Biocatalysis for sustainable chemistry and bioeconomy

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Abstract

The unique selectivity of enzymes, along with their remarkable catalytic activity constitute powerful tools for transforming renewable feedstock but also for adding value to an array of building blocks and monomers produced by the emerging bio-based chemistry sector. Although some relevant biotransformations run at ton scale demonstrates the success of biocatalysis in industry, there is still a huge untapped potential of catalytic activities available for targeted valorization of new raw materials, such as waste streams and CO_2. In some instances, scientific advances have already delivered specific segments of innovation that need to be integrated with economics, environmental and technological information according to a systemic vision. Computational tools and effective big-data analysis are expected to pave the road for the successful implementation of biocatalysis within these new scenarios of bio-based chemistry and bioeconomy.

Keywords: industrial biotechnology; biocatalysis; bioeconomy, renewable feedstock, bio-based chemistry; sustainable chemistry

Biocatalysis beyond pharmaceutical and fine chemistry applications

According to A. Bommarius, “Biocatalysis is the general term for the transformation of natural and non-natural compounds by enzymes” [1]. Therefore, biocatalysis is generally referred to the use of enzymes and microorganisms in chemistry. During the last decades, biocatalysis has delivered sustainable technologies and selective enzymes that have promoted the transition of chemistry towards processes that are environmentally benign. Conversely, scientific advances in biocatalysis have been boosted primarily by requests coming from the pharmaceutical industry and fine chemistry sector, which make use of processes often characterized by low atom efficiency and high production of waste [2].

The biocatalyst market for specialty enzymes, where pharmaceuticals are the prominent application, is accounted only for $230 M. Although these figures do not take into account own production and usage of enzymes by companies, it is evident that specialty enzymes represent a limited portion of the global industrial enzyme market, which in 2013 was valued at around $4.4 B (Figure 1) and by 2020 it is expected to reach 111.7 kt by volume [3-5]. This growth is motivated by the increasing industrialization, the growing environmental concerns but especially by the evident benefits coming from the use of enzymes in multiple technical applications in the food, environmental, biomedical and energy sectors [6].

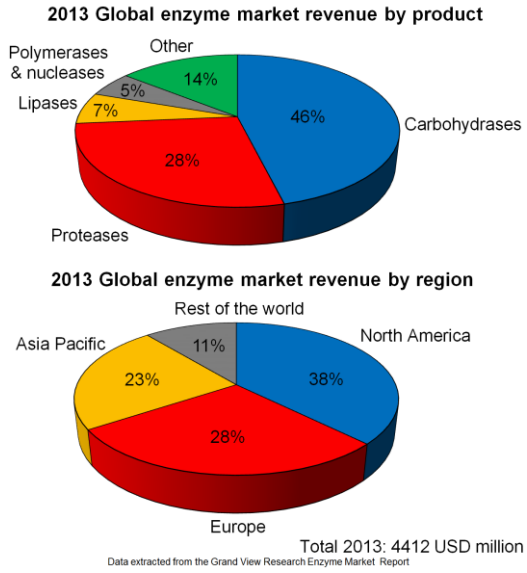

Figure 1. Global enzyme market revenue by product (top) and by region (bottom) in 2013.

However, it must be noted that previous growth expectations resulted too optimistic: in 2009 a study from Freedonia group [7] forecasted global enzyme market to reach $7.0 B yet in 2013, whereas it was later calculated in about $4,8 B [8]. Actually, it is quite difficult to analyze enzyme market, since three companies (namely Novozymes, Du Pont and DSM) hold the 70% of the shares and several enzyme consumers have their own production facility or regulate their supplies by means of joint ventures with producers. At the same time, new players like China and other eastern countries are becoming important enzymes producers, also gaining new technologies and innovation, and their real role within the market is not always taken into account. In general, it is evident that there is a wide potential for fermenting biocatalytic proteins worldwide and for expanding the benefits of biocatalysis to different segments of the chemical sectors. These include specialty chemicals intended for the so-called “B to B” (business to business) applications, which represent the starting materials in several processes (e.g., production of pharmaceuticals, pigments and crop protection chemicals). In addition, there are the so-called consumer chemicals, addressing the “B to C” (business to consumer) market of cosmetics, detergents and perfumes [9].

The future impact of enzymes and biocatalysts on the different segments of chemistry will be influenced not only by the urgency to innovate process technologies in order to meet sustainability criteria: new economic (i.e. lack of resources), political (i.e. United Nations Framework Convention on Climate Change) and regulatory scenarios set the basis for the gradual replacement of petrochemical feedstock by new platforms of bio-based chemical intermediates and polymer building blocks. Many examples are already evident and available on the global market, produced by fermentation of biomass components or recovered as side products of biomass processing (e.g. glycerol derived from biodiesel synthesis). Indeed, there is a potential synergy between processes leading to biofuels and the success of the new platforms of chemicals or precursors for fine chemicals and this switch to sustainable resources influences the whole chemistry production chain (market

pull). Furthermore, the ongoing revolution in life sciences has a huge expanding effect on the possibilities to develop sustainable, environment-friendly, energy saving, clean bioprocesses.

Besides these non-technological factors, it is important to consider the “technology push”: advances in the field of genomics, with impressive automated sequencing possibilities, fast *in silico* screening, and highly efficient use of metagenomics databases expand the chances for tailoring biocatalysts properties while reducing laborious and expensive laboratory practices [9].

This picture has inspired the present review article, which intends to discuss some emerging applications of biocatalysis. The selected examples demonstrate that biocatalysts are key enabling tools for bioeconomy and bio-based chemistry, provided that biocatalysis evolves beyond the conventional aapproaches for developing processes in the fine-chemistry and pharma sectors. The new solutions must take into account stringent technological, economic and environmental constraints at the same time. A new systemic approach is necessary, where the entire biocatalytic process is planned and optimized as a whole rather than resulting from the assembling of discrete fragments of innovation. Within such vision, big data exploitation and new computational methods for analysis, function automation and optimization are expected to become routine elements in biocatalysis research and innovation.

Will biocatalysis boost bio-based chemistry?

A recent report commissioned by the Biobased Industries Consortium estimated the European bioeconomy market to account for €2.1 T in 2013, while the annual U.S. bioeconomy market is estimated at about $330 B [10]. The main EU market shares are represented by food and beverage sectors (about 50%) followed by agriculture and forestry segment (21%), with the remaining part related to the so-called bio-based business,

including chemicals, pharmaceuticals, biofuels and bioenergy, which is the context where biocatalysis is applied [11].

The global bio-based chemical market in 2012 accounted for about 9% of the total sales of chemicals and it is expected to reach 11% of the worldwide chemical market by 2020, meaning around €350-400 B. Overall, the growth of the bio-based market should reach an annual rate of 8% over the preceding decade, with biopolymers, renewable chemicals, and industrial biocatalysts having the highest growth rate [12]. The question is whether and at what extent the technological and scientific potential of biocatalysis will intersect and boost the growth of the bio-based chemistry sector.

The interest of the chemical sector to develop bio-economy is global and it stimulates alliances with the biotechnological and rural sectors throughout the different bioeconomy value chains [13]. As extensively discussed by M. Franssen in a recent review [14], biomass and renewable feedstock contain in their chemical structure most of the functional groups that are currently introduced in fossil-based chemicals with high energy and capital costs. Economic analysis indicates that 7% of annual petroleum consumption ($88*10^6$ barrels per day in 2011) goes to chemistry sector, which makes use of six fundamental groups of chemicals, including methane, ethylene, propylene, C_4 olefins and a few aromatics [15]. On the other hand, enzymes are able to transform natural molecules into an array of functionalised chemicals or intermediates that are nowadays produced from fossil oil by the petrochemical industry, with the advantage that biocatalysts work optimally at much lower temperatures and milder conditions (Tables 1 and 2).

Oils and fats		Biocatalyst	Biotransformation	Product	Reference
Unsaturated fatty acids		Lipase	Epoxydation	Epoxyacids	[123]
	Oleic acid	*Candida anctartica* Lipase B (Novozym 435)	Epoxydation	Epoxy-stearic acid	[128] [129]
	Rapeseed oil	*Candida anctartica* Lipase B (Novozym 435)	Epoxydation	Rapeseed oil fatty acids	[130]
Olein fatty acids		*Candida anctartica* Lipase B	Esterification Amidation	Fatty amides	[131]
Palm olein		*Candida anctartica* Lipase B (Novozym 435); Lipozyme		Fatty amides	[132]
Saturated fatty acids		*Candida anctartica* Lipase B (Novozym 435);	Transamidation	Alkanolamides (amide surfactants)	[133]
Vegetable and waste oils		Lipases	Transesterification	Biodiesel (FAME)	[126] [127]
Vegetable oils	*Jatropha curcas* oil	*Burkholderia cepacia* lipase	alcoholysis	Biodiesel (FAME)	[77]
	Pistacia chinensis seed oil	*Rhizopus oryzae* lipase	Transesterification	Biodiesel (FAME)	[78]
	Babassu and palm oils	*Thermomyces lanuginosus* (TLL); Pseudomonas fluorescens (PFL) lipases;	Transesterification	Biodiesel (FAME)	[115]
	Soybean and rapeseed oil	*Thermomyces lanuginosus* lipase (TLL); *Candida anctartica* lipase B; *Pseudomonas cepacia*; *Rhizopus oryzae* lipase	Transesterification	Biodiesel (FAME)	[111] [116] [119] [120] [128]
	Olive oil	*Pseudomonas gessardii* lipase	Hydrolisis	TGA	[83]
	Palm oil	*Rhizopus niveus* lipase; *Candida anctartica* lipase B	Interesterification	Cocoa butter substitute	[104] [106] [107] [117]
	Various vegetable oils	Various lipases	Interesterification	Human milk fat substitutes	[112] [113]
Waste oils and fats		Lypozyme RM IM; *Rhizopus oryzae* lipase; Novozym 435; Lipases LS-10A, *Candida sp.* lipase	Transesterification	Biodiesel (FAME)	[79] [80] [114][118] [108] [109] [110] [121]* [122]*
Slaughterhouse lipid wastes and vegetable oils		*Pseudomonas gessardii* lipase	Hydrolisis	TGA	[81]
Microalgal oil		*Burkholderia cepacia* lipase (immobilized)	Transesterification	Biodiesel (FAME)	[82]
Phytosterols		*Candida rugosa* lipase	Esterification	FFA	[84]
Fish oil		Lipases	Selective concentration of EPA and DHA	Omega-3 concentrates	[85]
		Porcine pancreas lipase	Re-esterification	Monoacylglycerols (MAG)	[105]
Glycerol		Lipase (Novozym 435)	Glycerolysis	Glycerol carbonate Mono- and diacylglycerols	[86] [87]
		TEMPO/laccase	Oxidation	Glyceraldehyde, glyceric acid, tartronic acid (for cosmetics and pharmaceuticals),	[88]

Table 1: Main examples of biocatalyzed transformations applicable in the oleo-chemical sector.

115

Carbohydrates		**Biocatalyst**	**Biotransformation**	**Product**	**Reference**
Cellulose					
	Microcristalline cellulose	CLEAs of *Trichoderma reesei* cellulase	Hydrolysis	Glucose	[89]
	Cellulose from corn cob (1st step)	Cellulase cellulase from *Trichoderma reesei* (immobilized) – 1st step	Hydrolysis	Glucose	[90] [91]
	Cellulosic hydrolysate (2nd step)	*Lactobacillus delbrueckii* (immobilized cells) – 2nd step		Lactic acid	
	Cellulose from sugar beet pulp	Cellulase	Hydrolysis	Cellobiose	[92]
	Cellulosic biomass sugars	Glucose isomerase	Hydrolysis	Ethanol	[96] [97]
	Peanut-shell hydrolysate	Xylose isomerase	Isomerisation	convert D-xylose to D-xylulose in ethanol production	[98]
	Cellulosic biomass Sugar cane biomass Waste woody cellulosic materials Switch grass (*Panicum virgatum* L.)	Cellulases and xylanases	Hydrolysis	Ethanol	[101] [102] [103]
Grain products and cane sugar juice or molasse		Xylose isomerase	Isomerisation	convert D-xylose to D-xylulose in ethanol production	[99] [100]
Starch		Amidases	Hydrolysis	Maltose, glucose	[93] [95]
		Aspergillus carbonarius starch digesting amylase	Hydrolysis	Maltose, glucose	[94]
		Glucoamylase and *Saccharomyces cerevisiae*	Saccharification and fermentation (SSF)	Ethanol	[138]
Lignocellulosic biomass		Cellulales; hemicellulases	Hydrolysis	Biofuels	[124] [125]
Glucose		Glucose isomerase	Isomerisation	Fructose	[147]
Galactose		*Aspergillus oryzae* β-galactosidase	Oligomerisation	Galactooligosaccharides (GOS)	[134] [135]
Lactose		β-galactosidase	Transgalactosylation	Galactooligosaccharides (GOS)	[136] [137]
Proteins and ammino acids from animal and plant sources					
Soybean flour Egg white		*Aspergillus Oryzae* Protease	Hydrolization	Aminoacids	[139]
Phenylalanine		Phenylalanine ammonia lyase (PAL)	Deamination	Cinnamic acid	[140]
alanine		Decarboxylase	Decarboxylation	Ethylamine	[141]
Glutamine and glutamic acid		Decarboxylase	Decarboxylation	γ-aminobutyric acid (GABA)	[142]
D,L-Methionine		*Aspergillus oryzae* acylase	Resolution	L-methionine	[146]
Lignin		Lignin peroxidases; manganese peroxidases; versatile peroxidases; laccases.	Oxydation		[143] [144] [145]

116

117 **Table 2**: Biocatalized transformations of proteins, aminoacids and lignocellulosic derived substrates.

When considering the transformation of biomass and bio-renewables, the introduction of complex functionalities translates into an increase of the value of chemicals, as compared to commodities and biofuels, so that there is more room for enzyme applications in the production of high value chemical products. Nevertheless, it is important to underline that food and beverages sector, which delivers low cost and high volume products, is the largest application of industrial enzymes. Indeed, it has been demonstrated that one kg of an enzyme can be produced at a cost around 100 € with the aid of technologies already available for genetic and fermentation optimization [14]. This concept is confirmed by a number of large-scale processes employing enzymes also for the production of commodity chemicals [16]. For instance, amylases, because of genetic engineering, were reported to have an impact as low as 1 cent per litre on ethanol production from starch.

Enzymes are employed in these large scale processes in crude form or as whole microorganisms, representing a big share of biocatalysis, since whole cells can be easily and economically produced through cheap fermentation methods. Notably, acrylamide is produced from acrylonitrile at a scale of 10^5 tons per year in industrial processes that make use of immobilised microorganisms endowed with nitrile hydratase enzymatic activity [16].

Looking at large scale biocatalyzed processes where immobilised enzymes are employed, the most significant example is given by production of high fructose syrup by means of glucose isomerase. In the oleo-chemistry sector, fats and oils are transformed into food ingredients but also in emollient esters and biodiesel, through reactions catalysed by immobilized lipases (Table 1).

The transformation of the soluble enzymatic protein into an insoluble heterogeneous bio-catalyst represents an advantage when the recovery of the enzyme is required either for preventing contaminations or for recycling the expensive catalyst. However, the immobilization process represents also an extra economic barrier for the large-scale applications. The impact of immobilization costs is connected to the kg of product produced

per kg biocatalyst basis, which is also referred as total biocatalyst productivity. Ultimately, productivity depends on the recyclability of the enzyme, and it is suggested that costs of few hundred $ per kg are acceptable for specialty chemicals, whereas in the bulk chemical sector the economic impact must remain below $10 per kg and if often close to 0.1$ [17, 18]. Interestingly, in 1990 immobilized enzymes accounted for almost 20% of enzyme market, while now they represent a much lower fraction [16]. Those data are affected by the fact that companies using immobilized enzymes for their processes often have an internal enzyme production, or they purchase the enzyme in free form and then immobilize it in their own facilities.

Although enzyme immobilization is considered as an effective route for increasing the stability of biocatalysts, recent trends indicate that enzyme producers or big chemical firms applying enzymes for their processes prefer to focus on enzyme engineering rather than enzyme immobilization. As a consequence, enzyme engineering has been indicated as the new real disruptive innovation in the field of biocatalysis [19], with a number companies (Codexis, Pfizer, Merk, Novartis) strongly investing in it.

Nevertheless, immobilization remains a compulsive choice of many enzyme applications, as in vegetal oil transformations catalyzed by lipases [20, 21]. These enzymes remain also active and efficient in low-water environments, so that they are employed in bulk oils. However, the enzymatic proteins would aggregate when suspended in the hydrophobic substrates, whereas the immobilization on solid carrier improves the distribution and accessibility of the biocatalyst. In this context, the application of biocatalysts in the oleochemical sector has the potential to be further expanded provided that robust and cheap immobilized lipases are made available.

Overall, the application of biocatalysts in the transformation of renewable feedstock suffers from stringent economic constraints that make optimization procedures - both in terms of

enzyme stability and process design - by far more critical when compared to the practise observed in the fine chemistry sector.

From conventional feedstock to waste and CO_2

Industrial Biotechnology (IB) and biocatalysis contribute already to bioeconomy within the biorefinery context, namely by transforming different conventional biomass and renewable feedstocks into chemicals (Tables 1 and 2) [14]. Research efforts are currently directed not only at the transformation of biomass through more efficient routes but also at the identification of new and non-conventional feedstock streams as opportunities. Although some technological breakthroughs are still expected to fill some gaps (e.g. lignin exploitation, reduction of CO_2), important technological advances are already available and these innovations have effective potential to reach the market in a few years [22-25]. In other cases, biocatalysts able to catalyze useful transformations are already available but they require further optimization for meeting technological and economic standards imposed by industry.

Food industry is a major driver of biocatalysis growth so that In the last decades, the oleo-chemistry sector has massively used lipases for transforming oils and fats not only into products for food industry, such as cocoa butter analogues, but also into valuable chemicals comprising lubricants, esters for the cosmetic sector, surfactants. More recently, the scalability of enzymatic synthesis of biodiesel has been also demonstrated (Table 1). Food industry also makes use of proteases for converting peptides and proteins into shorter peptides used as supplements, ingredients of infant formula or as pharmacological active agents [26]. Moreover, amino acids obtained through fermentation are massively used by fine chemical industry as chiral-pool: the stereoselectivity of different classes of enzymes allows for the bioconversion of these cheap starting materials into chiral building blocks that

194 are at the basis for the manufacturing of an array of expensive and chemically complex chiral
195 drugs and fine chemicals (Table 2).
196 Biotransformation of sugars represents probably the oldest case of biorefinery and, also in
197 this case, the first technologies were developed for the food and beverage sector already in
198 the ‘70s with the amylase catalyzed hydrolysis of starch along with glucose isomerization to
199 fructose. Currently, they represent the largest enzymatic processes implemented at
200 industrial scale. Starch industry has capitalized on the know-how generated for
201 polysaccharide processing and promoted the surge of the first generation of biofuel industry.
202 Only in the last two decades, the scientific advances enabled the design, production and
203 optimization of a pool of hydrolytic enzymes able to split the chemically heterogeneous
204 glycosidic bonds of cellulose and hemicellulose (Table 2). Conversely, starch based
205 biorefineries were replaced by second-generation biorefineries feed by non-food-based
206 ligno-cellulosic feedstock.
207 The lignin fraction resulting as a by-product from biorefinery processes constitutes a further
208 chemical platform for the production of chemicals. The abundance of lignin in nature (25 -
209 35% of lignocellulosic biomass) makes this biopolymer the ideal source of aromatic building
210 blocks, currently derived from fossil oil (Table 2). Despite extensive research employing
211 oxidative enzymes (e.g. laccases and peroxidases) for lignin depolymerization, the
212 recovering of aromatic building-blocks from lignin structure remains a major challenge
213 because of its amorphous and recalcitrant structure [27]. One of the few commercial uses
214 of lignin is represented by the production of vanillin starting from the residual lignosulfonates
215 of the pulping industry, although the petrochemical route is still more competitive.
216 Nowadays, most lignin (also that derived from second generation biorefineries using lingo-
217 cellulosic biomass) is burnt for energy production within the pulping industry, whereas
218 chemical routes for the valorization of lignin remain of limited practical relevance [28].
219 Nevertheless, the problem of supplying bio-based aromatic building-blocks has been

circumvented by the production of styrene starting from bio-ethanol, which is firstly converted into the intermediate butadiene [29]. Moreover, the direct production of aromatics through fermentation has been also reported as feasible [30].

The chemical and, more in general, the bio-based industry seeks feedstock flexibility to lower costs and also to avoid any competition for the use of soil for food production. That has promoted the interest towards residues and agro-waste streams as a source of biomass and, most importantly, high-value bio-components [31]. This is in agreement with the concept of “second generation biorefineries”, which should rely on integrated chemical and biotechnological innovations for converting biomass/waste/residues into valuable products in a hierarchical cascade, with processing chain including returning waste and nutrients to the land. For instance, enzymes, such as pectinases, are employed industrially for promoting cell-wall hydrolysis and facilitating extraction of the hydrolysis of antioxidants, pigments, enzymes, dietary fibers, fructans, and an array of nutraceuticals [32].

It must be noted that the food wastes generated worldwide amount at about $1.3*10^9$ tons and represent an important source of chemicals, nutrients and micro-components. [15]. Besides agro-waste, fishing activity and seafood processing lead to 30 up to 70% waste, where the fish waste has an oil/fat content of 19% on a dry weight basis and can be used for biotransformations into unsaturated fatty acids. Since lipase catalyzes the splitting of the mild conditions glycerides into fatty acids and glycerol at very mild conditions, side-reactions, and especially oxidation, can be prevented, preserving the precious unsaturated w-fatty acids chemical structure (Table 1).

Of course, the most abundant and inexpensive source of renewable carbon on earth is represented by CO_2, with 36,600 million metric tons of anthropogenic CO_2 emissions [33]. It has also the advantage of overcoming any competition with land use. Enzymes have been investigated as a route for reducing the cost barrier of carbon capture technologies, which are the prerequisite for further storage (via gas injection underground for long term storage)

or exploitation of CO_2 as a feedstock [34]. One of the many methods currently under development for CO_2 capture is based on the reactivity of amines, such as mono ethanol amine, in absorber columns at 40-60 °C to form carbamates. Carbonic anhydride is then released by heating the solution to a temperature above 100 °C. This energy intensive process (about 80% of operational costs) requires also large columns to process massive amounts of CO_2. Since the rate determining step for desorption is CO_2 hydration to bicarbonate, studies are under development for the use of immobilized carbonic anhydrase, to increase the rate of CO_2 desorption. Carbonic anhydrase enzyme is involved in many biochemical processes in nature, such as detoxification pathways, respiration, pH homeostasis and photosynthesis [35]. The enzyme allows the reduction of energy related to the desorption step because catalyses the fast hydration of CO_2 at lower temperature. The advantage is two folds: lower consume of energy and smaller volume of absorber columns. Because operational temperatures are relatively high, carbonic anhydrase is generally employed in the immobilized form, which displays higher stability also upon prolonged storage improves and enables the recycling of the biocatalyst.

When talking about CO_2 as a feedstock, the enzymatic reduction of CO_2 could, ideally yield C_1 molecules such as methanol while contributing to the valorisation of a form carbon, which actually represents a major environmental concern [36]. Redox enzymes have the advantage that they are able to catalyze reactions in which conventional chemical catalysts fail. Unfortunately, the use of such enzymes is limited by the high cost of the cofactors (e.g. nicotinamide adenine dinucleotide, NAD+) necessary for redox reactions. Major efforts in the field are directed towards the *in situ* regeneration of cofactors by means of electrochemical regeneration but also cells. Hybrid enzymatic/photocatalytic approaches were reported for the bioconversion of CO_2 to methanol catalyzed by three dehydrogenases (FateDH, FaldDH, and ADH). The enzymes, encapsulated into cages of alginate and tetraethoxysilanes, consume three mol of NADH, which is then regenerated by exploiting a

visible-light-active photocatalytic system made by TiO_2. The electron (hydride) is then transferred from a H-donor such (e.g. water–glycerol solutions) to NAD+ with the assistance of a Rh(III)-complex. Globally, the process allows for the production of 100-1000 mol of methanol starting from one mol of NADH [36].

One emerging field of research is the study of bioelectrochemical systems (BESs), which are based on interactions that are established between electrodes and specific biocatalysts [37]. These electrogenic or electrically-active bacteria are able to perform 'extracellular electron transfer' (EET) and they have been isolated from different environments, including extreme environments. The use of whole microorganisms in bio-electrosynthetic systems is generally preferred because enzymes adsorbed on electrodes lack of long-term stability, although they provide higher reaction specificity and controllability [38].

BESs work as any other electrochemical cell (e.g. a battery), where an anode and a cathode are connected through an external wire that closes the electrical circuit. Optionally, a membrane separates the two electrodes. Electrogenic microorganisms oxidize organic substrates at the anode and then transfer electrons from inside their cell to the electrode. At the same time, the microorganisms release protons in the solution, where the two electrodes are submerged, and also CO_2, which can be captured. The electrons flow to the cathode, where a reduction reaction occurs.

A first type of BESs is represented by the Microbial fuel cells (MFCs), which operate under aerobic conditions. When electrons reach the cathode they combine with oxygen and protons to produce water. MFCs produce electric power, which derives from the external circuit that carries the electronic flow.

Alternatively, when the cathode operates under anaerobic conditions, the electrons reduce protons to form hydrogen. This BES configuration is referred as MEC or Microbial Electrolysis Cell and it requires, besides the energy produced by the same microorganisms,

some extra energy supply to accelerate the kinetics of substrate conversion or to drive reactions that are thermodynamically unfavourable (Figure 2).

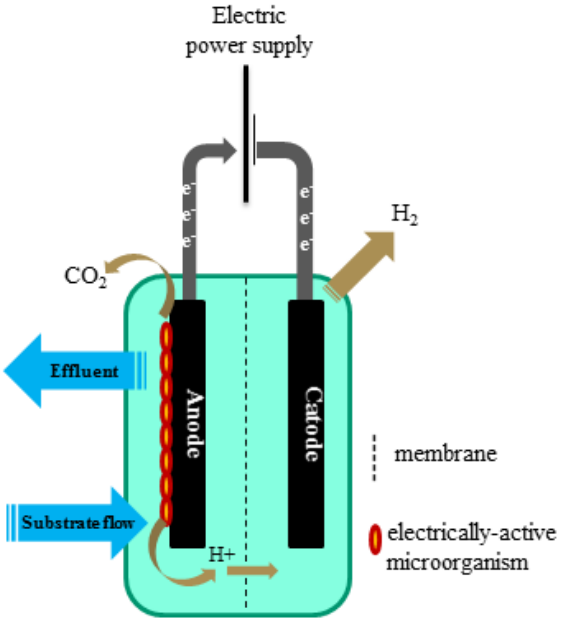

Figure 2: A schematic representation of a Microbial Electrolysis Cell. An electrically-active microorganism adsorbed on the anode surface oxidizes the chemical components present in a wastewater. The reaction liberates carbonic anhydride, protons and electrons, which flows towards the cathode. Under anaerobic conditions, protons are finally reduced to H_2.

In principle, electrogenic microorganisms can boost both MFCs and MECs by oxidizing organic components or contaminants present in wastewater [39]. Although MFCs in principle represent a route for generating large amount of energy from various waste streams, at the state of the art the electricity generated by MFCs is of scarce economic value and cannot compete with other energy sources derived from biomass degradation, such as biogas [37]. On the other hand, MECs could be a promising means for producing renewable hydrogen, an attractive and sustainable energy carrier. MECs have the advantage that they require a limited amount of energy to treat wastewater and the final energy balance is positive since the energy contained in the hydrogen produced through the process counterbalances the

electric power supplied for microbial electrosynthesis operation. They have also a four-fold higher hydrogen productivity as compared to conventional processes based on microbial fermentations and are efficient in the treatment of diluted concentration of organic components at very mild temperature (also < 20°C).

The evidence that electric power can drive microbial metabolism has inspired the concept of “Microbial Electrosynthesis” that goes beyond H_2 production but addresses even the synthesis of multi-carbon chemicals [40]. Nevin and co-workers described the reduction of CO_2 to acetate using a film of *Sporomusa ovata* cells deposited on an electrode, which directly supplied the microorganism with the electrons required for the reduction. The system was conceived as an artificial form of photosynthesis because it was powered by solar energy [40], thus realizing a fully renewable microbial electrosynthesis.

Recent analysis indicate that one major challenge for making bioelectrochemical systems of practical applicability is to elucidate and improve the mechanisms used by microorganisms to transfer electrons to the electrodes. The studies reported so far indicate that there are two main methods exploited by microorganisms for transferring the electrons. In one case the cells adhere physically to the electrode and there is a direct exchange with the electron surface. Other electronically-active microorganisms do not attach on the electrode surface but rather exploit chemical compounds that act as long-range shuttle.

Biocatalysis for valorization of bio-based building blocks: the case of bio-based polymers

Despite the green chemistry perspective and the perfect fitting in a circular economy context [41], enzymes for industrial-scale applications are in many cases still too expensive to be implemented as election catalysts when compared to the traditional chemical catalysis. From an economic perspective, enzymes could easily enter the market for applications that require mild and selective processing not feasible using traditional chemistry [42]. In this

context, application of biocatalysis to polyester synthesis and modification can be regarded as an opportunity for increasing the value and the competitiveness of bio-based polymers. Analyses indicate that the value of the renewable plastic market will increase up to $ 5.2 billion by 2030 [43]. The growth of bio-based polymer market is motivated by the need of plastic industry to decrease the environmental cost of fossil-based plastics. The analysis of UN Environmental Programme indicate that over 75% of the natural capital cost of plastic use in the consumer goods ($75 B per year) is derived from the extraction and manufacturing of plastic feedstock [44]. It appears evident that the problem requires solutions addressing not only the efficient management of plastic waste but also the migration towards more sustainable plastics.

IBs contribute to the replacement of petrol-based polymers and plastics through well-established fermentation technologies able to deliver an array of bio-based monomers usable, for instance, in polyester production. Polylactic acid represents already a success case, with a production of about 180 000 tons per year [45].

Research efforts aim not only at replacing the existing fossil-based polymers (drop-in products) but also at the design of a new generation of polymers and materials that must compete in terms of performance with the well-established fossil -based products. On that respect, an enzyme can be exploited to catalyze *in vitro* synthesis of polyesters under mild conditions but also to perform targeted hydrolysis while retaining bulk properties of the polymer. In the latter case, the objective is the insertion of functional groups on polymer surface, thus enlarging the spectrum of advanced applications.

A number of studies at laboratory [46-48] or pilot scale [49] have demonstrated the feasibility of enzymatic polycondensation and ring opening polymerization. As an example, the synthesis of aliphatic functional polyesters carrying lateral chemical functionalities, such as vinyl [50-52] and hydroxy groups [53-54] has been performed via enzymatic catalysis at temperatures of 50-70 °C. Such mild conditions prevents the undesired isomerization and

crosslinking reactions of the polymeric chains observed in the traditional chemical polycondensations, which are carried out at temperatures above 150 °C. Moreover, enzymatic catalysis brings remarkable advanced when a mild and limited surface functionalization of polymers is needed. Poly(L-lactic acid), poly(ethylene terephthalate), polyamide 6,6 and polyurethanes [42] are only a few of the polymers that were enzymatically treated in order to create functional surfaces with an increased hydrophilicity while maintaining the bulk properties of the material. These polymers was further functionalized in a second reaction step using enzymes [55], making the surface prone to the anchoring of bioactive molecules [56] or endowed with surface properties useful for packaging formulation or clothing applications [57]. If lipases are the election enzyme for synthetic reactions, all these hydrolytic processing approaches were found out to be optimally carried out using another class of hydrolytic biocatalysts, namely cutinases. They are fungal enzymes responsible in nature for the hydrolysis of cutin, the complex hydrophobic polyester that protects plant cell wall [58, 59].

Efforts are still needed for transferring the concept of enzymatic synthesis and modification of polyesters at the industrial level. Firstly, it is necessary to enlarge the portfolio of enzymes and biocatalysts. Secondly, studies indicate that applications of biocatalysis in the context of bio-based chemistry require more integrated strategies, where process engineering, environmental and costs issues are accounted and optimized at the same time [59].

Concerning process design, enzymatic polyester synthesis is preferentially carried out in solvent-free systems, thus reducing both environmental and economic costs. The viscosity of reaction mixtures is the undesired consequence so that mass transfer represents the major limitation to polymer chain growth. Actually, similar problems are encountered also in a number of different solvent-free biotransformations of renewable feedstock, such as in the synthesis of long chain esters starting from fatty acids. It is evident that classical stirred tank reactors employed in chemicals manufacturing are inappropriate for achieving efficient

mixing and mass transfer. Recently, new generations of reactors base on thin-film formation [49] or bubble column reactors [60]. Thin-film processes have the advantage that the biocatalyst is not damaged because no mixing system is necessary (Figure 3). The thin film is generated by applying centrifugal force to the system and it has been demonstrated that that does not cause any detrimental effect to the immobilized enzymes. Since the reaction mixture is spread over a large and thin surface, heat and mass transfer are optimal and the removal of volatile by-products is facilitated.

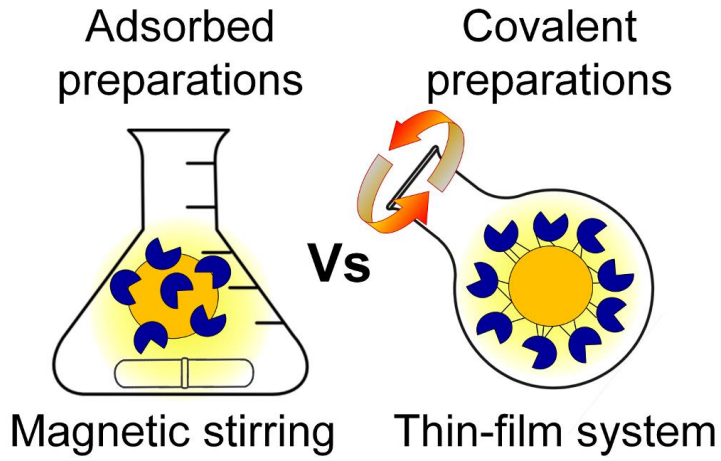

Figure 3: On the left: schematic representation of a conventional magnetic mixing system for biocatalyzed reactions using lipases immobilized by adsorption. As a consequence of the mechanical stress the biocatalyst is damage and the protein is released in the medium. On the right: a biocatalyzed reaction employing lipases covalently immobilized on solid carriers with a thin-film reaction system operated at laboratory scale.

Polyester synthesis requires the use of immobilized enzymes to enable the recovery of the expensive biocatalyst (generally lipase B from *Candida antarctica* or various cutinase enzymes) and to avoid the contamination of the product with the enzymes. This has been accomplished by anchoring covalently the enzymes to different organic carriers [49, 50]. The "thin-film concept" can be conveniently applied to different ester and polyester synthesis under solventless conditions characterized by high viscosity. The concept was experimentally validated at 10 kg scale using a turbo reactor that has been operated according to a two-step solvent-less process for the polycondensation of adipic acid with

1,4-butanediol [49]. The first step is catalyzed by the enzyme and leads to the formation of oligomers. At that stage, the product mixture is sufficiently fluid to allow the recovery of the immobilized biocatalyst. Afterwards, the temperature is increased up to 90°C and the polymer elongation is driven thermodynamically through the removal of co-product (i.e. water). This technical solution prevents the exposure of the biocatalyst to mechanical stress and preserves its long-term activity. It must be note that the turbo-reactor configuration makes unnecessary the application of vacuum, since the water is easily removed from the thin-film even at 90°C (Figure 4).

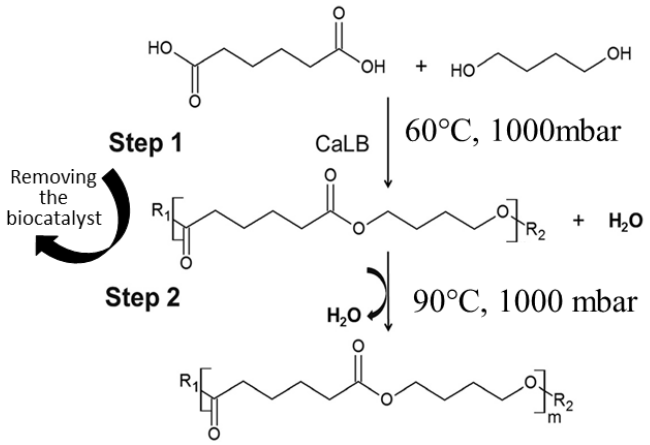

Figure 4: Scheme of the synthesis of poly(1,4-butylene adipate) catalysed by lipase B from *Candida antarctica* using a turbo reactor that create a thin-film of the viscous reaction mixture. The first step of the synthesis involves the biocatalyst and leads to the formation of pre-polymers. After the removal of the biocatalyst, the polycondensation is thermodynamically driven by the evaporation of water, the side product of the reaction.

In principle, this type of reaction system is applicable to a wide variety of large-scale biotransformation of viscous substrates under solvent-free conditions.

Towards more sustainable and inexpensive industrial biocatalysts

As mentioned before, when dealing with commodity chemicals having low added value, cheap and recyclable biocatalysts are the determinants of success of enzymes applications. For instance, biodiesel can be produced by using biocatalyst technology instead of basic chemical catalysts, with the advantage of reducing energy consumption, preventing undesirable side products and, conversely, reducing expensive downstream processing. A detailed analysis of enzymatic biodiesel production concluded that the productivity of the immobilized enzyme is a key requirement for the economic viability of the process. The study suggested for the biocatalyst a target cost of $25 per ton of biodiesel, which is comparable to that of chemical catalysts [61].

One factor that often appears to be underestimated throughout the scientific literature dealing with biocatalysis regards the environmental impact of the biocatalyst itself and, more specifically, of the immobilized enzymes. In the perspective of achieving bio-based products meeting sustainability certifications, Life Cycle Assessment (LCA) methodology is gaining increasing relevance among the scientific community and it is recognized as an effective method for the evaluation of environmental burdens associated with productive industrial processes. An interesting LCA study applied a “cradle to gate” approach to evaluate three different processes for enzymatic biodiesel production [61]. The Authors estimated the environmental impact of the three catalysts, namely an immobilized biocatalyst, a soluble enzyme and an alkali catalyst. The results showed that immobilized biocatalyst has a lower environmental impact on the biodiesel production, compared to alkali and soluble biocatalyst. Actually, the environmental impact of the immobilized biocatalyst depends

458 strongly on its recyclability and re-use for different cycles [62, 63]. Therefore, biocatalyst
459 productivity is the major factor affecting both environmental economic and environmental
460 impact of immobilized biocatalysts. On that respect, a productivity of 5-10 tons of product
461 per kg of immobilized enzyme has been indicated as an acceptable target.
462 Another study analyzed the impact of enzymes immobilized on fossil based methacrylic
463 carriers and produced industrially for application in the pharmaceutical sector [64]. LCA
464 methodologies pointed out that the major contributions to acidification, eutrophication, and
465 photochemical smog formation come from the media used for the enzyme fermentation
466 (yeast extract soybean protein) and secondly from the immobilization processes. Actually,
467 the immobilization resulted as the major contributor in terms of global warming potential,
468 with 16 to 25 kg of carbonic anhydride eq per kg of immobilized biocatalyst. Data indicated
469 that the preparation of the immobilized biocatalyst is energy intensive, since it consumes
470 from 117 to 207 MJ of non-renewable energy per kilogram of immobilized enzyme. The
471 global warming potential is due to the methacrylic carrier, which emerged as the
472 environmentally most crucial factor associated with the production of the immobilized
473 biocatalyst. That was associated to the fossil-based raw materials (i.e., glycidyl methacrylate
474 and ethylene dimethacrylate) employed in their production.
475 The conclusions of these studies shed light on the recent analyses that demonstrate how
476 immobilized biocatalysts has not met – so far - the initial optimistic expectations. Actually,
477 only four types of biotransformations employing immobilized biocatalysts are carried out at
478 large industrial scale [16]. Nevertheless, there is a huge biocatalytic potential that needs to
479 be re-considered and optimized in the perspective of producing bio-based chemicals and
480 fuels from renewable resources also by means of immobilized enzymes [14].
481 Overall, there is a need for a more holistic analysis of environmental and cost constraints in
482 the development of immobilized biocatalysts for bio-based chemistry applications. It is
483 noteworthy that the cost of commercial methacrylic carriers is in the range of a few hundred

€ per kg of resin. Scientific literature reports a wide number of studies where renewable materials or biopolymers are used as economical and sustainable alternatives to fossil-based immobilization carriers [65].

Carbohydrate-based biopolymers represent the group that has been most widely investigated. One notable example is given by the immobilization of penicillin G acylase on chitosan for antibiotic processing on a large industrial scale [21].

Recently, rice husk (RH) has been suggested as a carrier for immobilization of biocatalysts. This natural and robust composite material is made of lignin, cellulose, hemicellulose and SiO_2 [59, 66]. The milled material requires minimal pre-treatments and it is applicable in both physical and covalent immobilization protocols and under various process conditions. Although the material appears considerably less homogeneous in shape and size, as compared to commercial fossil based methacrylic carriers (Figure 5), studies confirmed that lipase covalently immobilized on RH can be recycled and are particularly suitable for application in viscous solvent-less systems.

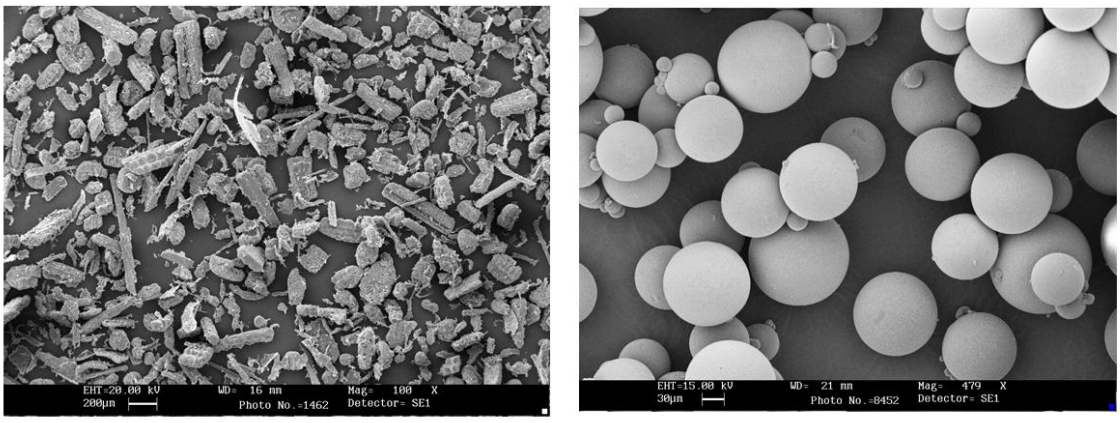

Figure 5. Scanning electron micrographs of rice husk fibres after milling (on the left) as they are used for enzyme immobilization. On the right, for comparison, the image of commercial methacrylic beads used as enzyme carriers.

Experimental data demonstrated the applicability of hydrolases immobilized on RH in the bulk synthesis of emollient esters as well as in solvent free polycondensation of bio-based monomers for the synthesis of polyesters [66]. RH display remarkable mechanical and chemical robustness and, more importantly, it is available worldwide in virtually unlimited amounts (globally 120 Mt per year).

Concerning the greenness of RH as enzyme carrier, some preliminary analysis underlined that RH can be re-utilized at the end of its proposed industrial application [66]. For instance, it has been bio-degraded *via* anaerobic digestion to produce bio-methane and biogas [67]. As a renewable composite material, RH can be re-used in the building sector and various manufacturing applications, in accordance with the principles of the circular economy [66]. Exhausted RH can be also used as ruminant feed or in pet foods as a source of fiber, applied as fertilizer or medium for gardening. RH, and bio-products in general, have the subsidiary advantage that they undergo to less stringent legislative constraints. Thus, even after chemical modification, they are currently exempted from the European REACH registration [68].

Although the sustainability of biocatalyzed processes and bio-based products can be assessed by applying LCA methodologies, it must be noted that they imply very extensive inventory of all inputs and outputs of the production system as well as their environmental impact assessment. On that respect, it would be desirable to develop simpler metrics to be used as a decision-making tool at an early stage of product synthesis. Some examples are the E-factor conceived by Roger Sheldon and other atom efficiency criteria [65, 69].

Outlook: success will pass through big-data access, multi-sectoral integration and contamination

Nowadays, research and innovation activities are facing a shift towards multi-disciplinary integration. While the complexity of processes is increasing, information needed for facing

the new challenges are dispersed in multiple and heterogeneous data sources. The optimal results can be achieved by addressing different domain approaches and analyzing a wide number of variables, objectives and constraints related to various disciplines.

Biocatalysis is an inherently multidisciplinary discipline, embracing know how spanning from molecular biology to enzyme technology, chemistry, and chemical engineering. Impact and success of biocatalysis in bio-based chemistry will strongly depend on the ability of the biocatalysis scientific community to transform the huge amount of *information* currently scattered in a multiplicity of databases (DBs) into useful *knowledge* [70-72].

More specifically, biocatalysis is expected to achieve objectives that require from one side tools able to mine big databases and then methodologies for analysing the massive amount of scientific data already available (Figure 6). A number of extremely powerful and refined bioinformatics tools for data analysis are becoming accessible, in principle, to any end-user, as most of them are based on open source software [72-74]. However, concerning integration and optimization, biocatalysis still appears as a relatively novel field as compared to other disciplines (e.g. mechanical engineering, material science), which make extensive use of numerical optimization and advanced algorithms able to integrate several actions and software into automated workflows. These computational integration tools allow for the design of reproducible and cost-effective and repeatable processes while the computational platforms operate by taking unbiased decision [75]. It must be mentioned that some initial attempts have been made by integrating software and calculation for *in silico* screening and rational design of enzyme mutants [76].

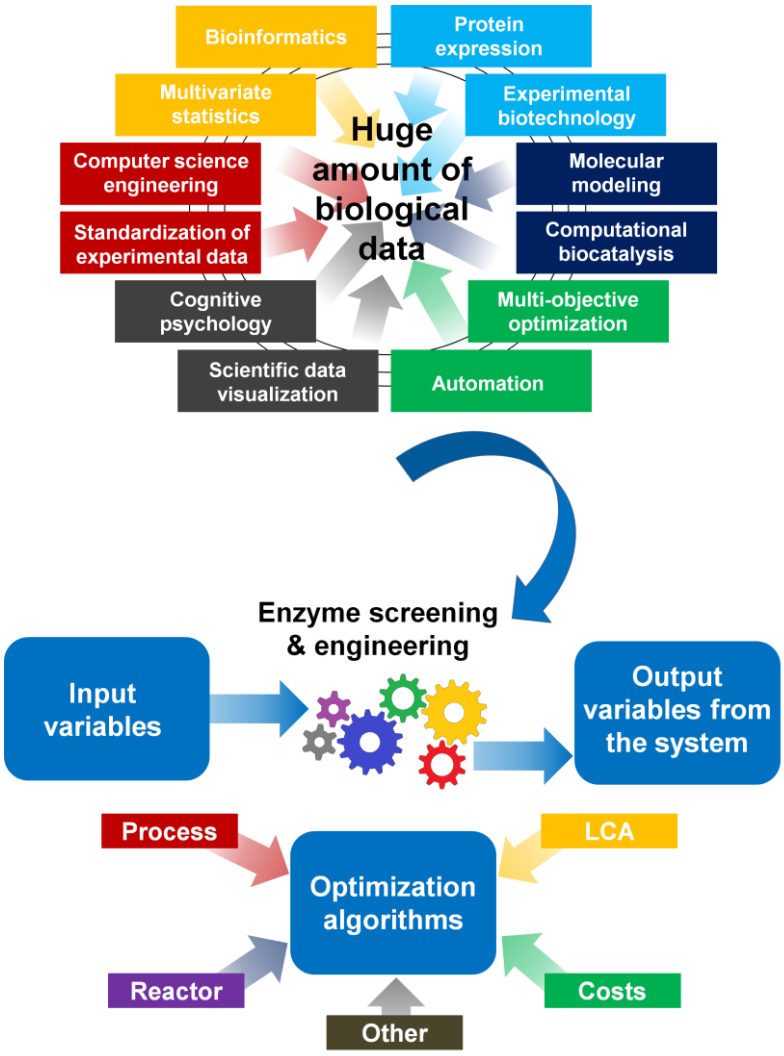

Figure 6: A systemic vision of integrated strategies for developing biocatalytic processes applicable to bio-based chemistry and biorefineries.

In conclusion, biocatalysis applied within the bio-based sector requires a closer integration, not only of scientific and technological factors but also of constraints and information coming from economic, social, legislative and environmental analysis (Figure 6). Actually, in many cases scientific advances provide already solutions that might reach the market in the next years, provided they are effectively optimized. Therefore, there is an urgent need of more integrated strategies able to solve highly complex problems that cannot be faced through a simple assembling of discrete steps of innovation.

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Abbreviations

kt	kilo tonnes
B	Billion
T	Trillion
M	Million
LCA	Life Cycle Assessment
MFCs	Microbial Fuel Cells
MECs	Microbial Electrolysis Cells
RH	rice husk
DBs	Databases
KET	Key Enabling Technology