Chapter 8
Producing Value-Added Products from Organic Bioresources via Photo-BioCatalytic Processes

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Abstract The interplay between light and bioprocesses represents an opportunity to develop high-value products from organic waste. In the past decade, the field of green chemistry was overturned by applications of photobiocatalysis, despite being investigated since the early 1900s. New developments allow fine-tuning control and accelerated kinetics of enzymatic redox reactions by light. Indeed, solar irradiation can be deployed either to directly activate or to ensure the in-situ regeneration of reducing equivalent promoting redox enzymes activity. Till now, organic wastes are only partially utilized as biomass growth support in biorefinery processes and its fully exploitation is far to be achieved. Photobiocatalysis exemplifies a strategic way to design new biotransformation processes. In this context, the production of high-value molecules is achieved by using organic waste as primary chemical precursors that provides electrons upon oxidation, also widely defined as sacrificial molecules. In this chapter, the organic wastes recovery though photobiocatalytic processes, including enzymatic systems, electron reservoirs and final acceptors, are discussed. In addition, a special focus will be given toward the light-driven valorisation of organic by-products involving whole-cell biotransformation approaches. These technologies are considered as the new frontiers in the biorefinery field.

Keywords Photobiocatalysis · Redox enzymes · Biomass · Light · Pigments · Biorefinery

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8.1 Introduction

At its onset, life on Earth started with sparkles of energy ultimately caused by electromagnetic radiations originating from the Sun. Sunlight driven reactions were probably the very first happening on primordial Earth and could have contributed on generating the reservoir of prebiotic molecules, necessary for the incumbent evolution of life [1]. Since then, light had become the basic energy input for the Earth ecosystems, and its energy conversion through photosynthesis produce today 100–200 Gton/year of reduced carbon compounds, which represents a vast resource to look at if we want to harness the very first source of renewable energy available, with the goal of guiding the transition of our society out of the fossil-based era.

For long, scientific challenges reproducing in vitro the biological photosynthesis able at splitting water for production of viable electrons, had been endured in what is known today as artificial photosynthesis. Thought with limited success, in terms of quantum efficiency, this had laid the fundaments of new domain of studies among which the Photobiocatalysis (PBC) is emerging.

The PBC concept sees the use of light-excitable photosensitizers coupled with biocatalysts, i.e., redox enzymes, fueled directly or via electron's conveyor pigments, ultimately sacrificing an inexpensive source of electrons. PBC provides us with a basic tool to initiate and expand research on the biggest challenges within chemistry, physics and biology: a completely renewable/green technology capable of converting light energy into chemical energy. In a PBC system the light energy is used to promote the electron transfer among the catalytic components and to allow thermodynamic challenging reactions.

The substrate-to-product conversion in a classic PBC setup is usually achieved by the concerted action of four main elements: (i) photosensitizer (PS), (ii) electron mediator (EM), (iii) biocatalyst (i.e., enzymes) and (iv) electron donor (or sacrificial molecule). Photosensitizers are single molecules or complexes able to absorb light and generate photoexcited electrons, and their exposure to their specific wavelength is considered the starting event in PBC. Then, the excited photosensitizer (PS*) can either reduce the biocatalyst (usually a single redox enzyme) with a direct electron transfer or alternatively activate it by an indirect electron transfer through the reduction of an electron mediator (usually a prosthetic group of enzymes). In both cases, the presence of a sacrificial external electron donor is needed for the regeneration of the photosensitizer (or to fill the electron void) and thus assuring the photocatalytic turnover (Fig. 8.1). Depending on the specific system, the electron mediator can be either a small artificial redox molecule or the natural cofactor of the enzyme catalyzing the reaction. Inspired by this basic architecture, various PBC systems have been developed to cope with specific reaction conditions, component incompatibilities or electron transfer optimization. For example, an additional mediator can be added to improve the indirect electron transfer from the photosensitizer to the biocatalyst. The use of a whole-cell approach, in which the biocatalyst is the entire recombinant cells expressing the enzyme of interest, can bypass the addition of expensive cofactors or improve the overall system stability.

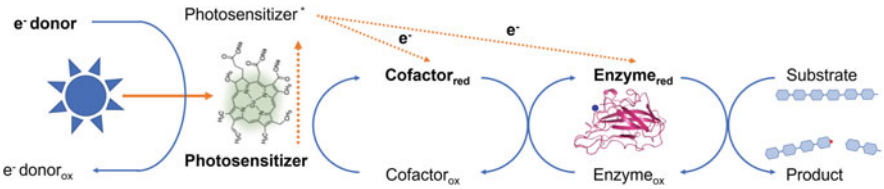

Fig. 8.1 Schematic diagram of photobiocatalysis process via direct or indirect photoelectron transfer from photosensitizer to enzymatic active site

This chapter will provide the reader with insights on the basic working principles of photobiocatalysis and on the features of the main bioresources that can be valorised through it. Some examples of the latest advancements in the still young PBC field are reported, mainly as proof-of-concept studies at small scales, yet representing valid greener alternatives to actual harsh chemical or fossil-based processes (summarized in Table 8.1). We will start by introducing the first elements for PBC as choice of light sources and photosensitizers aiming at practical application of the technology at lab scale and discuss possible up-scaling. Then we will report the most important examples based on light-driven enzymes couple to common biowaste sources of their substrates (summarized in Table 8.2), indeed a unique point of view not yet covered in the literature.

8.2 Light Sources and Pigments

Exposure to appropriate light energy induces changes in the electronic structure of a photosensitizer thus promoting its transition from the *ground state* (more stable) to an energy-rich state namely *excited state*. At the excited state, a charge separation occurs resulting from the formation of electron-hole pair in the conduction and valence band of the photosensitizer, respectively. In a photocatalytic process, the light-driven charge separation is used as driving force for thermodynamically challenging redox reactions. As only the absorbed light is effective and photosensitizer compounds display intrinsic absorbing properties at specific wavelength, the choice of the appropriate light source is of primary relevance for the accomplishment of the photoinduced reaction. The light spectrum currently exploited in PBC systems spans form 280–700 nm, being composed by UV light (mainly UVB and UVA) and visible light, strictly depending on the aimed photosensitizer, electrochemical features and the process applications. Beside the type of light source, another parameter influencing photoconversion is the light energy supply in terms of quantity (light intensity) and duration/period (continuous or intermittent). The formation of electron-hole pairs linearly increases with the light intensity until a certain low threshold, but at high photon intensity, the rate of electron-holes recombination outcompetes the rate of redox reactions between the photosensitizer and the catalyst. Therefore, a tunable light dosage is not only necessary for the economy of the process but also for its

Table 8.1 Summary of PBC systems reviewed in this chapter. Photoactivation mode (direct/indirect), enzyme, cofactor, photosensitizer and substrate class are listed

Direct/indirect electron transfer	Enzyme	Cofactor	Photosensitizer	Generic substrate	Ref.
Direct	*Tt*AA9E, *Tt*AA9H, *Ta*AA9A	—	Chlorophyllin, thylakoid, Water-soluble chlorophyll-binding protein	Polysaccharide	[12, 13, 56]
	*Bs*AA10, CPB21	—	V-TiO_2, chlorophyllin	Polysaccharide	[14]
	LAC3	—	V-TiO_2, chlorophyllin	Cyclic alkene	[15, 60]
	Commercial Laccase	—	Laccase/carbon dots decorated with phosphate groups complex	Low-molecular weight mediator	[61]
	*Aae*UPO	—	Carbon nitride (CN-OA-m)	Cyclic alkene	[6]
	*Aae*UPO	—	Sodium AnthraquinoneSulfonate (SAS)	Cyclic alkene	[70]
	*Aae*UPO	—	Nitrogen-doped Carbon Nanodots (N-CNDs)	Cyclic alkene	[71]
	*Ci*VCPO	—	Sodium AnthraquinoneSulfonate (SAS)	Cyclic alkene	[70]
Indirect	CHMO	NADPH	Intact cyanobacterial photosystem (whole-cell)	Cyclic alkene	[21]
	CHMO	NADPH	Au-TiO_2, g-C_3N_4	Cyclic alkene	[8]
	CHMO, HAPMO	NADPH	Sodium AnthraquinoneSulfonate (SAS)	Ketoalkene	[76]
	*Cv*FAP	FAD	FAD	Fatty acids	[78, 79]
	YqjM	NADPH	Intact Cyanobacterial photosystem (whole-cell)	Alkenes	[81]
	*Yers*ER	FMNH	Iridium complex [Ir-$(dmppy)_2$(dtbbpy)]PF_6	Alkenes	[82]

intrinsic electrochemistry. Moreover, the use of intermittent light has been seen to reduce the electron-holes recombination phenomenon and prevent the overproduction of highly reactive ROS species thus improving the quantum yield and stability of the photobiocatalysis conversion [2]. Here we provide the reader with an overview of the most common light sources investigated up to date.

Table 8.2 A summary of main bioresources suitable for PBC conversion and valorization. PBC set up and obtained products are listed

Substrate	BioResource	Enzyme	Light/PS	Products	Ref.
Cellulose	Lignocellulose, pulping paper	LPMO – *Tt*AA9E-H, *Ta*AA9A	Chlorophyllin - Sunlight, LED, Bulb, 150-200 μE, continuous and intermittent	Cellooligosaccharides various DP, Cello-aldonic acids, keto-cellooligosaccharides	[12, 13, 15, 58]
Hemicellulose	Agricultural waste, food waste, lignocellulose	LPMO – *Tt*AA9E	Chlorophyllin - Sunlight, LED, Bulb, 150-200 μE, continuous	Xyloglucans oligosaccharides, xyloglucan aldonic acid	[12]
Chitin	Fishery industry, food waste, restauration sector	LPMO – *Bs*AA10, CPB21	Chlorophyllin, V-TiO_2 - visible light - 42 W cm^{-2}, continuous	Chitooligosaccharides and their aldonic acids	[14]
Lignin	Pretreated lignocellulose	LPMO – *Mt*AA9I-A, *Tt*AA9E w/ Laccase	Chlorophyllin - Sunlight, LED, Bulb, 150-200 μE, continuous	Water soluble lignols, carboxylate lignols, lignin oligomers, lignin nanoparticles	[12, 64, 98]
Lipids	Fatty acids and PUFAs, algal production, agricultural oil, WCO	*Cv*FAP	LED - 68 μmol m^{-2} m^{-1} - continuous	Decarboxylated fatty acids, biodiesel	[78, 79]
Terpenoids	Lignocellulose, microbial communities	*Ci*VCPO	Sodium Anthraquinone Sulfonate (SAS) - white light bulb - continuous	Halogenated terpenoids and cyclized halogenated terpenoids	[70]
Aromatic hydrocarbons	Lignocellulose, microbial communities	*Aae*UPO	Sodium Anthraquinone Sulfonate (SAS) - white light bulb, – continuous	Alcohol and ketone form of aromatic hydrocarbon	[70]

8.2.1 Sunlight

Natural sunlight is an ideal starter for highly sustainable PBC processes being renewable, economical and offering a complete array of suitable electromagnetic radiation. However, its supply is discontinuous due to changes between day and night, seasonal turnover and latitudinal variations that are important constraints for developing robust industrial processes. The sun electromagnetic spectra radiation reaching the Earth's surface is also influenced by the weather conditions, whereas on a cloudy day, direct sunlight is less intense and short wavelengths are strongly reduced [3]. Sunlight-fueled approaches are well developed for biomass and high-valued compounds production using photoautotroph microorganisms as in the case of microalgae cultivation in open-ponds and outdoor photobioreactors. The effect of discontinuous sunlight supply on outdoor plants productivity can be modelled and mathematical predictions are a powerful tool for adaptation to other tunable parameters e.g. for preventing cell self-shading or photoinhibition mechanisms [4]. Photosynthetic organisms are naturally provided with adaptative mechanisms enabling them to cope with fluctuating illumination, while artificial PBC systems are more sensible to environmental light variations. It is thus necessary to intensify investigation on the electrochemical properties of photosensitizers to further develop more stable and efficient molecules for assuring constant photoconversion yields under variable conditions. Xanthene-based organic dyes are reported to successfully mediate in vitro light catalyzed production of molecular hydrogen (H_2) through a cyanobacterial hydrogenase under ambient daylight irradiation [5]. The bio-inspired simplified system activated by visible light and stable H_2 productivity has been reported [5] despite daily light intensity variations, proving the possibility of its application outside standardized laboratory conditions.

8.2.2 Gas Discharge Lamps

Gas discharge lamps are widely used light sources composed of tiny glass ampules containing two electrodes and a mixture of noble gases at low pressure. The composition of the gas mixture determines the emission spectrum of the lamp. Neon bulbs are characterized by emission wavelengths in the red-orange (above 600 nm) range while argon lamps are shifted towards blue-violet (below 500 nm). Fluorescent lamps are typically mercury-based and a phosphor coating is used to produce visible light that appear white to the human eye. Despite fluorescent light is perceived as a homogenous beam, its spectrum is quite discontinuous, displaying discrete high intensity emission peaks related to the noble gas employed. Generally, gas discharge lamps present a substantial time delay in the full intensity emission since the current supply, this can hamper their application in PBC reaction with an intermittent illumination setup.

8.2.3 Light Emitting Diode—LED

Light emitting diodes (LEDs) are a relative recent light source having huge interest in industrial applications as their price is continuously decreasing and the technology improving leading to remarkable decrease in electricity consumption for amount of light produced. The light emission spectrum of a LED is narrow enough to be depicted by a single wavelength. The actual range of semiconductor materials suitable for this technology allow a wide diversification of LED colors and, a broader overall emission spectrum can be achieved by LEDs combinations (like RGB LED array) or by recent white LED. Having a spectral width in the range of 20–50 nm, LED devices allow a fine control of photocatalytic conversions where combination and alternation of irradiation with different wavelengths is required to drive specific reactions [6]. LED are nowadays replacing conventional lighting systems, such as incandescent bulbs and fluorescent tubes, because of the lack of infrared emissions ascribed to heating of the radiated surfaces thus reducing the need of cooling systems for the reactor volumes. When intermittent illumination is needed, LED technology represents a reliable light source being able to assure on/off cycles within a time range in the order of milliseconds. Notably, the use of LED in pulsed mode results in an extended lifespan and diminished power dissipation and heating generation. Lastly, the main challenge for LEDs implementation in photobiocatalytic processes at industrial scale, is the low in-depth penetration power of the emitted beam. This limitation must be taken in account especially for applications where UV-LEDs are used to drive biocatalytic conversions in high turbidity streams.

8.2.4 Pigments Photo-Oxidation/Reduction

Nowadays several photosensitizers have been identified for PBC processes that are either biologically derived and thus renewable, or chemically synthesized. Generally, after absorbing light, the photosensitizer is promoted to its excited state (PS^*), and interacts with other molecules via electron transfer, energy transfer or atom transfer pathways. In PS^*, an electron in the highest occupied molecular orbitals (HOMO, or valence band) is promoted to the lower unoccupied molecular orbital (LUMO, or conduction band) leading to charge separation. PS^* is thus a strong redox agent and tend to undergo electron transfer by donating it to an appropriate electron acceptor (reductive quenching) or by receiving it from an electron donor (oxidative quenching). The photosensitizer is then regenerated via a second electron transfer that restore the ground state. The electron transfer direction and thermodynamic feasibility depends on the redox potentials of the involved species and competes with other relaxation mechanisms such as the radiative deactivation by emission of less energetic photon and the nonradiative deactivation by heat dissipation [7]. Thus, to achieve efficient photoconversion, the photobiocatalytic system must be careful designed by coupling the redox partners favoring the electron transfer over other

concurrent deactivation mechanisms. After light excitation, intersystem crossing can promote the PS to its triplet excited state ($^3PS^*$) allow the direct energy transfer to ground state substrate causing the formation of triplet excited substrate ($^3SUB^*$). This event is commonly occurring after light exposition of chlorophyll in aerobic condition. The excited pigment interacts with molecular oxygen leading to the formation of highly reactive oxygen species which then disproportionate forming H_2O_2. Light-driven in-situ production of hydrogen peroxide is a mechanism extensively used to activate peroxygenases in photobiocatalytic conversions [8, 9]. Each photosensitizer possesses only one ground state but multiple excited states with specific energetic levels, physiochemical features, and lifetimes. Excited triplet states are usually preferred than singlet states due to their longer lifetime (in microseconds and nanoseconds order, respectively) making the electron transfer more efficient. Lasty, a PS^* can interact with other molecules by atom abstraction from a suitable substrate usually displaying an accessible hydrogen atom. A common chemical structure features of photosensitive molecules is the presence of large and stable π-aromatic configuration which assure electron delocalization making them excellent electron donors/acceptors [10]. In current PBC reactions, the successful use of a wide range of photosensitizers is reported, including natural and natural-derived pigments or complexes, organic dyes, transitions metal complexes and carbon-base nanomaterials (Fig. 8.2).

8.2.4.1 Natural Chlorophyll and Derivatives

Taking inspiration from photosynthesis, which is the most powerful natural process for storing light energy into chemical energy, a wide number of PBC setups have implemented porphyrin-based molecules as the light harvesting component. Photoactive porphyrins display a large and rigid tetrapyrrolic conformation coordinating a transition metal (e.g. Mg, Fe), in which the extended conjugated system determine a strong absorption in visible electromagnetic spectrum (Fig. 8.2a). Modifications in the peripheral decoration or in the transition metal allow a fine tuning of their physiochemical properties such as the modulation of the ground- and excited-state optical and electronic features, excited state stability and absorption shift [11]. The abundance in natural feedstock and the plasticity of these chromophores attracted the interest of researchers working on sustainable PBC processes. Water-soluble chlorophyl complexes and chlorophyl copper substituted derivative, (chlorophyllin), were used to photoactivate oxidative enzymes active on polysaccharides [12–14], while a zinc-based porphyrin was shown to perform multielectron transfer to laccase enzyme via long stable triplet excited state [15].

8.2.4.2 Complexes Phycobilisomes, Thylakoids, Cell Lysates

In higher photosynthetic organisms (plants) the light energy absorption take place in well organized and compartmentalized environment (the chloroplast) in which series

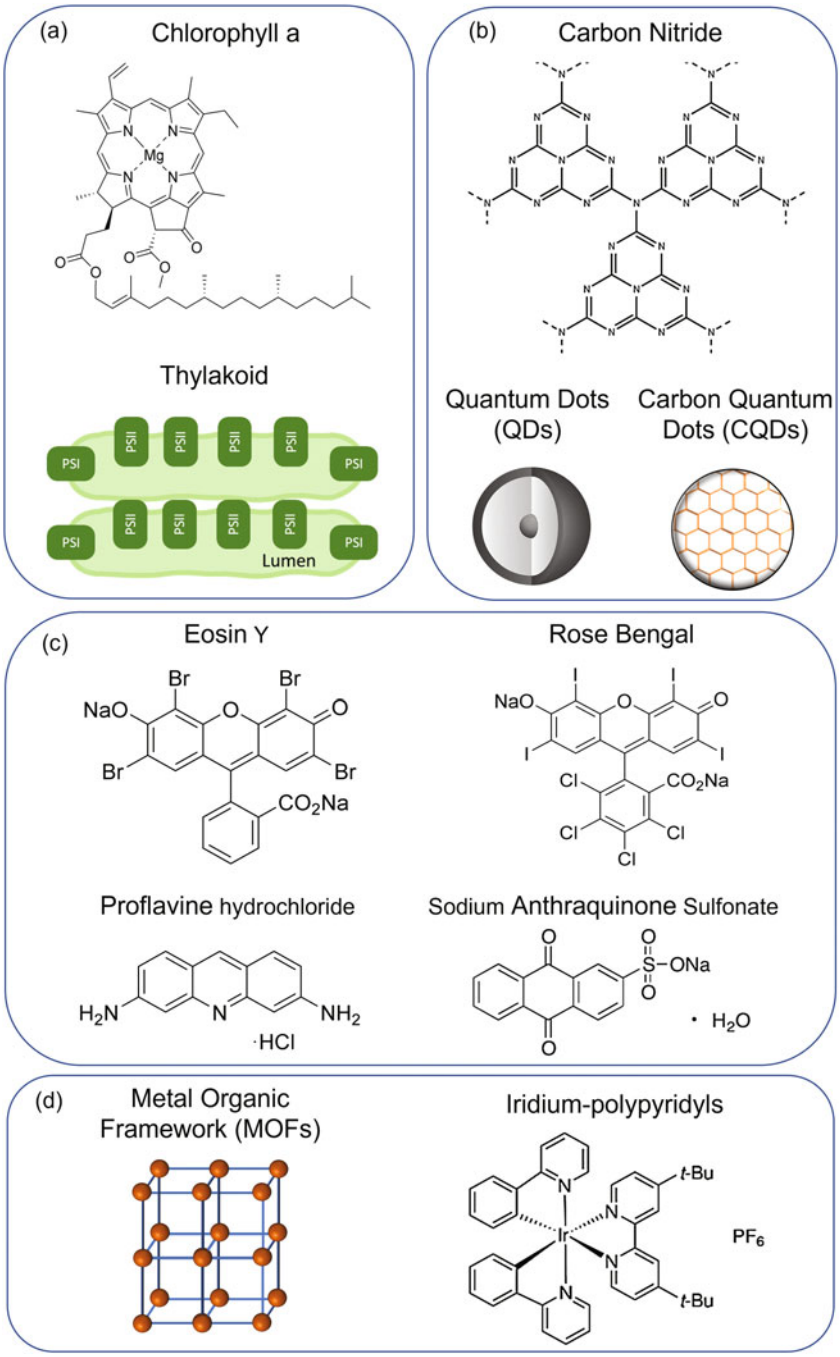

Fig. 8.2 Structures of commonly used photosensitizers grouped by class: (**a**) natural chlorophyll; (**b**) Quantum dots and functionalized materials; (**c**) microbial derived xanthenes; (**d**) metal complexes

of light harvesting complexes (photosystems) concentrate the incident photons and the electron transport chain maximize the charges separation across the membrane of the thylakoids (Fig.8.2a). Exploiting this kind of structure can be beneficial for some reactions in which the orientation of the electron flux and the presence of a compatible interface between the photocatalyst and the biocatalyst are needed [16]. Isolated photosystems have been used to create in vitro chromophore-enzymatic complexes for efficient H_2 at the only expense of water oxidation [17, 18]. Many attempts are reported to artificially reproduce photosynthetic processes and mimic chloroplast organized and stable environments. A bio-based artificial photosynthetic system was produced by Park and co-workers using lignocellulosic derivatives to encapsulate hydrophobic porphyrins and regenerate enzymatic redox cofactors [19]. Other whole-cell biocatalytic approaches demonstrated the possibility to exploit natural photosynthesis to drive the production of target compounds via metabolic engineering of cyanobacteria. Photosynthetic direct electron transfer or reducing agent production (in form of NAD(P)H) can be employed for enzymatic production of bioactive-compounds, organic molecules and their oxy-functionalization [20–22].

8.2.4.3 Microbial Derived Xanthenes

Alongside photosynthetic pigments, other biobased compounds present ideal features for green photobiocatalytic applications. Xanthenes and xanthenes derivatives are organic dyes with heterocyclic structure able to absorb light in the central region of visible spectrum (500–600 nm) and, when photoexcited, are able to undergo reductive and oxidative quenching. Eosin Y and E, Rose Bengal and flavin-based compounds are the most widely used in light driven reactions being able to direct activate the catalytic site of redox enzymes [23, 24] to sustain the photoregeneration of several redox cofactors or to photoactivate substrate for further enzymatic conversion [25]. Photophysical and electrochemical properties of xanthene can be tailored by specific structure modifications and peripheral functionalization with, for example, halide groups (Fig. 8.2c). Aromatic heterocycles with broad absorption properties are abundant secondary metabolites naturally produced by plants and microorganisms during symbiotic and pathogenic interactions [26]. New natural structures are constantly discovered and characterized, thus representing an unlimited source of green photosensitizer building blocks.

8.2.4.4 Metal Complexes

Photocatalysts containing precious metals like iridium, platinum and ruthenium (Fig. 8.2d) are broadly used to drive challenging redox reactions due to their high chemical stability and long-lived excited states. However, the actual need for more environment friendly and affordable conversion strategies resulted in the development of new metal complexes with on Earth more abundant first-raw transition metals such as copper, zinc and nickel and titanium. UV absorbing titanium oxides

are the most exploited photosensitizers especially for in situ generation of hydrogen peroxide, electron donor transfer [9, 14, 27] and reducing agent regeneration [28] for oxidoreductase activation. TiO_2 nanoparticles can also be doped with other metal oxides (like Cu_20) to improve their stability and modulate the absorbance range [29] or be incorporated in bioderived carrier, such as cellulose, for application in photocatalytic-biodegradation coupled processes [30]. A subsequent evolution of metal complex is the design of metal-organic frameworks (MOFs) in which metal clusters are coordinated with organic ligands allowing multidimensional structures (Fig. 8.2d). Structure and properties depend on the nature of the ligand (valence and functional groups) and of the coordination preference of the metal. MOFs can thus be designed to host stable pores which improve the contact surface area with the reactants, target goal especially for application in H_2 storage and CO_2 capturing and fixation. Ligand electron delocalization and introduction of antenna groups ($-OH$; $-NH_2$) can broaden and shift from UV range the absorption spectrum and favor charge transfer kinetics of MOFs rendering them suitable photosensitizers [31].

8.2.4.5 Quantum Dots and Functionalized Materials

Developments in nanomaterial science led to the design of practical semiconducting nanoparticles called quantum dots (QDs, Fig. 8.2b). QDs find application in a broad range of technologies like microelectronic, biomedical, bioimaging and solar cell, just to mention few. Due to their unique photophysical characteristics, QDs are successfully used as photosensitizer in PBC. QDs absorption spectra, emission quantum yield and excited state lifetime are all features depending on the particle size and atom composition allowing high degree of freedom in their design and adaptability. Common sizes span from 2 to 10 nm in diameter, with smaller crystals absorbing high light intensity (UV – blue spectrum region) and bigger size being excited at longer wavelengths. Contrary to organic dyes and pure pigments, QDs are more prone to photobleaching, thus showing a higher stability over repeated cycles of excitation. Moreover, nanocrystals optical and photo-physic characteristics can be further shaped by varying their composition using different transition metals for their fabrication [32]. Besides, metal based QDs, more sustainable and less toxic semiconducting nanoparticles are now made upon carbon structure (Fig. 8.2b). Carbon quantum dots (BQDs) can be synthesized either with a top-down approach by fragmentation of graphite and carbon nanotubes structures or by bottom-up processes where small precursors (e.g., sugars and citrate) are assembled via hydrothermal or solvothermal treatments. This latter production approach allows their straightforward synthesis from simple carbohydrate-rich natural biomass such pear juice, peach blossom and agricultural waste hydrolysates [33–35]. CQDs are suitable photosensitizers for PBC thanks to their high solubility in aqueous solution, biocompatibility, light harvesting and electron transfer properties. All these characteristics can be tuned by surface groups modifications and modulated by the environmental pH conditions [36].

8.3 Biocatalysts- Enzymes and Whole Cell Applications

The term biocatalyst usually refers to the specific isolated enzyme that catalyzes the aimed substrate-to-product conversion. Commonly, PBC conversions are based on the use of enzymes belonging to the oxidoreductase class (EC 1) which catalyze the electron exchange between a donor and an acceptor molecule. To perform the reaction, these enzymes often require the presence of reduced nicotinamide (NAD(P)H) cofactors or prosthetic groups such as metal-ions, flavins and heme groups. Oxidases and oxygenases use O_2 as an electron acceptor, the latter directly incorporate at least one oxygen atom into the substrate. H_2O_2 is the co-substrate of peroxygenases, while dehydrogenases use molecules other than oxygen as electron acceptors [37]. Many approaches have been proposed for the regeneration (reduction) of monooxygenases or for their cofactor, spanning from chemical, electrochemical to enzymatic approaches but all at the expenses of a valuable donating electron molecule [38]. When considering applying these enzymes in biotechnological processes, electron donation can pose a limitation due to the high costs of these reducing agents. A renewable and inexpensive source of electrons that can regenerate these molecules, or that can bypass it and directly donate the electrons to the enzymes, will finally enable to exploit all the potentials of these enzymes. In a broader definition a biocatalyst can be used to depict the whole organism able to express (produce) the enzyme and ideally sustain its activity over time by continuously providing suitable cofactors, as a part its endogenous metabolism [39].

8.3.1 Pigment Mediated Photo Excitable Enzymes Without Cofactor

8.3.1.1 Lytic Polysaccharide MonoOxygenases - LPMOs

Lytic Polysaccharide MonoOxygenases (LPMOs, EC 1.14.99.-) discovered in 2010 [40], are redox enzymes able at cleaving by oxidation the β-1,4-glycosidic bond in numerous polysaccharides. This had abruptly changed all our understanding of polysaccharide degradation. The new scenarios see LPMOs creating rupture in the fibers tension via oxidative cleavage of polysaccharide chains leaving new entry sites for the further depolymerization actuated by cellobiohydrolase enzymes, that were thought to operate in synergy only with endoglucanases [41]. LPMOs were firstly identified in bacteria (*Serratia marcescens, Sm*AA10A) but shortly after, numerous LPMOs-coding sequences were found in many different organisms including archaea, eukaryotes and viruses. Up-to-date, according to the CAZy classification [42] LPMOs are grouped into 7 families of Auxiliary Activity enzymes (AA9-11, AA13-15 and AA16). The AA9s, AA11s, AA13s, AA14s and AA16s are widespread in eukaryotes, while the AA15s are also found in insect and viruses and the AA10s are mostly common in bacteria [43]. The highly conserved catalytic site

host a single copper atom (type II copper) coordinated with a T-shaped histidine–brace [44, 45]. The enzyme activation requires the electron transfer from an external donor to reduce the catalytic copper which then can oxidatively cleave the glycosidic bond using either O_2 or H_2O_2 as co-substrate [46, 47]. This cleavage leads to the production of oxidized oligosaccharides and their non-oxidized counterpart. Upon enzyme reduction, the catalytic site performs a hydrogen abstraction from carbon in position 1 or 4 in the pyranose ring. The resulting radical intermediate is then hydroxylated causing the subsequent break of the glycosidic bond [48, 49]. The oxidation at C1 or C4 position leads to the formation of aldonic acids or 4-ketoaldose gemdiols, respectively. Moreover, some LPMOs are able to release double oxidated products acting on both sides of two different monomers [50].

The first industrial application of LPMOs was their implementation in cellulolytic cocktails for the digestion of pretreated lignocellulosic biomass to obtain molasses of monosaccharides suitable for any bio-productions upon biological fermentations: biofuels, bioplastics and biochemicals [51]. Among other factors, the optimization of LPMOs activity clearly depends on the chemical nature of the electron donor and on its redox potential. Several reducing agents such as small molecules, enzymatic partners, mono- and poly-lignols, and photoexcited pigments have been reported to activate LPMOs [12, 27, 52–55]. In the first study of light-driven cellulose oxidation by LPMOs, the water-soluble form of chlorophyll (chlorophyllin) and isolated thylakoids were used to activate the monocopper enzyme via direct electron transfer (Fig. 8.1). Furthermore, the oxidized pigment was continuously regenerated at the only expense of ascorbic acid. The Photobiocatalytic system dramatically increased the product yield of the fungal *Tt*AA9E, and more interestingly revealed a secondary activity on a different substrate (xyloglucan), not detectable under dark conditions [12]. A more recent work highlighted a possible double role played by the light supply in boosting the activity of LPMOs. Along with the electron delivery to the catalytic center, the excited photosensitizer promotes the in situ generation of hydrogen peroxide that can be used as a co-substrate by the redox enzyme [14]. The importance and influence of other factors on the efficiency and robustness of this light-driven system are currently under investigations. The balance between all the reaction components (enzyme, photosensitizer and reducing agent) was assessed in a recent study where free or complexed chlorophyll (WCSP-Chl *a*) were used to mediate the light energy conversion. By optimizing the reducing agent concentration and the light intensity it was possible to prevent early enzyme inactivation which may occur in presence of an excess of H_2O_2 [13]. Finally, a beneficial effect on the system stability over time and an energy input gain is assured by the use of illumination on/off cycles [56].

8.3.1.2 Laccases

In nature, the degradation and modification of lignin, which is one of the most abundant and recalcitrant organic polymers, is enabled by the activity of powerful redox enzymes such as class II peroxidases, dye peroxidases and laccases. These

latter are an evolutionary old a family of extracellular copper-containing polyphenol oxidases (EC 1.10.3.2) widespread among the tree of life spanning from fungi, bacteria, plants and in a lesser extent in animals. Expression and secretion of polyphenol oxidases in fungi is also one of the first response lines when harmful conditions are "sensed" such as in the presence of bioactive compounds, xenobiotic toxins and antagonistic microorganisms. Moreover, laccases play a crucial in pathogenetic mechanisms by producing melanin and melanin-like pigments recognized as antimicrobial and virulence factors. The abundance of laccases coding genes reflects the wealth of architecture diversity, substrate specificity and kinetic properties displayed by this enzymatic class. Other biological functions of laccases are metal homeostasis/oxidation, morphogenesis, cell pigmentation, plant lignification and wound healing. Laccases molecular masses vary from 40 to 180 kDa having generally slightly acidic pH optima with the exception of bacterial laccases preferring alkaline conditions (e.g., SLAC from *Streptomyces coelicolor* with pH optimum 9.4.). Peculiar feature of laccases is the presence of cupredoxin-like domains hosting the binding site for type I copper (strongly distorted coordination sphere) and of one or two other domains supporting the coordination of one type II copper (planar coordination) and two type III coppers (oxygen bridged Cu-Cu dimer) [57]. The multicopper center allows the oxidation of a variety of phenolic substrates while performing the four electrons reduction of molecular oxygen to water. The substrate is oxidized at the type I copper site which then transfer the electrons to the tri-copper cluster in which the reductive generation of water takes place. The driving force for the substrate oxidation is the redox potential difference between the fully oxidized catalytic cluster and the phenolic compound. Based on their redox potential, laccases are categorized as low-redox-potential enzymes (mainly in bacterial and plants) and high-redox-potential enzymes (mostly found in white rot fungi). An expansion in substrate scope, including non-phenolic lignin subunits, is possible when low molecular weight mediators (such as ABTS; 2,2-azinobis-(3-ethylbenzothiazoline)-6-sulfinic acid) are firstly oxidized by the laccase and then target the substrate performing the final oxidation [58]. Thanks to their substrate versatility, temperature and pH stability, cofactor and hydrogen peroxide independence, laccases are ideal powerful oxidative enzymes for industrial biotechnological application in a wide range of fields like biofuel production, bioremediation, chemical, pharmaceutical and clothing industry, just to mention few.

Classic photoactivation mechanism supports single electron-transfer while the accumulation of multicharges or holes needed to activate metal-cluster enzymes remain challenging. The first complete reduction of a fungal laccase (from *Trametes* sp. strain C30) by light was achieved in 2011 by Tron and co-workers [59]. Excitation with white light of a Ru^{II}-polypyridine-type complex in presence of an exogenous electron donor induced the delivery of four electrons to the multicopper-cluster. The fully reduced laccase was then able to generate water molecules by reducing dioxygen. In a follow up work, the authors overcame the need for the external electron donor and coupled the laccase oxygen reduction with the oxidation of unusual olefin substrates. The model reaction led to the photo-epoxidation of *p*-styrene sulfonate (alkene) mediated by Ruthenium complex using O_2 both as

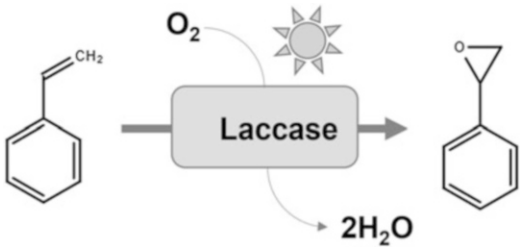

Fig. 8.3 Epoxidation of styrene to styrene epoxide by photoactivated Laccase in which the molecular oxygen (O_2) has the double role of electron acceptor and O atom donor

electron acceptor as well as O atom donor and laccase as electron sink (Fig. 8.3) [60]. Further investigations on laccase light activation led to the development of a tunable enzymatic-photosensitizer hybrid system showing higher catalytic activity compared to free laccase. Hydrophilic carbon dots decorated with phosphate groups (PCDs) were connected through noncovalent bonds to the type I coppers in the laccase catalytic center, resulting in a hybrid complex with improved oxidative activity and stability. Photoexcited PCDs possess strong electron donating and accepting properties which can favor the electron transfer between the low-molecular weight mediator (ABTS) and the laccase multicopper-cluster. More interestingly, the laccase/PCDs hybrid was sharply regulated by light illumination specifically responding to the provided light intensity and to on/off activation cycles [61]. Lignin removal from lignocellulose biomass is a high energy demanding and fundamental step in carbohydrate derivatization and refinery. The laccase/mediator systems are now exploited for lignin deconstruction in biotechnological processes and more recently the new concept seeing lignin as a precious reservoir of biochemicals is attracting the research interest for their customization. Low-molecular weight lignin (LMWL) pools derived from laccase/ABTS oxidation of steam-exploded sugarcane bagasse and wheat straw have been demonstrated boosting the LPMOs oxidative breakdown of cellulose via long-range electron transfer [62]. Another possible synergism between the two oxidative activities (of laccases and LPMOs) has been recently envisioned when the two enzymes work simultaneously. The phenolic radicals produced by laccases can interact with molecular oxygen forming hydrogen peroxide which in turn promotes LPMOs catalysis [63]. Moreover, lignin valorization via grafting desired compounds in lignin fraction is a further biotechnological route where laccase/mediator systems can be applied. It has been showed that laccase oxidation via ABTS efficiently deconstructs lignin polymers, prevents possible subsequent repolymerization event between phenolic radicals forming relative stable adduct with phenolic lignin dimers [64].

8.3.1.3 Unspecific Peroxygenases – UPOs

Recently discovered Unspecific PerOxygenases (UPOs, EC 1.11.2.1) are extracellular fungal enzymes grouped under a unique family of peroxygenases due to their broad substrate scope. Few UPOs have been currently isolated and biochemically

characterized, the first one, *Agrocybe aegerita* (*Ae*UPO) was found within the Basidiomycota phylum. Current phylogenetic analysis show the presence of diversified putative UPOs sequences in almost 30 classes of Basidiomycota, Ascomycota and fungus-like Oomycota, with some of the hosting species living in extreme pH conditions or saline environments [65]. Like the well-known Cytochrome P-450 and Chloroperoxidases (CPOs), they host in the active site an heme-thiolate prosthetic group with an iron atom coordinating a proximal cysteine residue. UPOs are small monomeric proteins with molecular masses and isoelectric points varying from 32 to 46 kDa and from 3.8 to 6.1, respectively. Because of the high degree of glycosylation, UPOs are appreciated for their water-solubility and stability. Furthermore, the large variety of reactions they can catalyze is attracting the research attention for the design of new biocatalytic routes [66]. At the only expense of hydrogen peroxide (used as electron acceptor) UPOs catalyze the formation of alcohol products starting from short and medium-chain alkanes, and unlike CPOs, also from aromatic compounds and recalcitrant heterocycles [65]. Over 300 possible molecules are reported as a possible substrate for UPOs catalysis, including naphthalene, toluene, pyrene and p-nitrophenol among the aromatic rings and pyridine, dibenzofuran and various others as recalcitrant heterocycles.

Vinyl monomers are industrially important commodity chemicals used to produce polyacrylate, polystyrene, adhesive, protectives coatings, resins, rubbers, and other copolymers. Avasthi et al. [67] reviewed recent catalytic transformation strategies used to produce three important vinyl monomers such as acrylic acid (AA), methacrylic acid (MA) and styrene (ST) which precursors (itaconic acid, glycerol, allyl alcohol, lactic acid and acrolein) can be biomass-derived. Styrene is derived from oxidative dehydrogenation of ethylbenzene. Ethylbenzene can be obtained from selective lignin depolymerization[68]. Ethylbenzene, together with other valuable arenes (benzene, toluene and xylene) are now obtained from lignin [69].

Kroutil and co-workers [6] developed a photocatalytic process for the stereoselective conversion of ethylbenzene to 1-phenylethanol. Highly pure (S)- or (R)-enantiomers were obtained by simply tuning the visible light wavelengths used to activate a carbon nitride (CN-OA-m) photocatalyst. When the carbon nitride is irradiated with green light (528 nm), electron holes with lower oxidation potential are formed and hydrogen peroxide is photocatalitically produced from the water molecules present in the solution. On the contrary, the irradiation of CN-OA-m with more intense photons (440 nm) leads to the formation of a stronger oxidant excited state affording the C-H bonds oxidation to produce acetophenone. In situ generation of H_2O_2 supports the asymmetric hydroxylation of ethylbenzene catalyzed by the unspecific peroxygenase from *A. aegerita* (*Ae*UPO), leading to the formation of enantiopure (R)-1-phenylethanol (99% *e.e.*) (Fig. 8.4). An alcohol dehydrogenase (ADH-A) from *Rhodococcus ruber* is instead employed to enantioselectively convert acetophenone to (S)-1-phenylethanol. The two light-driven reactions are wavelength specific enabling a fine control of the entire photobiocatalytic system by simple governing the emission light stimuli. More interesting, a protective effect against *Ae*UPO deactivation was revealed upon green light illumination compared to

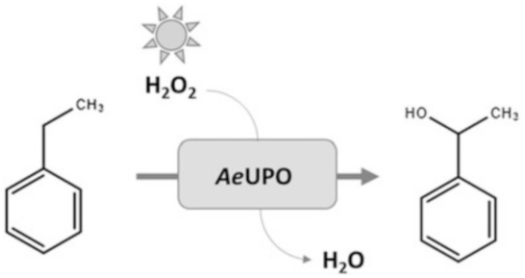

Fig. 8.4 *Agrocybe aegerita* (*Aae*UPO) oxidation of ethylbenzene to 1-phenylethanol driven by in situ photocatalytic production of H_2O_2

blue light due to minor production of ROS species. The substrate scope of *Ae*UPO was also partially investigated with a series of methyl and halide substituted ethylbenzene. This elegant proof-of-concept pointed out, one more time, the importance of the rational design to assure a full exploitation of the photobiocatalytic system and to maximize its stability. Overoxidation of cyclohexane is a desirable reaction for the production of ε-caprolactame (key precursor of nylon polymers). *Ae*UPO can be used for the conversion of cyclohexane into the corresponding ketone via a self-sustainable PBC system exploiting visible light energy. The excited state of the organic dye sodium anthraquinone sulfonate (SAS) was used to both yielding H_2O_2 via oxygen reduction, to drive the substrate hydroxylation catalyzed by *Ae*UPO, and to mediate the further oxidation of the alcohol into cyclohexanone [70]. The same PBC system was also able to sustain the halogenation of thymol (a common natural terpenoid) catalyzed by a vanadium-dependent chloroperoxidase from *Curvularia inequalis* (*Cv*VCPO) [70]. In the two above mentioned works, substrate solubilization is required to afford the reaction in an aqueous solution. A study by Selin and colleagues [71] overcame the need for additional solvent supplementation, thus decreasing the number of pre-processing steps. In this case, an engineered *Ae*UPO (*Aae*UPO PaDa-I) was immobilized into calcium alginate beads assuring good enzymatic stability in organic media. In situ hydrogen peroxide generation was concomitantly provided by light excitation of nitrogen-doped carbon nanodots and the strategy afforded the production of cyclohexanol in neat cyclohexane solution.

8.3.2 Cofactor Dependent Enzymes

Monooxigenases are a powerful class of enzymes that catalyze the insertion of one oxygen atom (oxidation or hydroxylation) into a wide variety of organic substrates for their functionalization. Monooxygenases can selectively oxidize alkenes, ammonia, methane, cyclohexanones, styrene, dyes and phenols, just to mention few of their substrate [38]. To do that these enzymes have to activate molecular oxygen, and this activation occur only if coupled with donation of electrons to molecular oxygen, and some di-copper monooxygenases share similarity with mono-copper LPMO in the mechanism of activity [72]. Almost all monooxygenases depend upon cofactors

to obtain the electrons (i.e. mostly NADH and NADPH) which implies that this reducing agent equivalent molecule is oxidized then must be regenerated [73].

8.3.2.1 Baeyer-Villiger MonoOxygenases - BVMOs

Baeyer-Villiger monooxygenases (BVMOs) are FAD-containing redox proteins which activate O_2 for the incorporation of one oxygen atom into the substrate and reduce the other forming H_2O (EC 1.14.13.X). BVMOs are largely widespread among the tree of life and currently more than hundred eukaryotic and prokaryotic representatives have been isolated, characterized and crystallized for structure determination. Generally, they catalyze the conversion of ketones and cyclic ketones into esters and lactones, respectively. The binding of a NAD(P)H cofactor and the following two electrons flavine reduction are needed for initiate the catalysis of BVMOs, representing the first reductive half-reaction. During the second half-reaction, dioxygen is activated through the formation of an unprotonated peroxyflavin intermediate that attacks the carbonyl group in the substrate. In the absence of a suitable substrate, the uncoupling reaction leads to the production of hydrogen peroxide, as commonly happens for all monooxygenases. The broad substrate scope and variety of specific reactions catalyzed by BVMOs render this enzyme highly attractive for biotechnological applications but only recent research advances made them suitable for industrial uses [74]. One example is the Cyclohexanone monooxygenase (CHMO) from Acinetobacter sp. NCIMB 9871 which shows an impressive substrate scope as well as exquisite chemo-, regio-, and enantioselectivity and is currently employed for the enantioselective sulfoxidation of pyrmetazole to produce esomeprazole [75]. The full exploitation of BVMOs potential at industrial scale lies on some limiting factors such as poor enzyme stability in solution (being intracellular enzymes), sustainable cofactor regeneration and substrate/product inhibition.

A common strategy assuring continuous cofactor regeneration is the use of a whole-cell approach where the biocatalysis is supported by an engineered microorganism overexpressing the aimed enzyme. In 2017, Böhmer and co-workers [21] showed an interesting solution to cope with BVMO cofactor regeneration avoiding the depletion of energy-rich organic molecules, such as glucose in the case of whole-cell approach with heterotroph, or preventing the use of artificial photocatalysts when the reduction of NAD^+ is mediated by light (Fig. 8.5). By engineering the

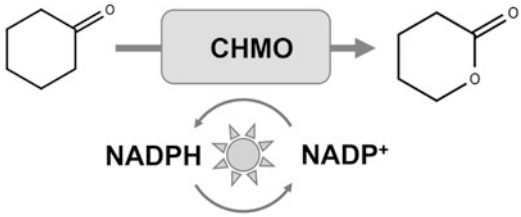

Fig. 8.5 Cyclohexanone MonoOxygenase (CHMO) catalyzed oxidation of cyclohexane to ε-caprolactone driven by photocatalytic regeneration of NADPH cofactor

cyanobacteria *Synechococcus elongatus* with the NADPH-dependent Baeyer-Villiger cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*, the authors were able to exploit the natural photosynthetic production of reducing power for the CHMO activity. The cyclohexanone substrate was completely converted into δ-valerolactone within 48 h without affecting the cells viability, thus allowing for further re-cultivation after the biotransformation. Whereas the inhibitory effect of high substrate concentration can be relieved by adopting sequential or simultaneous reaction configurations where the true enzymatic substrate is produced over time by a preliminary (photo)catalysis. The combination of the photocatalysis and enzymatic conversion was developed by Hollmann and colleagues [76] by designing a universal two-phase system for the C-H functionalization of simple alkanes. In this approach the organic phase acts as substrate reservoir while the aqueous phase contains the photocatalyst and the selected enzymes together with the relative cofactor, when needed. The reaction was taking place at the interface between the organic and the aqueous phase preventing the incompatibilities between the photo-organo catalyst and the biocatalyst, such as premature substrate degradation and enzyme inhibition caused by light-driven ROS generation or high substrate concentration. The light energy is directly used to drive small molecules oxidation into aldehydes or ketones by mean of the organophotocatalyst SAS (sodium anthraquinone sulfonate), and specifically in the above-mentioned work, cyclohexanol was oxidized to cyclohexanone. The further substrate functionalization is then catalyzed by CHMO or HAPMO (4-hydroxy-acetophene) yielding ε-caprolactone or phenyl formate, respectively. The compartmentalization into two phases was beneficial for the system productivity compared to what has been achieved by the same system but in a homogeneous environment. Alternatively, a photo-biocatalytic cascade reaction can be designed to build a hybrid system where the catalytic substrate preparation is followed by the enzymatic conversion with a whole-cell approach [8]. The one-pot two-step reaction consists in a first oxidation of the cyclohexane to obtain the relative lactone using in situ photocatalytical generation of H_2O_2. Secondly, the whole-cell approach is used to support the biocatalytic Baeyer-Villiger oxidation of the cyclohexanone into the desired ε-caprolactone employing a cell suspension of *E. coli* expressing the cyclohexane monooxygenase from *Acinetobacter calcoaceticus* (*Ac*CHMO). Here, the photooxidation of cyclohexane mediated by Au-TiO_2 or g-C_3N_4 assured a clean and specific production of the cyclohexanone intermediate and the use of the cell suspension instead of the free extract contributed to the *Ac*CHMO stabilisation.

8.3.2.2 FAP – Fatty Acid Photodecarboxylase

A new natural photo-enzyme named Fatty Acid Photodecarboxilase (*Cv*FAP, EC 4.1.1.106) was discovered in the microalgae *Chlorella variabilis*, by Sorigué and colleagues [77]. This FAD-dependent enzyme belongs to the GMC (glucose-methanol-choline) oxidoreductase family and, contrary to the other members, it is involved in the lipid metabolism by converting fatty acids (FAs) into alkanes in

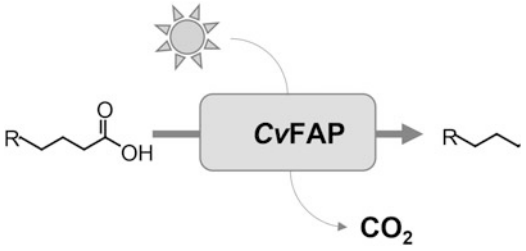

Fig. 8.6 Light-driven *Cv*FAP (Fatty Acid Photodecarboxilase) decarboxylation of palmitic acid

response of blue light (Fig. 8.6). This discovery pointed out that in nature the light-driven catalysis does not only relay in the photosynthetic processes or in the repair of UV damages in DNA, but it is also exploited in metabolic pathways. The catalytic site of *Cv*FAP is composed by a narrow hydrophobic tunnel harboring the FAD cofactor and allowing the stabilization of the fatty acid substrate. WT *Cv*FAP is able to oxidatively remove the carbonyl group of a wide range of fatty acids but showing higher efficiency for C16–C17 chains. The turnover number measured for palmitic acid decarboxylation is $0.86 + - 0.13\ s^{-1}$ with a quantum yield higher than 80% [77]. The discovery of FAP is revolutionizing the paradigm of biofuels production. The classical approach is based on the conversion of fatty acids in the corresponding methyl and ethyl esters (FAMEs and FAEEs). The transesterification process carried out in mild condition is an equilibrium reaction in which significant molar surpluses of alcohols are required to arise the conversion yield and subsequent purification steps are needed to remove the formed soap. The possibility to enzymatically decarboxylate FAs offers a sustainable alternative for the conversion of waste bio-oils into biofuels having even a slightly higher specific heat of combustion (ca.9%) compared to classical FAMEs.

Huijbers and co-workers [78] developed a bienzymatic two-step cascade for the conversion of non-edible oils and fats into biofuels. The process allows the enzymatic hydrolysis of triolein to free oleic acids and glycerol using a lipase from *Candida rugose*. The subsequent intermediates are then subjected to photodecarboxylation to obtain the corresponding C1-shortened alkanes. The irreversible reaction of photodecarboxylation is catalyzed by a natural photoenzyme derived from *Chlorella variabilis* (*Cv*FAP) which is activated upon blue light illumination leading to the photoexcitation of the FAD cofactor present in the active site. *Cv*FAP is characterized by a high substrate scope and good tolerance toward organic solvent (up to 50% DMSO). Higher activity and robustness were achieved performing the catalysis in presence of the cell crude extract rather than with the purified enzymatic preparation. In another approach spores of *Bacillus subtilis* were used as cell factories for the recombinant expression on *Cv*FAP and physical support for the enzyme immobilization. The *Cv*FAP was cloned in frame with an abundant endogenous coating protein assuring the localization in the outer layer of the spore coating. Microbial oils and olive oil were used for the hydrocarbon production in a one pot bienzymatic reaction. Commercially available lipase catalyzed the release of

free fatty acids and their decarboxylation was conducted under blue light irradiation by *Cv*FAP [79].

8.3.2.3 Ene-Reductases

Ene-reductases (EC 1.3.1.31), belonging to the Old Yellow Enzyme (OYE) family, are flavin mononucleotide (FMN)-containing redox enzymes catalyzing the reduction of C=C double bonds of a wide range of substrates at the expense of a nicotinamide cofactor. Several biocatalytic processes have been developed to sustain the regeneration of the natural reducing agent (NAD(P)H), to expand the enzymatic scope as well as combine various reactions to diversify the product range. Asymmetric chemical reduction yielding chiral products is an essential reaction for the synthesis of pharmaceutical and valued-chemical compounds. The stereoselective saturation of C–C double bonds in alkenes is currently achieved employing precious metal catalysts and hydrogen gas. Although the chemical conversions boast a perfect atom efficiency, new biocatalytic approaches represent a valid alternative when milder conditions, metal-free reactions and highly enantiopure products are desired [80]. Increasing research efforts are resulting in the enzymatic alkene reduction using ene-reductase as a key biocatalyst.

A first attempt for the sustainable regeneration of reducing power using water and light was proved by Köninger et al. [81] by developing a whole-cell biocatalytic approach based on a recombinant cyanobacteria (*Synehocystis* sp. PCC6803) overexpressing the enoate reductase YqjM from *Bacillus subtilis*. A broad range of cyclic prochiral alkenes were enantioselectively reduced by the viable biocatalyst upon irradiation with visible light and at the only expense of water oxidation (Fig. 8.7). Despite the high enantiopurity of the products (> 99% for 2-methylsuccinimide) and the fine light control of the reaction, further process optimizations are envisioned to achieve industrially relevant product yields. However, the cell tolerance toward high substrate concentration and the repression of side reactions leading to product (or substrate) degradation are still the common main challenges for most of the whole-cell technologies. In a recent work by Wang and colleagues [82] the light energy supply is exploited to both drive the ene-reductase activation and the substrate isomerization from a less to a higher reactive form. Based on the light dual utilization concept, the authors conceived a chemoenzymatic system aimed to convert mixture of alkene isomers into an enantiopure product using

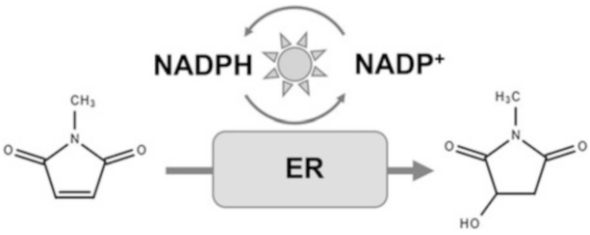

Fig. 8.7 Photocatalytic cofactor regeneration for Ene-reductase (ER) reduction of 2-methyl-*N*-methylmaleimide to (*R*)-2methyl-*N*-methylsuccinimide

blue light irradiation. Mixtures of alkene substrates are often low isomeric homogeneous, and some isomers (*E*) are preferentially targeted by ene-reductases than the (*Z*) counterpart causing a loss of conversion yield. A photosensitized energy transfer process mediated by an iridium catalyst, is used to drive the alkene isomerization from *Z*-alkenes to *E*-alkenes. Those reactive intermediates are then subjected to asymmetric reduction by specific ERs via photoinduced electron transfer for the regeneration of the flavin coenzyme ($FMNH_{red}$). The chemoenzymatic system has been tested for the conversion of several aryl alkenes with specific reductase enzymes, all of them yielding products with high optical purity (> 95% ee, in average). An improved final yield was observed when the two light-driven reactions worked in a cooperative manner rather than sequentially performed. This is likely due to the benign competition among chemo and enzymatic reactions that prevents the non-specific alkene reduction mediated by the photoexcited free FAD cofactor.

8.3.3 New Proposed System for Conversion of Biomass and CO_2 to Value-Added Bio-alkanes

Very recently Lin and colleagues [83] envisioned a circular cascading system for the production of bio-based fuels combing the latest bioeletrochemical technologies such as electro-fermentation, microbial CO_2 electrosynthesis and photobiocatalysis. Through electro-fermentation, renewable feedstocks can be converted in short chain carboxylic acid (such as acetic and butyric acid) and CO_2 which are then subjected to chain elongation to obtain e.g., caproic acid via microbial CO_2 electro synthesis. Finally, the specific calorific value of medium chain carboxylic acids is enhanced by removing their carbonyl groups yielding relative alkanes through the photoenzymatic catalysis step using *Cv*FAP. The application of an electrical field in fermentation processes allow a fine regulation of the microbial redox metabolism leading to an increased carbon usage efficiency and improved products selectivity compared to traditional fermentation [84]. In this context, the working electrode (acting as electron donor) favors cathodic reactions resulting in the reduction of the substrate and the product enrichment in volatile fatty acids [85]. CO_2 is then used as principal substrate for the sequential carbon chain elongation of short chain carboxylic acids by a microbial electro synthesis driven by reducing power externally supplied from a cathode with higher potential [86]. The entire biorefinery process is envisioned to end up with the photobiocatalytic oxidation to alkanes performed by FAP enzymes under mild conditions. Despite the recent discovery of these photoenzymes, several studies have resulted in an improvement of its catalytic stability and in substrate preference tuning [87].

8.4 Bioresources for Photobiocatalysis and Applications

The PBC processes are particularly attractive for their potential applications to bioresources and biomass transformations, a key strategy to enhance the circularity aspects of our modern biobased society oriented towards the zero-waste goal. The transition from a fossil-based to a greener society is not only linked to consumption of bioderived or renewable goods, but also aims at increasing the sustainability of current productions methods still often entirely based on petrol-derived catalysts, toxic metals additives, and non-renewable energy inputs. Therefore, PBC based on natural pigments, sorted directly from the biomass or produced by microorganisms, are particularly attractive in these regards. Imagining a self-sustainable PBC process, an ideal bioresource should then be made of: (i) the substrate for the photoactive enzyme, or contain platform molecules (i.e. glucose) to be converted by a cell-based PBC system; (ii) moreover, should have sacrificial molecules to recharge the PS or to work as electron donors; (iii) and optionally a natural pigment, either sorted directly from the bioresource (i.e. chlorophyll-derived from green grasses), or produced by the cell. Finally, combinations of bioresources and/or waste streams could allow the instauration of the above-mentioned conditions, and if the same bioresource could sustain the endogenous production of the enzymatic catalysts, then it would further increase the entire sustainability of the process towards stand-alone conditions as well. The coupling of photocatalytic and biodegradative process is a relatively recent strategy allowing efficient removal of pollutants contained in waste waters [88] but still differs from PBC as the photoelectron are not directly transferred to the enzymatic active site, and needing of a porous carrier for instauration of a microbial film. The process is known as intimate coupled photocatalysis and biodegradation ICPB, and already found several fields of applications and a patented technology (WO2009023578A1) for general abatement of organics, phenolics removal [89]; and tannery waste waters often containing dyes [90, 91]. Therefore, such waste waters could be blended with lipids rich or lignocellulosic rich slurries for achieving simultaneous removal of toxicants and conversion of the primary organic waste into the desired products.

Several available bioresources or organic wastes could sustain entirely or partially a PBC system and could be divided as naturally occurring bioresources or anthropogenic biowaste. In this paragraph we classified them following the chemical class of the major substrates that are target of enzymatic conversion, specifying the type of PBS that could be sustained by the specific biowaste, their source and sector of application (Fig. 8.8 and Table 8.2).

8.4.1 Lignocellulose

Nonedible lignocellulose bioresources are common in the biorefinery sector for production of bioethanol, lignols and second-generation hydrolysates/molasses.

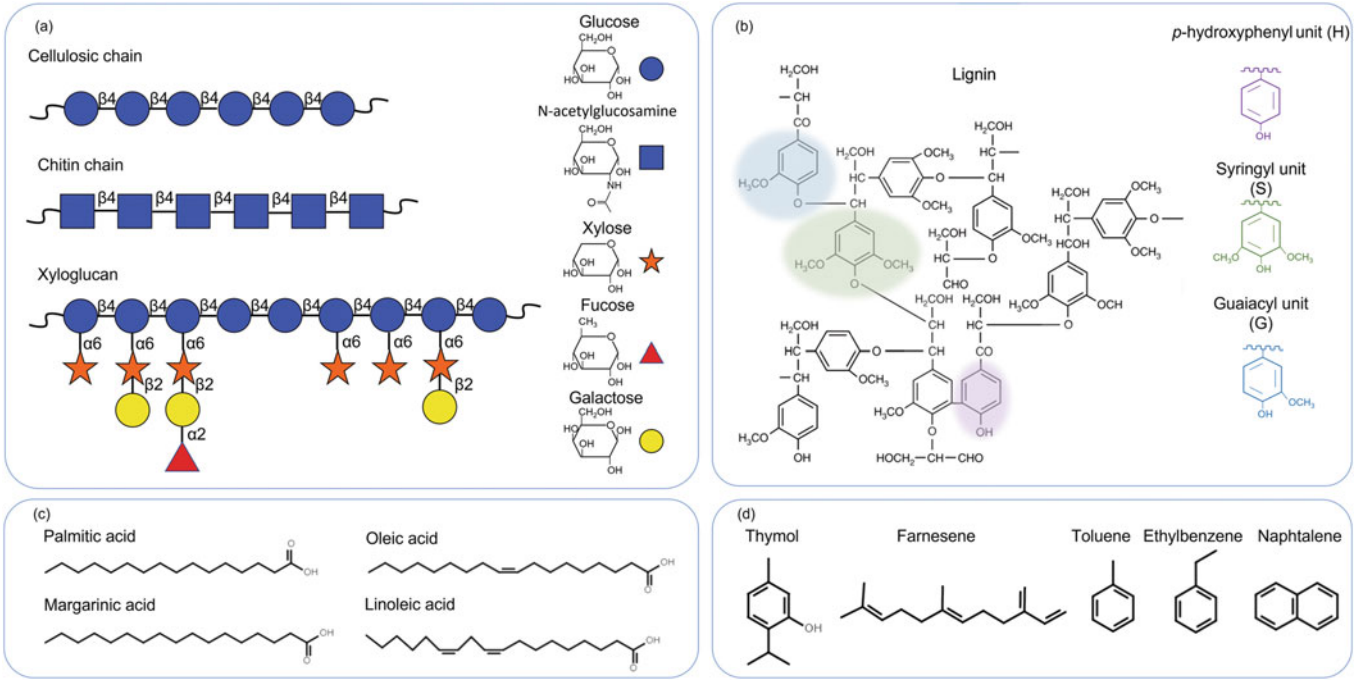

Fig. 8.8 Structure of suitable substrates for photobiocatalysis from bioresources grouped by class: (**a**) monosaccharidic building blocks (right) and polysaccharides (left) from plant biomass; (**b**) lignin units (right) and a representation of a natural lignin (left); (**c**) fatty acids; (**d**) terpenoids. Adapted with permission from [92]. Copyright © 2015, Elsevier

The main source are woody materials, agricultural waste and residues of dedicated crops (i.e., sugarcane bagasse). They originate from plant cell walls that are composed of a limited chemical diversity mainly carbohydrate polymers and phenol-based hetero-molecules: respectively cellulose, hemicellulose and lignin. Polysaccharides are made of relatively few types of monosaccharide building blocks (glucose, xylose, mannose, arabinose and galactose among the others, Fig. 8.8a). Moreover, their composition is further reduced after chemical-physical pretreatment needed for preparing the lignocellulose for subsequent enzymatic hydrolysis steps. Often only glucans- and xylan-based polymers dominate the entire saccharide fractions of pretreated lignocellulose, but still organized in recalcitrant ultrastructure surrounded by phenolic heteropolymer. This highly cross-linked molecule, referred to as lignin does not have a proper polymeric organization as distinct repeating units are not evident, but for easiness of definition is synthesized from three main phenolic units: para-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) (Fig. 8.8b). It is polymerized in a 3D structure enveloping the polysaccharides, filling the voids among them, finally providing inaccessible barrier. The composition of lignin varies among plant species. Softwood (gymnosperms) is rich in G units (G:S:H 94:1:5 in % of total lignin [93]) while angiosperms in general have more G and S lignin (wheat straw, G:S:H 45:46:9 in % of total lignin [94]) and an overall lower amount compared to softwoods.

The role of lignin during enzymatic hydrolysis has been subject of extensive studies often showing negative effects to glycosyl hydrolases inhibition occurring through irreversible non-productive adsorption mainly [95, 96]. However, this paradigm had been recently challenged thanks to seminal discoveries that have seen the lignin derived phenolics being able at activating key redox enzymes, i.e. LPMO [52, 62, 97], that consequently increase the activity of glycosyl hydrolases. In addition, lignin derived molecules could even provide electrons to photo-reduced photosensitizers, also able at donating photo-excited electrons to LPMO, i.e. chlorophyllin [12], therefore making lignin the ultimate donor of electrons in the role of sacrificial molecule for PBC [12]. Because lignin is regarded often as the waste of biorefineries, this confers it the label of the most attractive sacrificial molecule to be used for powering photocatalytic system owing to its wide range of redox potential, availability and low costs [98].

Of note, is that all evidence on the role of lignin as LPMO activator has always dealt with pretreated lignin often using hydrothermal or organosolvent strategies, that tend to preserve the native phenolic structure [12, 99], compared instead to the highly oxidizing technology like alkaline based methods which are found detrimental for preserving the reducing ability of lignin [100]. Potentially photoactive enzymes, such as laccases and peroxydases, have been found that depolymerize lignin [101], laying a major route for future development in the light driven conversion of lignocellulosic biomass. To summarize, the potential uses of lignocellulosic biomass in PBC based on LPMO enzymes are linked to the productions of either cello-oligosaccharides and xylan-oligosaccharides using waste lignin as electron donors and leaving a semi-treated lignocellulose material to ease subsequent enzymatic hydrolysis for molasses productions.

On the side of freshly harvested lignocellulosic resources, it is worth mentioning the green grasses, an emerging source of various components of interest also for PBC. Often green grasses are treated immediately after mowing, leaving the chlorophyll derived pigments still active in donating electrons to redox enzymes although still anchored to the semi-lysates thylakoids membranes from chloroplasts [12]. This is due to the gentle screw-press separation which fractionates the liquid part of green grass (green juice) containing water soluble proteins, thylakoids, sugars and cell lysates, from the fibers, mostly composed of water insoluble polysaccharides and minimal fraction of phenolics. The fiber so obtained could undergoes subsequent physical-chemical pretreatment and later being reunited with the green juice to sustain a PBC process based for example on photoactivable redox enzymes acting on the cellulose fibers for production of oligosaccharides (Table 8.2). Alternatively, other enzymes able at receiving electrons from photoexcited thylakoids could be coupled.

8.4.2 Chitin

Chitin is considered the second most abundant polysaccharide in nature after cellulose, with which it shares many chemical and structural similarities, so that often it is referred to as the "animal cellulose". The backbone of chitin polymers is made of N-acetylglucosamine linked through a β-1,4-glycosidic bond, thus featuring both amorphous and crystalline regions, it can also be isolated in form of nanofibrils. Today, many industrial process and specialty chemical applications are based on chitin rendering this molecule attractive for biotechnological purposes from food additives (edible protective films) to plant elicitors and bio-stimulants. Common into the cell walls of fungi and exoskeleton of insects and mollusks, chitin is often sorted in quantity from different man-made wastes: industrial fishery, fungal cultures, and food-waste. In biocatalysis research, chitin had been a precursor of several discoveries regarding degrading enzymes that later had been also confirmed or applied to cellulose degradation, as for example the discovery of the LPMO class (namely the CPB21 enzyme [40]. Therefore, after proving the photoactivation of LPMO on cellulose substrate, it was also found that similar PBC system could be applied on chitin-active LPMOs AA10 giving an array of native and oxidized chito-oligosaccharides, also confirming an higher turn-over rate when exposed to light and using chlorophyllin as PS [14].

8.4.3 Lipids

Lipids and fatty acids represent a wide class of substrates for photoactive enzymes either in PBC mode or whole cells PBC. They are extracted from bioresources or produced as metabolites from growing cultures that often contains also natural

photosensitizers and sacrificial molecules. The major source of renewable lipids for biofuels applications are agricultural dedicated productions (first-generation crops, *i.e.,* palm seeds, soybean, rapeseed), or their direct or processing waste (*i.e.,* not-edible olive oil pomace and their black waste waters, second generation biofuels); then algal productions (advanced biofuels or third generation); and finally, food industry waste (*i.e.,* waste cooking oil - WCO). Those mentioned represent the more common sources of lipids worldwide but obviously there exists many other sources. Particularly at local scale one could find more abundant availability from man-made activities or local biomass productions, and often with already established chains of disposal and collections that could enhance further the feasibility and circularity of their bioprocessing [102].

8.4.3.1 Algal Culture

Algal cultures produce fatty acids for carbon storage purposes (up to 50% in dry weight) or alternatively as secondary metabolites. Starting from CO_2 algae could virtually photo-convert this into any given substrate either naturally or via genetic modifications. For example new cyanobacterial strain, *Synechococcus* sp. PCC 11901, was discovered recently and engineered to produce free fatty acids in yields over 6 mM (1.5 g L^{-1}), an amount produced autotrophically and comparable to that achieved by similarly engineered heterotrophic organisms [103]. The literature is vast on bioresources production form algal cultures [104] (the reader is also redirected to Chap. 12 of this book).

The fatty acids produced by algae are a mixture of saturated and monounsaturated, and different lengths often ranging commonly from 14 to 20 carbons, called polyunsaturated fatty acids (PUFAs). Often their particular ratio or profile is unique for each strain and/or cultivation conditions [105]: among others linoleic, linolenic and arachidonic acids are the most commonly found algal PUFAs [106]. Phycobilisomes are the light harvesting antennae supercomplexes of cyanobacteria and red algae and consist of three types of phycobiliproteins: allophycocyanin, phycoerythrin, phycocyanin [107]. These phycobiliproteins are responsible for absorbing light in the region of 500-650 nm usually not permitted by chlorophyll pigments. Anchored on the surface of thylakoids, they can be used as PS for donating electrons to redox enzymes. Thylakoids of the cyanobacterium *Synechococcus* sp. PCC 7002 were used in combination with LPMO enzymes for successful oxidation of cellulose fibers [12]. Finally, an array of secondary metabolites is usually produced by algal and microbial cultures of which several could serve as sacrificial molecule once the cells are lysate: organic acids (ascorbate, gallate, 3HAA, etc.), phenolics, aromatics.

8.4.3.2 Food Waste

An important source of lipidic substrates is society-made linked to the food waste of various sectors. As for example, we analyze here the waste cooking oil, that in many parts of the world is separated from the general household waste through a dedicated collection system, therefore enabling potentially a series of tailored bioprocesses. The United States generates approximately ten million tons of WCO annually [108], while considering urban scale a particular indicative case is that of Hong Kong. There three million tons of municipal waste is produced every year and based on data reported by Karmee et al., the lipid fraction could sum up to approximately 400 thousand tons, which is attractive for local biodiesel production [102]. The chemical variety of waste cooking oil or lipid fraction of municipal waste can variate greatly among various regions, and in Shanghai in 2010 was reported to be made of linoleic, oleic and palmitic acid (55%, 21% and 8% respectively) (Fig. 8.8c) [109].

8.4.4 Microbial Production of Relevant Substrates

Continuous development on genetic engineering technologies, like CRISPR-Cas for example, are easing synthetic biology approaches for isolation of unique cultures for production of chemicals. Virtually no limits could be posed to the variety of molecules achievable with genetically modified strains. Today several de novo enzymes could be introduced in various hosts, for reconstituting entire biosynthetic pathways or creating new enzymatic cascades for producing the desired molecule. In photobiocatalysis these approaches are becoming popular either for producing the substrates to be used for photoactive enzymes or to form entire photocatalytic living cells, where the photosensitizers, enzymes, and substrates are assembled to produce the final product upon light energy and a carbon source (often CO_2). This latter approach is described in paragraph 2 dedicated to enzymes and whole cell applications of PBC, here we limit our discussion in the use of biologically derived substrates for PBC.

8.4.4.1 Terpenoid and other natural hydrocarbons

Terpenoids from plants represent probably the largest class of compounds in terms of chemical diversity. Many terpenoids are the building block for the production of several commodities such as whole are considered platform chemicals. Also, many have optimal reducing ability for donating electrons to exhausted photosensitizer, besides being themselves photoactive or photo-reducing. Olive oil waste waters and solid waste of olive processing often contain several terpenoids [110] and the reducing ability or antioxidant is retained for long time after harvesting or processing [111]. Many interesting compounds are today biorefined enzymatically from this

agricultural waste. Although natural sources are available, many studies are now focusing in producing terpenoids using microbial cells factories using the principle of synthetic biology purposely for increasing yield and ease downstream separation. Often the strategy adopted involves also photoactive enzymes (*i.e.* P450 enzyme) along the biosynthetic pathway reconstructed in microbial hosts [112]. The latter if also naturally containing photosynthetic complexes or modified to express some could indeed represent a self-sustaining cell-based photobiocatalytic process [24].

Farnesene is linear sesquiterpenoid (Fig. 8.8d) of various biological function and substrate of the OYEnzy or ENE reductases which catalyze the double bond rearrangement also in other alkene so allowing production of special stereoisomers of various molecules. Upon improvement by addition of farnesene synthase gene from *Artesemia annua*, S. cerevisiae strain could produce farnesene at a yield, when fed with glucose a conversion of 0.12 g/g was achieved [113]. Few other aromatics hydrocarbons are also produced microbiologically. In nature toluene [114] and naphthalene [115] (Fig. 8.8d) have been found in the environment of their relative ecosystems being produced for specific ecological purposes that are still not elucidated yet. However, the existence of the biosynthetic pathways in distinct organisms poses the fundaments for developing future biotechnologies for the enhanced bio-productions of such chemical commodities for direct use as substituent of actual petrol-based production, or as common substrate of photo-catalytically active enzymes for subsequent transformation into other necessary molecules. Styrene is a cyclic alkene (Fig. 8.8d) that can be oxidized to its epoxy form by monooxygenases like laccases [60]. Polystyrene it is probably one the most produced plastic in the world. It has been demonstrated that styrene can also be formed by micro-organisms from renewable substrates such as glucose [116].

8.5 Conclusions and Future Outlook

The idea of converting light-energy directly into chemical-equivalent, biofuels and biomaterial is the Holy Grail for a sustainable fossil-free society. To that end photobiocatalysis applied to the transformation of society-made waste and bioresources represent the closest technological option in our hands. Although the goal is still far as generally accepted, PBC is at its infancy, yet today we can count several scalable enzymatic systems on which could be focused on for future stable application. The scalability of the technology then is influenced upon few basic barriers being the choice of the sacrificial molecule and photosensitizer; the sourcing and composition of organic substrate to be converted; and other engineering aspects like, light supply, volumes/surface, bioreactor design etc.

There exist several classes of molecules that can be used as photosensitizer in PBC, from organic biologically extracted pigments, synthetic molecules based on metal, and semiconducting nanoparticles of which either industrial application already exists (i.e., chlorophyllin) or which its production is facile (carbon and graphene-based nanoparticles). The conversion of the light energy into chemical

energy primarily depends on the ability of a photosensitizer to absorb a photon, be promoted to its excited state, and donate the electron at the conduction band to an external acceptor before return to the ground state. This would leave the organic photosensitizer oxidized or photobleached preventing a new round of photoexcitation almost immediately after light exposure, yet given to its abundance in case of biologically derived (thylakoids etc.) this would be a tolerable cost. Instead, when using metal complexes for photoexcitation, this causes electronic holes that need to be quenched, although more expensive than biologically derived carbon nanoparticles could still undergo several cycles before exhausting.

It is therefore evident that in any photosensitizer option that one could choose, there is a need for refilling the electron photoexcited, and a sacrificial molecule must always be added to PBC. In nature, this is achieved by water molecule splitting, that is considered the most successful photo-biocatalytic example, representing though the final aim of artificial photosynthesis or PBC. Meanwhile, since we have not yet achieved the goal, still an equivalent electron donor has to be sacrificed. Their choice is crucial for the sustainability of the PBC as their chemical and economical value must be lower than the of the final product. Therefore, photobiocatalysis systems based on organic waste, carrying their own pool of sacrificial molecules otherwise not exploitable by any other bioprocesses (*i.e.*, low concentration), are becoming attractive.

Regarding the waste composition as emerged here, several biowastes are found suitable for PBC processes, some bringing all the components needed, substrate, pigments, sacrificial molecule or possibly enzymes. The major problematic issues associated with the use of bioresources in general is then linked to the solid particles, fibers, and membranes that cause light scattering and shading which impair a uniformed transmittance of light. To that end, slurry clarification by dedicated decanter units or membrane-based technology are a valid option as already used at commercial scale for cellulosic ethanol biorefinery. Another beneficial aspect of these strategies is also avoidance of hydrophobic adsorption of PS or enzymes on fibers and lignin.

Although many successful PBC systems have been reported in the literature (Table 8.1), the conversion yields and reaction volumes are still performed on laboratory scales making it difficult to obtain a reliable estimate of their scalability. The lack of studies investigating the robustness and reproducibility of PBC reactions in increasing volumes hamper the transition from "proof-of-concept" set up to the design of platforms at industrial relevant scale, and this is where the next research should be focused. However, the design and development of PBC large-scale reactors may benefit from what it has been already done concerning the optimization of photobioreactors for microalgal cultivation, sourcing then from decades of research for a quick adaptation of the technology to PBC.

Overall, the nascent field of photobiocatalysis holds all the premises for rapid development from lab-scale to commercial scale technology, especially if coupled with transformation of inexpensive substrates today considered waste, into added value chemicals, platform chemicals, and or material. Key to this success will be the adaptation of mature technologies developed for biorefinery and algal cultures.

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