	Catalyze the transfer of a functional group from one compound (donor) to another compound (acceptor)
3	Hydrolases	Catalyze the hydrolysis of various bonds
4	Lyases	Catalyze the cleavage of C-C, C-O, C-N and other bonds by otherwise than by hydrolysis or oxidation.
5	Isomerases	Catalyze either racemization or epimerization of chiral centers; isomerases are subdivided according to their substrates.
6	Ligases	Catalyze the coupling of two molecules with concomitant hydrolysis of the diphosphate-bond in ATP or a similar triphosphate.

Table 1: Enzymes involved in polymer reactions.

EC Number	Biocatalyst type	Polymer Synthesis	Polymer modifications	Polymer Hydrolyses
1	Oxidoreductase	Yes	Yes	Yes
2	Transferase	Yes	Yes	No
3	Hydrolase	Yes	Yes	Yes
4	Lyase	No	No	Yes
5	Isomerase	No	Yes	No
6	Ligase	No	No	No

Table 2: Enzymes involved in polymer reactions.

donor, or also a heteropolysaccharide such as oligosaccharide, protein, or nucleic acid, containing numerous glycosidic bonds [24].

Glycosyl transferases are divided in two principal groups depending on the belonging metabolic pathway: if the donor is a mono- or di-phosphonucleotides sugar is defined as Leloir enzyme (from Luis F. Leloir, the scientist who discovered the first sugar nucleotide and who received the 1970 Nobel Prize in Chemistry for his work on carbohydrate metabolism); otherwise when the donors are molecules like polyprenol pyrophosphates, polyprenol phosphates, sugar-1-phosphates, or sugar-1-pyrophosphates, the glycosyl transferases belong to non-Leloir group enzymes (Figure 1) [25]. Despite their potential, the glycosyl transferases use in oligosaccharide synthesis is limited for several reasons [26] First, the phosphorylated substrates have high cost, so their industrial use is not economical advantageous. Another problem is related to the glycosyl transferase structure: these enzymes are usually transmembrane proteins and are present in nature in small amounts, conditions that make difficult their purification and production. Only in the last few years these problems are partially overcame: nowadays several enzyme producers companies sell both cloned glycosyltransferases useful for industrial purposes, both nucleotide activated sugar produced by using enzymatic or biological techniques.

More accessible for industrial use are glycosyl hydrolases (endo- and exo-glycosidases) not only because they are expressed in all living organisms being responsible for the cleavage of glycosidic linkages, but also allowing the use of relatively inexpensive substrates. Reaction scheme with a glycosidase enzyme is showed in Figure 2: the glycosyl–enzyme intermediate can be intercepted by either water, to give the hydrolysis product, or by a glycosyl acceptor in in order to form a new glycoside or oligosaccharide.

Glycosidases can be classified as retaining or inverting enzymes depending on the stereochemistry of the bond being broken during hydrolysis. The active-site of the enzyme contains two carboxylic acid residues: in inverting glycosidases, the first one acts as an acid catalyst and the other one as a base catalyst. The retaining reaction mechanism involves a nucleophile group and an acid/base catalyst and allows the synthesis of polysaccharides.

In addition glycosidases are stereoselectives (they catalyze the formation of either α- or β-glycosidic linkage) and they can produce a numerous regioisomers depending on the acceptor structure. For this reason they are not useful for the synthesis of more complex oligosaccharides [27]. In this last case the better industrial choice is the much more specific glycosyl transferases. A list of the most used and commercial available glycosyl transferases and glycosyl hydrolases is shown in Table 4 [28-47].

The main limitation to glycosidases use in polysaccharides synthesis is the low reaction rate and the presence of side products, especially those due to the inverse hydrolysis. These limitations have been overcome recently by the introduction of 'glycosynthases'. These enzymes are mutant retained glycosidases in which the replacement of the nucleophilic residue into the catalytic site with a non-nucleophile moiety shifts the reaction equilibrium in the synthetic direction with increased yields [48,49]. This approach represents an important progress because glycosynthase can be derived from both endo- and exo-glycosidases being useful for the production of numerous oligosaccharides and glycoconjugates [50-55]. Moreover, direct evolution can be addressed to obtain increased rates and different specificity. In this case, very useful are the glycosynthases obtained from thermophiles: Mullegger et al. reported the engineering of the thermostable β-glucuronidase of *Thermotoga maritime* to generate both a glycosynthase and a thioglycoligase by a point mutation in the catalytic site of the enzyme. With this enzyme it is possible to produce uronic acid-containing glycoconjugate, such as pectins, glycosaminoglycans, heparin, heparan sulfate, chondroitin sulfate and hyaluronan, useful as therapeutic agents [56,57].

Furthermore, mannose derivatives, and also glycoconjugates are produced with synthetic enzymatic way catalyzed by glycosynthases. The production of branched (1→3)-β- and (1→6)-β-linked oligosaccharides by *Sulfolobus solfataricus* glycosynthase is very useful because they are the building blocks of the β-1,3 –1,6-glucans, elicitors in plant during the defense response against pathogens [58,59].

The recent work of molecular evolution of the glycosynthase shows also that it is possible to synthetize not only various polysaccharide structures, but also generate unnatural crystalline polysaccharides with new morphologies [60]. This new approach is very attractive for the possibilities of synthetize new biomaterials, such as polysaccharides functionalized with chemical group like azide or composite polymer materials.

Oligosaccharides and polysaccharides enzymatic synthesis has great relevance in the industrial fields not only to produce substrates for research laboratories, but also to target biopolymers in pharmaceutical fields. The bond of particular oligosaccharides on a polymer can make materials biologically active due to the biological recognition. In eukaryotes, bioactive oligosaccharides can be found on the cell surface where they are involved in several pathways such as cell growth and development, cancer cell metastasis, anticoagulation, immune recognition and response, cell–cell communication and initiation of microbial pathogenesis [61,62]. These molecules have consequently been recognized as a medicinally relevant class of biomolecules, resulting in the development of therapeutic agents based on specific oligosaccharide structures or mimics thereof [63].

Natural polymers such as polysaccharides, proteins, polyesters, etc. are synthesized in nature utilizing enzymes, so the same enzymes can be utilized to degrade them in a way based on chemical clocks. The rate of the hydrolysis reaction is programmed via selection of the detailed chemical environment around the ester bonds, such as side groups, crystallinity and hydrophilicity.

Polyesters production

Esterases are enzymes that catalyze the hydrolysis of an ester into an acid and an alcohol, while lipases are a subclass of the esterases, having as natural substrates long-chain fatty acid triacylglycerols. Both classes of enzymes are the most widely used biocatalysts in fine chemical applications. Microbial lipases are divided in three subcategories depending on the broken bound type in the reaction [64]. To the first group belong nonspecific lipases that break down acylglycerol molecules at random positions, producing free fatty acids and glycerol with monoacylglycerols and diacylglycerols as intermediates. Lipases (1,3-specific) catalyzing the release of fatty acids at positions 1 and 3 of the glycerol backbone are included in the second group and could be specific for an exact fatty acid. Finally, the lipases belonging to the third group preferentially catalyze the release of long-chain fatty acids containing a cis double bond in the 9-position from triglycerides. The scheme of the three lipase groups is shown in Figure 3 [65].

Lipases and esterases are stable in organic solvents and can catalyze the reverse reactions in non-aqueous media, resulting in esterification or

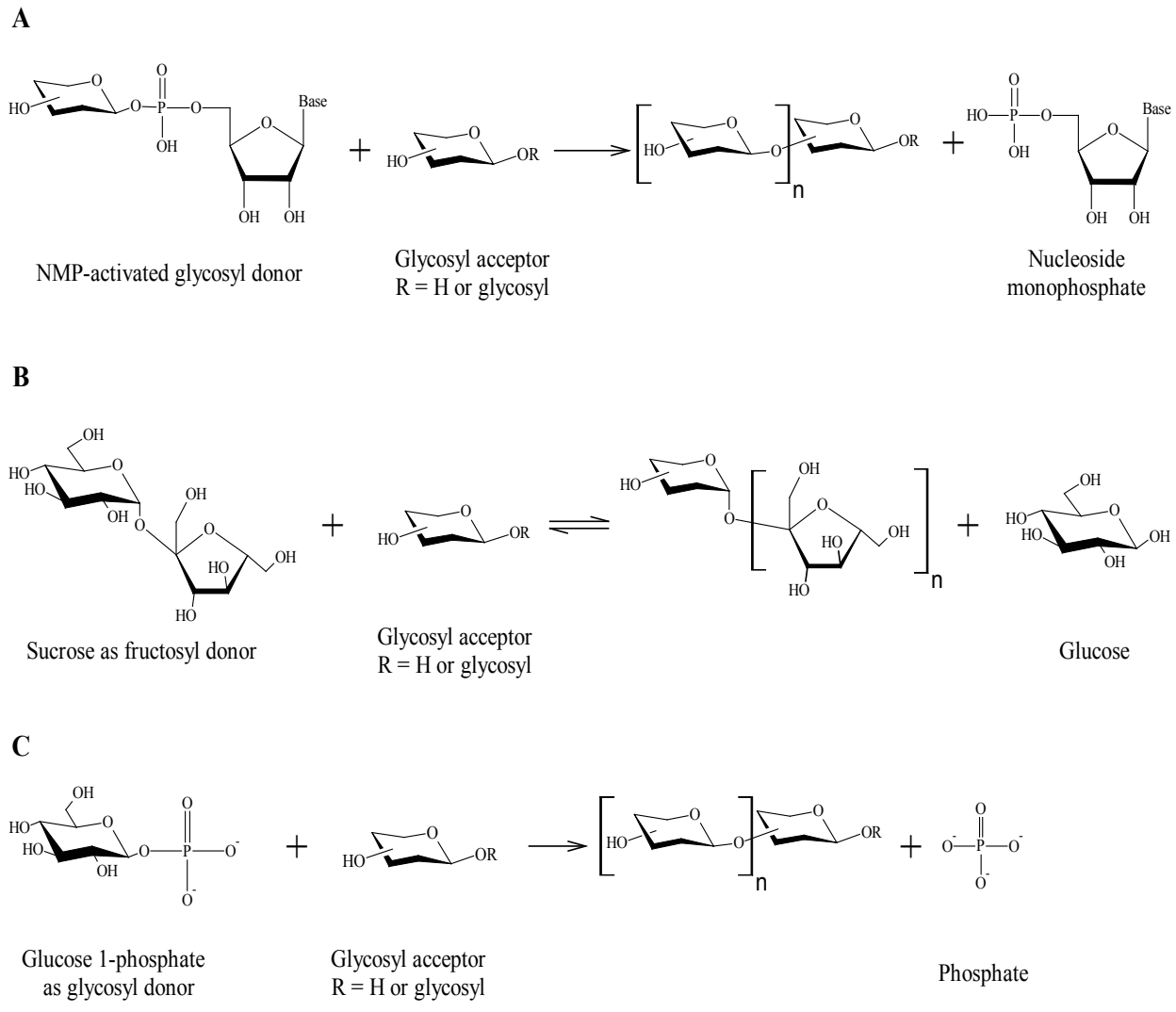

Figure 1: Reaction scheme of Leloir and non-Leloir glycosyl transferases. A: Leloir glycosyl transferases with a mononucleotide phosphate sugar (NMP) as a donor; B and C: non-Leloir enzymes with two different substrates: sucrose and glucose 1-phospate, respectively.

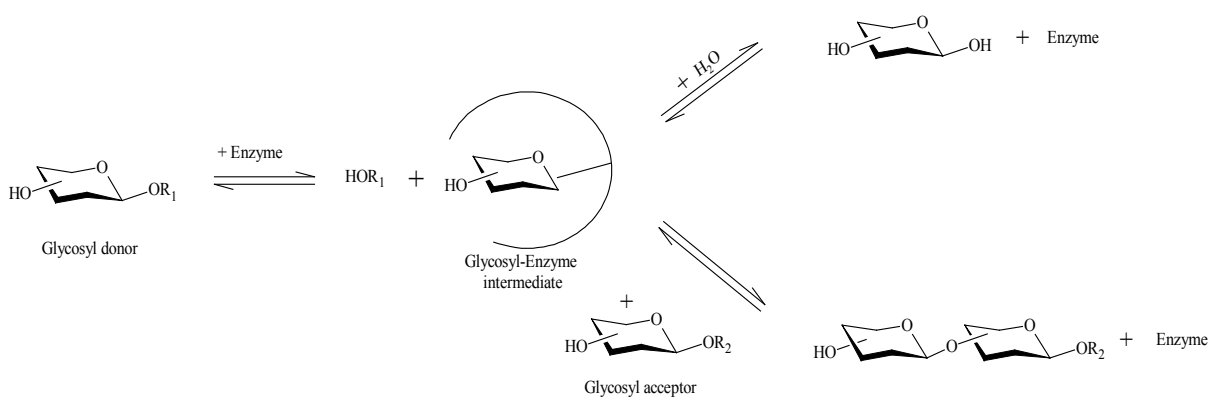

Figure 2: Scheme of a glycosidase catalyzed reaction.

transesterification. Precisely for these characteristics lipases have found applications in numerous fields, such as fat splitting, modification of fats and oils, detergent supplements, analytical procedures and mostly synthesis of organic compounds, especially polyesters.

Usually, enzymatic polyesters synthesis could be made via ring-opening polymerization of lactones (ROP) or via polycondensation, divided into polycondensation of diacids or their activated esters with diols and self-polycondensation of hydroxyacids or their activated esters (Figure 4).

The most widely used lactones or lactides in ROP include D- and L-lactide (LA), glycolide (GA) and ε-caprolactone (CL) (Figure 5) due to their suitable hydrolyzability and biocompatibility as polymers.

Aliphatic polyesters (like poly(ε-caprolactone) - PCL), are very important in the industrial synthesis because these materials are used for various biomedical applications, such as resorbable implant materials for tissue engineering and vehicles for drug or gene delivery. This is a consequence of the polymers biodegradability in the human body through hydrolysis into their corresponding hydroxy acids that can be metabolized. Their application ranges from sutures to bone fixation.

To date, various lipases or esterases have been employed in the ROP of ε-caprolactone, such as porcine pancreatic lipase, *Pseudomonas cepacia* lipase, *Candida antarctica* lipase B (CALB) and *Humicola insolens* cutinase (HIC), and between extremophiles, the *Archaeoglobus fulgidus* carboxyl esterase (AFEST) and the lipase from *Fervidobacterium nodosum* (FNL) [66-69]

However there are several problems regarding the low catalytic activities displayed by enzymes in non-aqueous media as shown by Kobayashi et al. and Dong et al. [70,71]. In these cases the authors reported the kinetic enhancement of *in vitro Pseudomonas sp.* lipase-catalyzed ring opening lactone polymerization. They show that the water content and the nature of the monomer are important to obtain a polymer production compatible with the industrial purposes.

Many studies involve the lipase B from *Candida antarctica* (CALB) as an efficient catalyst for hydrolysis in water and esterification in organic solvents. CALB is widely used in industrial applications for its high enantioselectivity, wide range of substrates, thermal stability, and stability in organic solvents [72]. In order to increase the polymerization rate, the associated company of Sigma-Aldrich, Novozymes, sells CALB as immobilized enzyme (Novozyme-435). Kobayashi et al. show not only that Novozyme-435 causes a much faster lactones polymerization respect to powdery lipases, but also that is possible to recycle the enzyme for many times [70]. In addition the mesophilic *Pseudomonas sp.* lipase showed a half-life of over 13 h at 90°C, whereas CALB has a half-life of only 2 h at 45°C [73,74]

Also in this case enzymes from thermophiles are the best candidates to perform the synthetic reaction at higher temperature and in presence of organic solvents.

Ma J. et al first reported the ring-opening polymerization of ε-caprolactone catalyzed by thermophilic esterase isolated from thermophilic microorganism *Archaeoglobus fulgidus* (AFEST). The synthesized PCL was of low molecular weight (M_n value of 1,400 g/mol), and for this reason the authors speculated its use as the soft segment of polyurethanes [75]. Moreover, compared with the reported lipases, AFEST had the highest affinity for ε-caprolactone as demonstrated by Michaelis - Menten kinetic analysis and molecular docking. AFEST

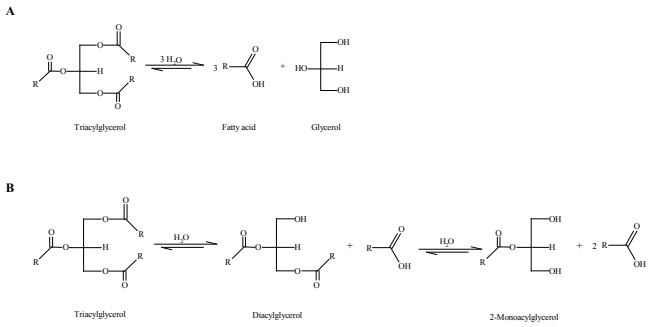

Figure 3: Lipase reaction mechanism. A: mechanism of non-specific lipases. B: mechanism of regiospecific lipases.

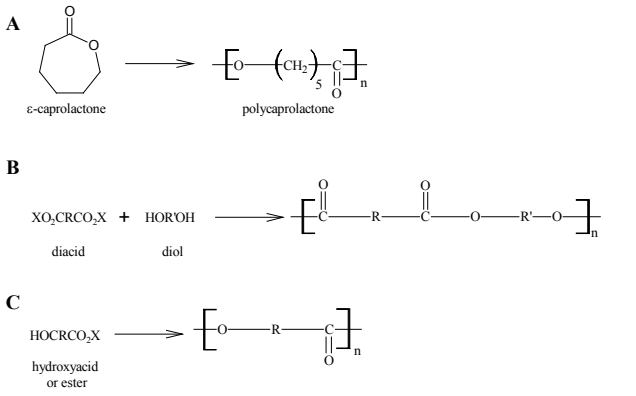

Figure 4: Polyester synthesis mechanism. A: An example of ring-opening polymerization for the synthesis of poly-caprolactone; B: polycondensation of diacids or their esters with diols; C: self-polycondensation of hydroxyacids or their esters.

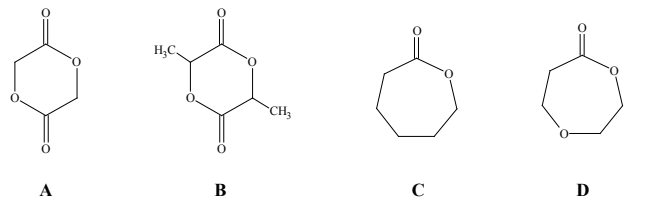

Figure 5. Most used monomers in ring-opening polymerization. A: Glycolide; B: D-, or L-lactide; C: ε-caprolactone; D: 1,5-dioxepan-2-one.

was improved by G. Li and Q. Li by immobilization on hydrophobic macroporous resin [76]. The use of immobilized enzymes not only can have a good effect on the enzyme stability, can produce economic benefits to the biocatalytic process through enzyme reuse, but also can be a method of election to scale processes from laboratory to industry. G. Li and Q. Li showed that by optimization of reaction parameters, PLC was obtained at an almost 100% monomer conversion rate and with a low average molecular weight (< 1,100 g/mol). Moreover, they obtained a good operational stability, with a monomer conversion value of more than 55% after four batch reactions. AFEST exhibited the highest activity towards p-nitrophenyl hexanoate, and no activities towards longer p-nitrophenyl esters (≥ C10) and triglycerides were detected [77].

The same authors, more recently, developed also another catalyst for polyesters synthesis: a thermophilic lipase from *Fervidobacterium nodosum* (FNL) an extremely thermophilic, glycolytic anaerobic bacterium [78]. FNL showed different and broader substrate specificity: it catalyzes the hydrolysis of p-nitrophenyl esters from C12 to C16, and triglycerides of short to mid-acyl chain (C4 to C10) and olive oil.

Polyesters derived from three monomers (lactide, glycolide, and caprolactone) are generally used for clinical purposes and are mostly characterized by degradation times ranging from days to years depending on formulation and initial Mw.

Aliphatic polyesters, poly(lactic acid) (PLA) and poly(hydroxyalkanoates) (PHAs), are a central class of biodegradable polymers, because hydrolytic and/or enzymatic chain cleavage of these materials leads to α-hydroxyacids which, in most cases, are ultimately metabolized by the host. For this reason, since the 1970s, FDA has approved their use for a variety of clinical applications, e.g. for surgical sutures and drug delivery systems, having good biodegradability, biocompatibility, and reasonably good mechanical properties [79-81].

Because lactic acid is a chiral molecule, existing in L and D isomers (the L isomer is the biological metabolite) the polylactic acid can be: 1) pure poly-L-lactic acid (L-PLA), 2) pure poly-D-lactic acid (D-PLA), and 3) poly-D,L-lactic acid (DL-PLA). All polymers in this family are insoluble in water but degrade by hydrolytic attack of the ester bond. That characteristic is central in the setup of the drug design because the rate of the ester bond hydrolysis is directly related to the pharmacokinetic.

However, the PLA homopolymer does not allow the binding of ligands: for this reason and to obtain a material with different characteristics, it is possible to co-polymerize the various monomers, e.g. in the case of copolymer poly(lactic acid-co-lysine). The ε-amino groups on the lysyl comonomer provide sites of ligand grafting in solution [82,83]. In this case it is possible to display the ligand in the bulk of the material, and provide the release on the surface during the process of polymer degradation and remodeling. Another good technique to incorporate ligand in biomaterials is the production of gels that are subject to cell infiltration. Borkenhagen et al. successfully demonstrated that bioactive migration promoting peptides, such as the YIGSR domain and the SIKVAV domain from laminin, incorporated within gels are very useful for nerve regeneration. In their work, the peptides were incorporated throughout the bulk of the three-dimensional gels to permit the infiltrating neuronal cells to contact the signals on all sides and at all times during the infiltration process.

Moreover, blending techniques are an extremely promising approaches that can be used to tune the original properties of the polymers [84]. For these reasons, several copolymers of poly(hydroxyalkanoates) (PHAs) are also widely available: PLA copolymers often consist of poly(D, L-lactic acid) (PDLLA; isomer copolymers), while PHAs have numerous copolymers available (more than 100 monomers in the PHA family have been reported). However, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and poly(3-hydroxybutyrateco-3-hydroxyhexanoate) (PHBHHx) are the most frequently examined, presenting advantages in biodegradability and biocompatibility [85]. In particular, miscible blends have been formed with polyoxyethylene (PEO), poly(epichlorohydrin) (PECH) and poly(vinylacetate) (PVAc). Partly miscible blends are formed with poly(methylmethacrylate) (PMMA), while compatible blends have been obtained with polycaprolactone (PCL), ethylene-propylene rubber (EPR), polybutylacrylate (PBA), and polysaccharides.

Another type of poly(hydroxyalkanoates) is polyhydroxybutyrate (PHB), a natural polymer produced by different bacterial strains like *Bacillus megaterium*. Pure PHB is thermoplastic with properties comparable to polypropylene, but its properties can be modified by blending or inclusion of other monomers [86-88].

PLLA and PHB are approved for use in medical applications such as sutures, scaffolding, and stents: Qu et al. implanted PLLA (26.4% crystalline) and PHB (65% crystalline) into rabbits to study the material degradation over a six-month period [89].

For tissue engineering and regenerative medicine, materials with a slower degradation should be selected [90]. PLLA has been widely used in ligament devices, degrading completely into lactic acid within a period of between 10 months and 4 years depending upon its molecular weight, crystallinity, shape, and implantation site [91]. Moreover, Chen et al. show that when PCL is combined with PLLA the degradation rate decreases due to PLC low stiffness. This result indicates also that it is possible to control the hydrolytic rate constant of the material, by co-polymerization or blending with different types of materials.

Several studies on development of ligament tissue biodegradable devices show that by using different fibers and varying diameter, architecture, and many commercially available materials it is possible to obtain wide range of degradation profiles and mechanical properties [79,92].

A list of the most used and commercial available lipases and esterases is shown in Table 5 [93-98].

Future Perspectives

Enzyme-catalyzed syntheses are becoming more and more important in contemporary industrial chemistry for both the increase of

Extremophiles	Habitat
Thermophiles	High temperature: Moderate thermophiles (45-65°C) Thermophiles (65-85°C) Hyperthermophiles (>85°C)
Psycrophiles	Low temperature
Acidophiles	Low pH
Alkalophiles	High pH
Halophiles	High saline concentration
Piezophiles	High pressure
Metalophiles	High metal concentration
Radiophiles	High radiation levels
Microaerophiles	Growth in <21% O_2

Table 3: Extremophiles and their habitats

Family	Enzyme name	EC Number	Sources	Microorganism classification	Industrial application	Ref.
Transferases	Hyaluronan synthase	2.4.1.212	*Streptococcus equisimilis*	Mesophilic bacterium	Biomedical applications, Biomaterials development; Production of synthetic hyaluronan	28,29
			Streptococcus pyogenes	Mesophilic bacterium		
	Cyclodextrin glucosyltransferase	2.4.1.19	*Bacillus stearothermophilus*	Thermophilic bacterium	Applications in many different fields: food, chemical, pharmaceutical and textile industries as well as in biotechnology, agriculture and environmental protection; Synthesis of α-cyclodextrin to improve the quality of baked goods; Hydrolysis of amylose with production of α-cyclodextrin	30-34
			Thermoanaerobacter spp	Thermophilic bacterium		
			Anaerobranca gottschalkii	Thermoalkaliphilic bacterium		
			Thermococcus sp.	Hyperthermophilic archaeon		
	4-α-Glucanotransferase	2.4.1.25	*Pyrococcus kodakaraensis*	Hyperthermophilic archaeon	Production of cycloamylose; Conversion of starch into a thermoreversible gelling derivative used in food industry	35,36
			Thermus thermophilus	Thermophilic bacterium		
	1,4-α-glucan branching enzyme	2.4.1.18	*Aquifex aeolicus*	Hyperthermophilic bacterium	Conversion of amylose into amylopectin; Application in sport drinks	37-39
			Rhodothermus obamensis	Thermophilic bacterium	Production of branched glycogen	
Hydrolases	β-glycosidase	3.2.1.21	*Thermus thermophilus*	Thermophilic bacterium	Synthesis of β-(1→3) disaccharides	40
	Chitinase	3.2.1.14	*Bacillus licheniformis*	Thermophilic bacterium	Degradation of chitin to chitobiose; N-acetyl glucosamine (chitobiase); chitosan (deacetylase) in food, cosmetics, pharmaceuticals, and agrochemicals industries	41,42
			Thermococcus chitinophagus	Thermophilic archaeon		
	Cellulase	3.2.1.4	*Thermoascus aurantiacus*	Thermophilic fungus	Hydrolysis of cellulose for biorefineries, pulp and paper, textile and food/feed industries	43-45
			Pyrococcus horikoshii	Hyperthermophilic archaeon		
			Thermotoga neapolitana	Hyperthermophilic archaeon		
	Glucoamylase	3.2.1.3	*Sulfolobus solfataricus*	Thermoacidophilic archaeon	Thermoreversible starch gels for food applications; Production of cycloamylose, ethanol, and sugars; Starch saccharification	46
	Xylanase	3.2.1.8	*Dictyoglomus sp.*	Thermophilic bacterium	Lignin removal in the various stages of bleaching	47

Table 4: Most used enzymes in polysaccharides synthesis

Family	Enzyme name	EC Number	Sources	Microorganism classification	Industrial application	Ref.
Lipases	Triacylglycerol lipase	3.1.1.3	*Pseudomonas cepacia*	Mesophilic bacterium	Production of esters for food industry as flavor and aroma constituents; Synthesis of polyesters and polyurethanes; Polymerization of ε-caprolactone; Biofuel production; Synthesis of enantiopure compounds	72,75,76,93
			Archaeoglobus fulgidus	Thermophilic archaeon		
			Candida antarctica	Mesophilic fungus		
	Cutinase	3.1.1.74	*Humicola insolens*	Mesophilic fungus	Synthesis of polyesters for drug delivery applications	67
Esterases	Carboxyl esterase	3.1.1.1	*Bacillus sp.*	Mesophilic bacterium	Synthesis of chiral drugs; Synthesis of optically pure compounds; Synthesis of polyesters	78,94-97
			Arthrobacter globiformis	Mesophilic bacterium		
			Bacillus stearothermophilus	Thermophilic bacterium		
			Pyrococcus furiosus	Hyperthermophilic archaeon		
			Fervidobacterium nodosum	Thermophilic bacterium		
	Feruloyl esterases	3.1.1.73	*Aspergillus niger*	Mesophilic fungus	Release of ferulic acid from plant cell wall into vanillin production process	98
			Aspergillus tubingensis	Mesophilic fungus		

Table 5: Most used esterases and lipases and their industrial application

commercially available enzymes and the development of new methods in biochemistry, including genetic engineering, that are expected to provide extremely pure enzymes at acceptable prices. In this contest, although the use of extremophilic enzymes in the industrial synthesis of polymers is very promising and interesting, in many cases is limited by several difficulties such as bacterial growth condition, enzyme purification and quantitative production.

The extremophiles peculiar characteristics concerning the maintenance of enzymatic activity also when the reactions are performed in harsh industrial conditions are too interesting to be loosened.

For all these motives in the last few years the research is more oriented to molecular engineering of well-known enzymes. With this approach it is possible to manipulate the kinetic characteristics of enzymes to make them more useful for any specific request. One approach to engineering enzymes is to make specific modifications, but this methodology involves an extensive knowledge of the relationship between sequence, structure and function. That kind of information, necessary for the application of rational engineering approaches, is available for only a tiny fraction of known enzymes. The glycosynthases described before are produced with this approach: the catalytic site and the kinetic mechanisms were well-known and a single DNA point mutation completely changes the specificity of the enzyme.

Another approach useful for modifying enzymes in the absence of such knowledge is the directed evolution. This method works in the same way as natural evolution, by combining mutation with selection or screening to identify improved variants. Of course, it is necessary

the creation of a library of mutated genes. The screening of the gene products allows identifying those showing improvement with respect to the desired property or set of properties. It is possible also to perform more cycles of mutation and screening in order to accumulate beneficial mutations. Most directed evolution strategies involve making relatively small changes to existing enzymes. This takes advantage of the fact that enzymes often have a range of weak promiscuous activities that are quickly improved with just a few mutations [99]. Aharoni et al. show that the evolution of a new function is driven by mutations that have little effect on the native function but large effects on the promiscuous functions that serve as starting point. Thus, an evolving protein can initially acquire increased fitness for a new function without losing its original function.

So, the increasing use of rational design in conjunction with directed evolution allows the generation of libraries containing a high frequency of sequences with the desired functional properties, useful for industrial purposes.

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