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Review

Advanced *Rhodococcus* Biocatalysts for Environmental Biotechnologies

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Abstract: The review is devoted to biocatalysts based on actinobacteria of the genus *Rhodococcus*, which are promising for environmental biotechnologies. In the review, biotechnological advantages of *Rhodococcus* bacteria are evaluated, approaches used to develop robust and efficient biocatalysts are discussed, and their relevant applications are given. We focus on *Rhodococcus* cell immobilization in detail (methods of immobilization, criteria for strains and carriers, and optimization of process parameters) as the most efficient approach for stabilizing biocatalysts. It is shown that advanced *Rhodococcus* biocatalysts with improved working characteristics, enhanced stress tolerance, high catalytic activities, human and environment friendly, and commercially viable are developed, which are suitable for wastewater treatment, bioremediation, and biofuel production.

Keywords: biocatalysis; environmental biotechnologies; actinobacteria; *Rhodococcus*; whole cell biocatalysts; cell immobilization

1. Biotechnological Advantages of Actinobacteria of the Genus *Rhodococcus* and Approaches to Obtain Stable Biocatalysts

Chemical contamination of the Earth is a global ecological problem. Billion tons of chemical wastes (petroleum hydrocarbons, heavy metals, synthetic polymers, plasticizers, pesticides, agricultural fertilizers, and dyes) enter the environment every year [1–8]. For instance, an estimated value of 250 million tons of plastics arrived in marine environments in 2015 alone [6]. The situation is complicated by novel groups of emerging persistent contaminants, such as microplastics (plastic fragments of sizes smaller than 5 mm), endocrine disruptors (hormonal drugs, bisphenol A, surface active alkylphenols, flame retardants, and plasticizers, for example, dimethyl, dibutyl, and dioctyl phthalates), and pharma pollutants [4,6,9–12]. Although green chemistry, recycling technologies, and careful attitudes to goods and natural resources are factors reducing chemical pollution, this problem is still far from being solved. Biological removal of contaminants (bioremediation *in* or *ex situ* with stimulated or introduced degrading microorganisms, biocomposting, biofiltration, detoxification with enzyme preparations, anaerobic digestion, activated sludges, and other techniques) is considered to be the most effective and ecologically safe clean-up approach. Biological methods do not employ aggressive reagents and adverse conditions and often provide complete mineralization of pollutants [1,9,11–15].

Actinobacteria of the genus *Rhodococcus* are well-known xenobiotic degraders [16–22]. These bacteria synthesize a wide range of disrupting enzymes, such as dehydrogenases, peroxidases, oxygenases, alkylsulphatases, nitrilhydratases, and phenolhydrolases. Oxygenases are most powerful of these enzymes because they are structurally and functionally diverse and have broad substrate specificities [17,21,23–30]. For example, 73 monooxygenases, 22 cytochrome P450 oxygenases, and 45 dioxygenases are encoded in the genome of the hydrocarbon-oxidizing *Rhodococcus ruber* strain IEGM 231, a microorganism able to utilize gaseous (C_2–C_4) alkanes. Moreover, diverse genes coding for monooxygenases (*mmoABC/prmA*,

alkB, flavin monooxygenase, and cyclohexanone 1,2-monooxygenase) were recovered in this strain [31]. In the genome of the polychlorinated biphenyl-degrading *Rhodococcus jostii* strain RHA1, 203 oxygenase genes with particular overrepresentation (19) of cyclohexanone monooxygenase genes were found [32,33]. This makes *Rhodococcus* able to assimilate a wide range of organic compounds: linear and branched alkanes C_2-C_{30}, cycloalkanes, benzene and its homologs, polycyclic aromatic hydrocarbons (PAHs), phenols, aromatic acids and their derivatives, halogenated hydrocarbons, including polychlorinated biphenyls (PCBs), nitriles, esters, isoprenoids, organic sulfides, nitrosubstituted organic compounds, N- and S-heterocyclic compounds, and synthetic polymers [16,18,21,24,34–38]. *Rhodococcus* strains are used for detoxification of dangerous chemicals, for example PAHs, PCBs, explosives, pharmaceuticals, endocrine disruptors, and pulp and paper wastes [18,20,21,34,38–40], in bioremediation [19,41] and plastic biodegradation [36,42,43]. Remarkably, rhodococci can attack polyethylene, one of the most abundantly produced and recalcitrant synthetic polymers [44].

Concerning biosynthetic capabilities of *Rhodococcus*, they are able to produce glycolipid biosurfactants [17,45,46], triacylglycerols [47], and polyhydroxyalkanoates (PHAs) [48,49]. *Rhodococcus* biosurfactants can be used as surface-active agents for the removal of hydrophobic pollutants from soil and water. They are nontoxic, even in comparison with other microbial surfactants, and show high desorbing, emulsifying, and washing activities towards hydrocarbons and their derivates [45,46,50]. Rhodococcal triacylglycerols are appropriate lipids for production of biodiesel, a renewable energy source intended to replace petroleum-based fuels [47], and PHAs are the basic compounds for production of bioplastics [51].

There are several biological features of rhodococci that make them attractive for environmental applications. Among 67 *Rhodococcus* species (List of prokaryotic names with standing in nomenclature, http://www.bacterio.net/rhodococcus.html), only one species, *Rhodococcus hoagii* comb. nov. (*formerly Rhodococcus equi*), belongs to opportunistic human pathogens (risk group 2), and *Rhodococcus fascians* is phytopathogenic [22,52,53]. It therefore requires no special occupational health precautions during application processes. Rhodococci are ubiquitous, they inhabit soils, surface and subsurface water bodies (rivers, springs, ponds, stratal waters, and marine environments), bottom sediments, and plant surfaces around the world [22,54–56]. As autochthonous microorganisms, they are ideally adapted to local environmental conditions including temperature, moisture, aeration level, ionic composition, presence of specific chemicals, and type of soil [54,56–58]. In some regions, the introduction of allochthonous microorganisms is restricted [59], which should be considered during *in situ* bioremediation. Rhodococci are frequently isolated from hydrocarbon-contaminated ecosystems where they often dominate. These bacteria have comparative advantages over other hydrocarbon-oxidizing microorganisms, such as intracellular pool of hydrocarbons and storage lipids, low levels of endogenous respiration, oligotrophy, direct passive transport of hydrocarbons into cells, lack of catabolic repression, and extremotolerance [54,56]. As a result, *Rhodococcus* strains are able to maintain their metabolic activities in a wide range of temperatures (−2–+40°C), pH (3–11), and NaCl concentrations (0.5–7.0%). Rhodococcal hydrocarbon-oxidizing activities are detected in many regions with cold and temperate climate, particularly in the Urals and Siberian parts of Russia, north Canada, the Arctic, Antarctica, and high mountains [19,56,59–62]. Moreover, rhodococci are resistant to toxicants like organic solvents and heavy metals. The organic solvent-tolerant *Rhodococcus opacus* strain B-4 survived for at least 5 days in *n*-tetradecane, oleyl alcohol, and bis(2-ethylhexyl)phthalate, which contained water less than 1% (w/v) [63]. Minimal inhibitory concentrations of heavy metals (Cd, Cs, Cr, Cu, Mo, Ni, Pb, Zn, and V) for *Rhodococcus* reached up to 250 mM; moreover, rhodococci were able to detoxify heavy metals and metalloids (As), changing their oxidation level [64,65].

Both *Rhodococcus* whole cells and purified enzymes are currently used as biocatalysts. Enzymatic catalysts guarantee high reaction selectivity and the absence of side reactions, while whole cells perform multi-step bioconversions and allow easy cofactor regeneration [66]. In any case, the most important parameter is the economic viability of a biocatalytic system, which depends greatly on the system's stability. Here, the term "stability" includes a constant rate of the biochemical process(es) catalyzed,

biocatalyst resistance to adverse conditions (toxic compounds, non-optimal temperature, salinity, or pH, starvation, and etc.), long-term storage without catalytic activity losses, and re-usability. The following approaches could be used to obtain stable *Rhodococcus* biocatalysts: (1) immobilization, (2) genetic modification, (3) cell acclimation, and (4) construction of multi-species consortia/associations. Immobilization of rhodococcal cells on or into the carrier is the most common approach since it is feasible and provides solutions for a number of technical problems. Genetically engineered microorganisms are considered unwelcome by many government agencies; acclimation is time-consuming; and development of multi-component biocatalysts requires proper studies of all biological agents used [67]. In this review, we focus on immobilization of whole *Rhodococcus* cells and briefly describe other approaches.

2. Guidelines for Immobilization of *Rhodococcus* on/into Carriers

2.1. Advantages of Immobilized Cells

Immobilized cells (IC) represent a group of heterogeneous catalysts and have the following advantages: a strongly concentrated biomass to provide effective concentration of catalytic units; the regulated contact area between "enzymatic factories" and a substrate; repeated usage; and facilitated separation of a biocatalyst. Immobilization minimizes cell losses and assures high cell loading without negative effects typical for the concentrated biomass, such as metabolic activity decrease and cell death. In comparison with suspended cells (SC), IC are protected from elimination by predatory protists, they are slow-growing and stress-resistant, have conventional storage and transportation form, high plasmid stability, and often increased catalytic activity [13,67–75]. Immobilization carriers can partly (ad)sorb the pollutant molecules, resulting in the decrease of their concentration and toxic impact. For example, the modified sawdust adsorbed up to 90% of heavy metal ions from contaminated water [76]. Furthermore, a diffusion distance between cells and a pollutant is shortened, and the (ad)sorbed pollutant molecules became readily biodegraded [77–79]. In contaminated soil, carriers act as bulking agents, facilitating aeration and moisture exchange, and therefore ensuring that microorganisms are sufficiently supplied with oxygen and water [78,80,81]. It is considered that, for heavy soils, addition of bulking agents is more important than choice of a bioremediation method (biostimulation or bioaugmentation); and examples are known of heavy soils that when supplemented with a bulking agent alone resulted in a significant increase of biodegradation rates [80–82].

However, regarding the aforementioned advantages, a suitability of whole cell immobilization for biocatalysts is still discussed. No differences between IC and SC and even partial losses of catalytic activities or stress resistance of bacterial cells after immobilization were revealed [69,83–86]. Each biocatalytic system is unique and its efficacy depends on many factors, such as biological properties of microorganisms and their response to a certain immobilization procedure, specificity of enzyme regulation in the cell, carrier features, and possible diffusion problems. It is generally accepted that immobilization favors the tolerance phenotype in bacteria, namely a high level of shock (heat, cold and osmotic) protein expression, a cytoplasmic membrane enriched with saturated fatty acids, and a specific cell wall glycoprotein profile associated with decreased cell envelope permeability. As shown by many authors, increased catalytic activities of IC could be a result of their enhanced tolerance to toxic substrates or extended cell loads achieved by immobilization [84,87]. For immobilized rhodococci, enhanced catalytic activities and increased tolerance were reported [13,40,68,88–91], as well as the lack of significant differences with SC [70,83,85,92–96]. In reference [70], the authors clearly demonstrated that, although immobilized *Rhodococcus* cells were similar to suspended ones and even underperformed them in catalytic activities, immobilization allowed a convenient (+4 °C, no humidity control) and long-term (12 months) storage of biocatalysts without losses in cell viability and functional activity.

In Figure 1, a general scheme for development of immobilized *Rhodococcus* biocatalysts is shown. It includes the following steps: (1) choice of the immobilization method; (2) selection of the most appropriate strain(s); (3) selection of a carrier and its improvement; (4) optimization of the immobilization process.

2.2. Choice of the Immobilization Method

There are two common methods used for immobilization of rhodococcal cells: (i) adsorption [13,68,89,90,97–100] and (ii) gel entrapment (synonyms: involvement, embedment, and encapsulation) [83,91,101]. Adsorption is a technically simple procedure, which has the main disadvantage, namely partial desorption and elution of cells from a carrier. However for some carriers, cells could be washed out completely and the carriers could be regenerated and reused [13]. Moreover, adsorption is preferred in bioreactor applications since no separate immobilization step is required and the cells are adsorbed directly in the reactor [88]. A promising modification of the adsorption method is realized in membrane bioreactors. This type of bioreactor is equipped with a hollow-fiber ultra-filtration membrane cartridge on which the cells are adsorbed, directly forming a biofilm [102]. Immobilization of bacteria in gel carriers prevents cell losses, however, this procedure is more laborious and substrate diffusion problems may occur [72,78].

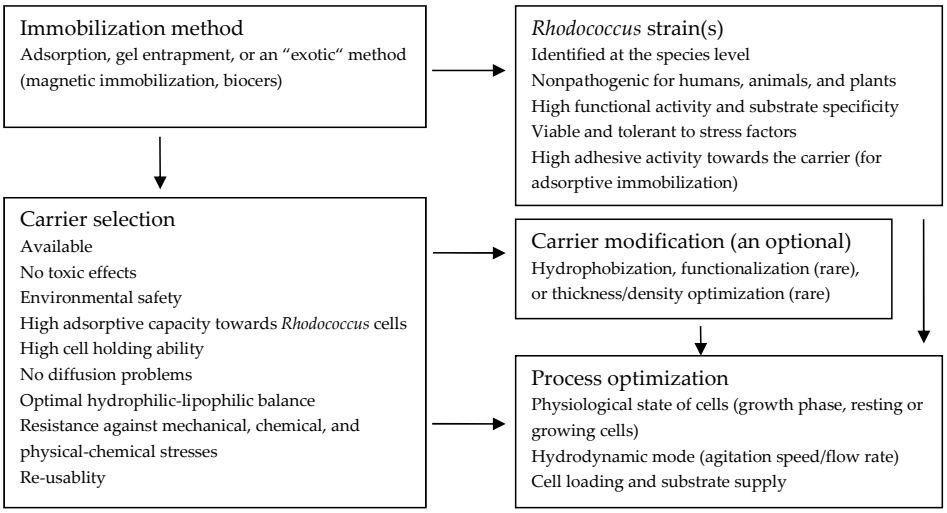

Figure 1. A general scheme to obtain stable biocatalysts based on immobilized *Rhodococcus* cells.

Recently, some advanced methods were proposed to immobilize *Rhodococcus* strains intended for environmental applications. In the work [96], *Rhodococcus* cells were magnetically held in the κ-carrageenan gel. For this, freshly prepared soft κ-carrageenan gel was mixed with *Rhodococcus rhodochrous* cells and Fe_3O_4 nanoparticles and then solidified. Magnetically immobilized rhodococci oxidized a target compound (2-chlorophenol) four times faster than SC or cells immobilized without magnetic nanoparticles. While particular mechanisms of these effects remain unclear, they are related perhaps to the increased contact area between cells and 2-chlorophenol, thus facilitating the substrate transfer into the cells [96]. In another work [70], rhodococcal cells were entrapped into porous ceramic-like materials. Cells were mixed with SiO_2 nanosol and a filler (ceramic powder), then casted and frozen in the mold. The biocomposite (the biocer) obtained had the advantages of both adsorbed and gel-entrapped cells. It was chemically inert, easily stored (at +4 °C in dry conditions for 6–12 months), possessed a superior mechanical, chemical, and thermal stability, and had high open porosity.

With *Rhodococcus* enzymes, the same methods plus covalent binding and cross-linked aggregation are currently used for their immobilization. For example, the azoreductase AzoRo from *Rhodococcus opacus* strain 1CP was covalently immobilized onto the functionalized mesoporous silica, and this method was more successful than cross-linking [103].

2.3. Selection of Bacterial Strains

Basic requirements to biotechnological strains include high functional activity and substrate specificity, lack of pathogenicity, and simple (inexpensive nutrients, no special conditions) maintenance

and cultivation. A wealth of relevant literature is associated with the proper description of these parameters. In this section, we focus on strain identification and adhesive properties of *Rhodococcus* strains used for immobilization.

In a number of works, *Rhodococcus* strains non-identified at the species level and indicated as *Rhodococcus* sp. are used [12,68,92,104]. As a result, possible pathogenic and phytopathogenic properties of these strains are unclear because they could belong to *R. hoagii* com.nov. (*formerly R. equi*), *R. fascians*, or be a representative of a new *Rhodococcus* species with unknown pathogenicity. Industrial application of such uncharacterized strains poses a serious risk for human health and the environment. Therefore, strict safety regulations are required to allow using only microbial agents with thorough defined biological features. For this reason, bacterial strains from well-known microbial collections (http://www.wfcc.info/ccinfo/) could be ordered, or genome sequence data should be obtained to properly identify the isolate(s).

High adhesive activities of bacterial strains and their ability to colonize surfaces are essential for adsorptive immobilization. Key biomolecules participating in *Rhodococcus* cell adhesion to solid carriers and mechanisms of their action are schematically shown in Figure 2. These are cell wall mycolic acids (specific long-chain α-branched and β-hydroxy fatty acids) and extracellular polymeric substances (EPS), basically exopolysaccharides. Mycolic acids greatly impact cell-wall hydrophobicity, and EPS mostly affect zeta-potential of rhodococcal cells. Additionally, EPS content can vary significantly, resulting in cell hydrophobicity changes, namely in a ratio between lipophilic and hydrophilic areas on the cell surface [92,105–110]. As a rule, hydrophobic interactions dominate electrostatic ones in the bacterial adhesion process [106], although the number of adhered rhodococcal cells is not necessary in a linear dependence on hydrophobicity or zeta-potential values [111].

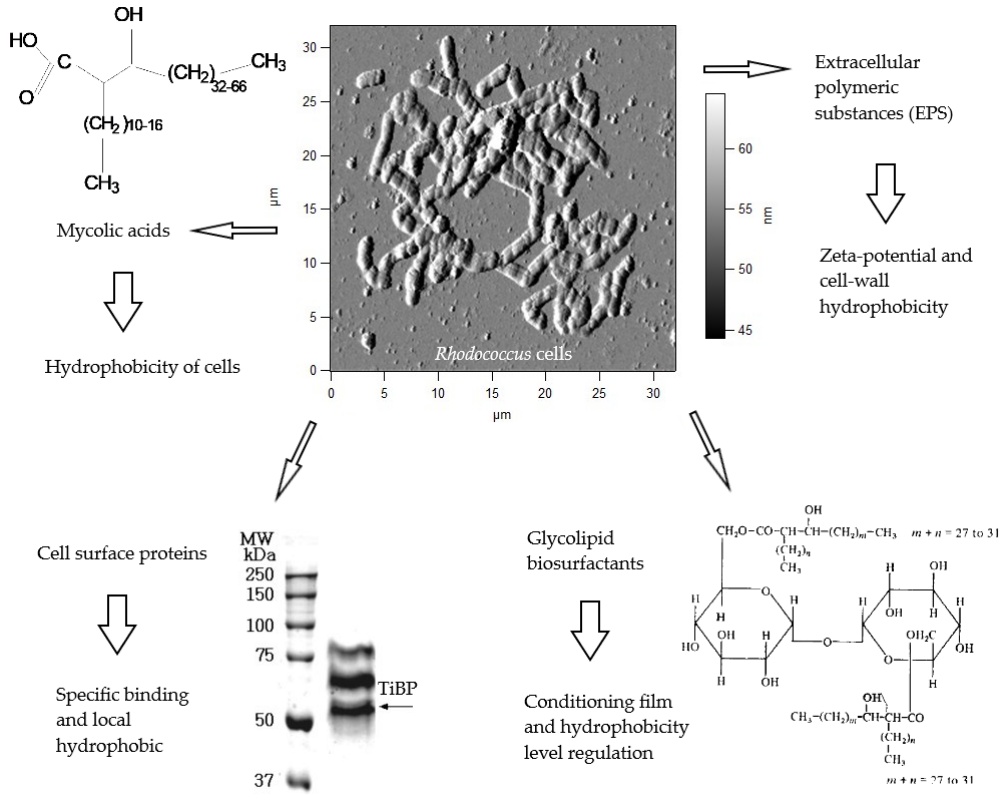

Figure 2. Key biomolecules participating in *Rhodococcus* cell adhesion to solid carriers and mechanisms of their action. An atomic force microscope image of *Rhodococcus* cells (the authors' original data, unpublished) is in the centre. A SDS-PAGE electrophoregram of a titanium-binding protein TiBP and a trehalose-dicorynomycolate structural formula are cited from reference [112] and reference [45], respectively.

Glycolipid biosurfactants and cell surface proteins represent other *Rhodococcus* adhesion mechanisms, which are less studied in comparison to mycolic acids and EPS. In our studies, stimulating effects of glycolipid biosurfactants on rhodococcal cell adhesion to solid carriers were revealed. These compounds were shown to act as a conditioning film, which changes the hydrophobicity of the carrier surface [90]. *Rhodococcus* sp. strain GIN-1 had a titanium-binding protein TiBP that was responsible for selective adhesion to TiO_2 and ZnO. TiBP resembled a homodimeric dihydrolipoamide dehydrogenase, leading to bonds between rhodococcal cells and minerals via hydrophobic interactions [112–114]. In reference [115], treatment with proteinase K resulted in a reduced rhodococcal ability to co-aggregate with *Acinetobacter calcoaceticus*, although not all *Rhodococcus* strains tested were sensitive to this procedure. Apparently, protein adhesins of rhodococci are strain-specific and are involved in the adhesion to certain sites on biotic and abiotic surfaces.

2.4. Selection of a Carrier for Cell Immobilization

Basic requirements for the carrier materials include lack of toxicity, economical efficiency, availability, environmental safety, optimal lipophilic-hydrophilic balance, and high adsorptive capacity towards bacterial cells. For environmental applications, suitable natural organic materials for *Rhodococcus* cell adsorption include peat and plant residues, such as sawdust, lemongrass leaves, orange peels, sunflower husk, chopped straw, cumin seeds, corn cob grit, and walnut shells. These materials are light, volumetric, inexpensive, available, biodegradable, and have an extended surface area and high water-holding capacity. Additionally, plant residues can release essential oils and organic acids utilized by immobilized microorganisms as extra nutrients [68,81,99,116]. Animal-derived organic wastes, such as poultry feathers and leather scrap, are also reported as *Rhodococcus* cell carriers [117]. Among them, feathers have advanced flotation properties, and therefore, are suitable for surface water applications. Inorganic adsorbents include minerals (macrostructured foam ceramics, ceramic beads, corund, foamed vermiculite, diatomaceous earth, and coarse sand grains), as well as coconut shell-based granular activated carbon. There are also synthetic polymers (nonwoven fibers, polyurethane foam, and polycaproamide fiber), which are chemically inert and easily regenerated [13,89,92,97,98,100,118]. Selectivity of synthetic polymers towards hydrophobic compounds and rhodococcal cells could be improved using various modifications like saturation of surface with functional (alkyl, nitryl, and carbonyl) groups, thickness variations, and changes in the fiber packaging density [97].

Various gel-type polymers, both natural (agar, agarose, alginate, κ-carrageenan, and gellan gels) and synthetic (hydroxypropylcellulose, poly(N-isopropylacrylamide), poly(acrylamide), PAAG, and poly(vinylalcohol), PVA) are used for *Rhodococcus* cell entrapment [83,85,91,96,101,119]. Moreover, the advanced macroporous cryogels (hydroxypropylcellulose, poly(N-isopropylacrylamide), cryo-PAAG, and cryo-PVA) were developed, having a spongy-like structure of large pores (50–200 μm) filled with water and surrounded by thin walls that favors diffusion of molecules and improves equilibrium sorption properties. Biological agents (cells or enzymes) are located inside the channels of such interconnected pores [91,120]. Cryo-PVA has additional advantages over other cryogels, namely high micro- and macroporosity, advanced water-holding capacity, perfect rheological properties, and relatively low biodegradability [77,101]. Among natural gels, κ-carrageenan has increased mechanical, chemical, thermal, and biological stability, and a loosened inner structure favoring substrate transport to bacterial cells [96].

Natural and biodegradable organic materials are preferable for *in situ* applications. They are environmentally compatible and can be left on site without the need to be removed and utilized. Inert inorganic carriers are perfect for contained systems, such as bioreactors and biofilters. These materials are easily regenerated and resistant to different stresses, for example a shear stress, overpressure, and mold damage [13].

Hydrophilic-lipophilic balance (HLB) is an important characteristic of *Rhodococcus* cell carriers since optimal thermodynamic conditions for biodegradation of hydrophobic organic pollutants

occur at the border of polar (water) and nonpolar (hydrocarbon) phases. At the interface, hydrocarbon-oxidizing bacteria have an access to hydrophobic oxidized substrates, as well as mineral salts and essential micronutrients dissolved in water [90]. Normally, unmodified carriers (e.g., polymeric gels and raw plant residues) are hydrophilic and located in the water phase. To change their HLB values, *Rhodococcus* cell carriers should be hydrophobized. In the work [98], ceramic carriers were covered with a layer of graphitized or catalytic fiber carbon in order to increase the hydrophobicity. We tested drying oil, silicon emulsion, *n*-dodecane, *n*-hexadecane, mixture of paraffins, and *Rhodococcus* biosurfactants for the hydrophobization of sawdust, cryo-PVA, and cryo-PAAG [88,90,116,119]. Hydrophobization lead to the changed carrier location in a two-phase system (hydrophobized materials were located at the interface), 2–18-fold increases in the hydrocarbon sorption by carriers, and a 13%–63% increase in hydrocarbon-oxidizing activities of immobilized *Rhodococcus* cells. Moreover, the hydrophobization procedure protected sawdust from mold damage, while hydrophobized gels (in particular, C_{12}-cryo-PAAG) retained more rhodococcal cells than intact gels. Interestingly, this procedure was ineffective towards the poultry feathers or leather scrap. Feathers were intrinsically hydrophobic and were located at the hydrocarbon-water interface, while leather scrap gravitated to the bottom, so hydrophobization did not improve its physical-chemical properties [121]. An optimal hydrophobizer concentration was found to be no more than 20% (w/w), and the carrier over-hydrophobization (appeared as carrier localization in the nonpolar phase and the sorption of $\geq$ 60% hydrocarbons) resulted in significant reduction of immobilized cell numbers [90,116].

Glycolipid biosurfactants produced by rhodococci seem to have optimal characteristics as carrier hydrophobizers because they are environmentally compatible, biodegradable, nontoxic, and hypoallergic, thus exceeding synthetic and many microbial surfactants in these parameters [50]. In our experiments, sawdust treated with *Rhodococcus* biosurfactants (5–10%) had a high (3.5×10^9 cells/g) adsorptive capacity towards bacterial cells, resulting in the *n*-hexadecane bio-oxidation rate of 35 mg /(l·h) [90]. Biosurfactants covered the entire carrier surface, smoothing its fine irregularities, but saving general surface topography, which allowed a homogenous distribution of bacterial adhesion sites. Rhodococcal cells were adsorbed as a uniform monolayer biofilm and occupied all available sites on the carrier surface (about 50% of the total area) [122] that provided maximal cell loading and unlimited molecular diffusion. In comparison, hydrocarbons (*n*-hexadecane, in particular) formed sporadic spots on the carrier surface, allowing rhodococcal adhesion on these spots in the form of dense cell clumps that resulted in a 20% decrease of their metabolic activities [90].

2.5. Process Optimization Strategies

One crucial parameter for *Rhodococcus* cell immobilization is a hydrodynamic mode. Rhodococci have morphological traits, significant for their immobilization, such as a complex morphogenetic cycle, cell polymorphism, and tendency to aggregate, especially in the presence of hydrophobic compounds. For example, at a low (0.6 mL/min) flow rate in a fluidized-bed column bioreactor, rhodococcal cells were aggregated rather than adsorbed on the sawdust carrier [123]. These cell aggregates sedimented at the bioreactor bottom and prevented a normal bottom-up liquid flow. In the shaking flasks, the low (60 rpm) orbital mixing rate resulted in the irregular cell immobilization curve due to cell aggregations at different parts of a flask [124]. However, at very high flow (2.8 ml/min) or mixing (210 rpm) rates, the sawdust carriers were destroyed, cell desorption was enforced, and cell (mycelium) fragmentation was initiated. Therefore, the optimal flow and agitation rates were found to be 1.2–2.0 mL/min and 110–160 rpm, respectively, which did not impact cell morphology and immobilization efficacy, and provided cells with sufficient oxygen and substrate supply [123,124]. Additionally, in aerated bioreactors, the rate of aeration is important and should be set adequately since very intensive air bubbling results in strong cell desorption from the carrier [13,67].

Other parameters that are relevant for immobilization include cell growth phase and physiological state, as well as the cell concentration. For example, exponentially-growing *Rhodococcus erythropolis* cells were adsorbed faster on Biolite than their stationary-phase counterparts [13]. It should be noted

that resting cells, i.e., washed from the growth medium and resuspended in the buffer, are more frequently used [70,83,85,90,91,96,118] than growing cells [13,67,93,102] due to easy control of their adhesion efficacy and catalytic activities. However, growing *R. ruber* cells were used, for example, in the propane-fed membrane bioreactor since its construction promoted the biofilm growth and a pollutant (N-nitrosodimethylamine) was degraded as a cometabolite [102]. Cell concentration effects were revealed in several publications [85,125], for example showing that a concentration of 10^9 cells/mL was optimal for the adsorptive immobilization of *R. ruber* IEGM 231. At this concentration, the number of IC reached 2.5×10^7 cells/cm^2 or 30% of the initial number of the suspended cells. Although the number of attached cells was 3–5 times higher at concentration 10^{10} cells/mL, rhodococci were strongly aggregated, thus preventing efficient substrate biodegradation [125].

3. Genetic Modifications of *Rhodococcus*

Genetic and molecular biology tools are used for manipulations with *Rhodococcus* strains in three directions: (1) modifications of nucleotide sequences to improve industrially important characteristics, (2) heterologous expression of *Rhodococcus* proteins in suitable microbial hosts, and (3) application of *Rhodococcus* cells as expression systems to produce recombinant enzymes.

Modifications of nucleotide sequences in rhodococci were recently used to obtain overproducers of biofuel components, acylglycerols and fatty acids [126,127]. The *ro00075* gene encoding phosphatidic acid phosphatase in *R. jostii* RHA1 was cloned in plasmid vectors containing strong promoters (either acetamidase promoter P*ace* or thiostrepton promoter P_{tipA}). These vectors were transferred into *R. jostii* RHA1 and *R. opacus* PD630 cells. The *ro00075* gene was overexpressed in transformants, providing enhanced dephosphorylation of phosphatidic acid to yield diacylglycerol, a key precursor for triacylglycerol biosynthesis. Transformation of the nonoleaginous *R. fascians* F7 with these vectors resulted in the 7 % increase and with a vector containing *ro00075* and *atf2* (coded for wax ester/diacylglycerol acyltransferase) genes—the 4-fold increase in the fatty acid content. Heterologous expression of the *ro00075* gene in *Escherichia coli* resulted in the 4-fold and 2-fold increases in the phosphatase activity and the diacylglycerol content in the host cells, respectively [127]. In another work [126], the metabolic pathway of D-xylose utilization was introduced into the same *Rhodococcus* strains to force the production of triacylglycerols from a cheap substrate (xylose was one of major components of the lignocellulosic biomass). To achieve this, a vector, which harbored the *xylA* gene encoding xylose isomerase in *Streptomyces lividans* TK23, the *xylB* gene encoding xylulokinase in *S. lividans* TK23, and the *tac* promoter, was transferred into *R. jostii* RHA1 and *R. opacus* PD630 cells. The transformed cells were able to use xylose as the sole source of carbon and energy with high production of lipids. However, the main challenges for industrial applications of the overproducers obtained are the presence of marker genes coding antibiotic resistance in vectors, risk of vector elimination, and putative application of a phytopathogenic strain.

Rhodococcus sp. T104 strain was used as a cloning and expression system for $phaZ_{Sex}$ gene encoding poly(3-hydroxybutyrate) depolymerase in *Streptomyces exfoliates*. A heterologous depolymerase was highly thermostable, keeping its functional activity at 40 °C and proposed for application in synthesis and degradation of bioplastics [128]. Three aromatic hydrocarbon dioxygenases, AhDOs, from *Terrabacter* sp. YK320, *R. erythropolis* TA42128, and *R. rhodochrous* K37 were expressed in host systems based on *Rhodococcus* sp. RD2 and *R. erythropolis* TA422, JCM 2892, and JCM 3201 under the control of the constitutive promoter P_{dfdB}. These enzymes participated in oxidative degradation of dibenzofuran and chlorinated dioxins and were difficult to obtain in classical *E. coli* expression systems [129].

4. Acclimation of *Rhodococcus* Cells to Toxic Substrates

Cell acclimation to toxic substrates is a preliminary step in many biotechnological processes performed with whole bacterial cells. A disadvantage of this procedure is the long time required. For *Rhodococcus*, acclimation time varied between 2.5 and 13.0 days [13,40,67,85,91,100,118,130]. At the same time, acclimation guarantees higher initial rates of biodegradation with or without a shortened

lag-phase. For example, consumption of 2,4-dinitrophenol by *R. erythropolis* HL 24-1 and HL 24-2 cells grown in the pollutant-free medium was preceded by a lag-period (≥1 h), which was eliminated when cells were preincubated with 2,4-dinitrophenol [85].

Two methods are currently used to obtain acclimated *Rhodococcus* cells: single batch cultivation in the presence of a pollutant, often at concentrations lower than working ones [40,100,130], or sequenced cultivation with a gradual increase of the pollutant concentration [13,67,91,118]. For example, *R. rhodochrous* IEGM 608 cells were pre-grown in the presence of 0.0007% isoquinoline during 3 days prior to biodegradation of a pharma pollutant, drotaverine hydrochloride [40]. M.B. Prieto et al. [13,67] cultivated *R. erythropolis* UPV-1 cells at increased phenol concentrations (0.2 and then 0.4 g/L) to obtain an acclimated culture. *Rhodococcus* bacteria used for detoxification of the NASA scrubber water rich in hydrazine, methylhydrazine (major constituents in a variety of rocket fuels and missile propellants), and citric acid, were serially conditioned with 5, 10 and 15% (v/v) scrubber water before the commencement of experiments involving full strength wastewater [118]. In reference [91], ten cultivation passages were carried out with increasing concentrations of the xenobiotic (phenol) to obtain an adapted *Rhodococcus wratislaviensis* culture.

5. Microbial Consortia/Associations Containing *Rhodococcus*

Mixed microbial cultures are promising for biodegradation of multicomponent pollutants, for example crude oil. In the work [130], two co-immobilized *Rhodococcus* strains with different substrate specificities were used for degradation of a model petroleum mixture. It was found that *R. ruber* IEGM 615 more efficiently oxidized *n*-alkanes C10–C16, while *R. opacus* IEGM 249 was able to degrade naphthalene. A nitrile-degrading strain *R. rhodochrous* BX2 was adsorbed together with a biofilm-forming, not degrading bacterium *Bacillus mojavensis* M1 [100]. The M1 strain was used as a biofilm producer and BX2 was incorporated into the M1 biofilm. This technical solution was successful because the association BX2+M1 provided complete detoxification of a target compound (cyanide), while single *R. rhodochrous* BX2 culture oxidized no more than 80% of this toxicant at the same concentration.

However, in many cases, interactions between microorganisms in consortia/associations are not so obvious. For example, *Rhodococcus* sp. SLG-6 was unable to mineralize di-*n*-octyl phthalate and biodegradation stopped after hydrolysis of ester bonds and production of phthalic acid. Although *Arthrobacter* sp. SLG-4 completely mineralized this compound, and the combination of these two strains resulted in a two-fold increase of biodegradation rates in comparison with *Arthrobacter* sp. SLG-4 alone [12]. A mixed biofilm formed by *R. erythropolis* PWD1 and *Pseudomonas putida* F1 strains was used to remove organic pollutants (in particular toluene) from waste gases [131,132]. The authors assumed that a mixed culture exhibited more robustness than pure cultures towards high pollutant concentrations. Inoculation of industrial wastewater with an algal-bacterial co-culture containing *Rhodococcus* sp. strain Ac-1267 and immobilization on capron fibers resulted in the formation of a stable microbial consortium and efficient removal of petroleum hydrocarbons, phenols, anionic surfactants, and heavy metals [104].

6. Environmental Applications of *Rhodococcus* Biocatalysts

Selected examples of *Rhodococcus*-based biocatalysts developed for environmental applications and shown in Table 1 suggest that most biocatalysts are based on whole cells, SC or IC. This can be related to biochemical complexity of the processes catalyzed, including multistep biodegradation/mineralization or biosynthesis. The only example of enzymatic *Rhodococcus* biocatalyst being a covalently immobilized azoreductase AzoRo from *R. opacus* 1CP is shown. The biocatalyst had high azo dye-degrading activity (38 U/mg) and stability (60 h operation) under unfavorable (pH 4) conditions but required supplementation with NADH, FMN, and formate dehydrogenase [103].

Table 1. Selected examples of *Rhodococcus* biocatalysts developed for environmental applications.

Application	Short description of a Biocatalyst	Operating Conditions	Efficacy	Ref.
Treatment of wastewater containing phenol	A biocer: whole cells of *R. ruber* DSM 7512 embedded into ceramics (alumosilicate + SiO_2)	Batch	Complete degradation of phenol (0.5 g/L) in 5 days; ≥ 5 cycles of re-using; stable for 12 months; stored at +4 °C for 6 months.	[70]
		A bench-scale flow dynamic system	Degradation of 80–100% phenol (0.5 g/L) in 6–8 days.	
Treatment of wastewater containing chlorophenols	Whole cells of *R. rhodochrous* DSM 6263 entrapped into κ-carrageenan mixed with Fe_3O_4 nanoparticles	Batch	Complete degradation of chlorophenols (0.25 mM) in 5 h; 6 cycles of re-using.	[96]
Treatment of wastewater and groundwater containing nitrophenols	Whole cells of *R. erythropolis* HL PM-1, SC [1]	Batch	Complete degradation of 2,4-dinitrophenol (0.06 g/L) in 6 h.	[85]
	Whole cells of *R. erythropolis* HL PM-1 embedded into agar beads	A plug-flow bioreactor	Complete degradation of 2,4-dinitrophenol (0.005 g/L) without loss of activity for 14 days.	
Treatment of wastewater containing phenol and hydrocarbons	Whole cells of *R. wratislaviensis* BN38 entrapped in hydroxypropylcellulose/ poly(N-isopropylacrylamide) cryogel, acclimated	Semicontinuous with a gradual increase of a contaminant concentration	Complete degradation of phenol (20 g/L in total) and *n*-hexadecane (20 g/L in total) in 5 days; more active than SC in 2.5 times.	[91]
Treatment of wastewater from phenolic resin manufacturing companies	Whole cells of *R. erythropolis* UPV-1 adsorbed on ceramic Biolite® beads, acclimated	A packed-bed two column bioreactor with aeration	The rate of phenol (0.1–0.8 g/L) degradation 0.4–18.0 g/(l·day); degradation of 100% formaldehyde (0.2 g/L); stable for 50 days.	[13]
Treatment of wastewater from phenolic resin manufacturing companies	Whole cells of *R. erythropolis* UPV-1 adsorbed on diatomaceous earth, acclimated	An air-stirred bioreactor with clarifier	The rate of phenol (0.1–1.2 g/L) degradation 0.1–11.5 g/(l·day); degradation of 100% formaldehyde (0.3–2.4 g/L).	[67]
Treatment of wastewater from paper and pulp industry	Whole cells of *R. rhodochrous* IEGM 107, SC	Batch	Complete degradation of dehydroabietic acid (0.5 g/L) in 9 days.	[38]
Treatment of wastewater containing pharma pollutants	Whole cells of *R. rhodochrous* IEGM 608 adsorbed on modified sawdust, acclimated	Batch	Complete degradation of drotaverine hydrochloride (0.2 g/L) in 25 days.	[40]
Treatment of wastewater containing toxic amides	Whole cells of *R. rhodochrous* NHB-2 entrapped in agar gel beads	A five-stage plug bioreactor	Degradation of 90–100% acrylamide, acetamide, or propionamide (100 mM) at 45 °C and pH 8.5 in 8 h.	[83]
Treatment of wastewater containing azo-dyes	Azoreductase AzoRo from *R. opacus* 1CP covalently bound to meso-cellular foams	Batch	38 U/mg towards Methyl Red(25 μM) at pH 4; stable for 60 h.	[103]
Treatment of groundwater contaminated with cyanides	Whole cells of *R. rhodochrous* BX2 + *B. mojavensis* M1 adsorbed on granular activated carbon, acclimated	A fluidized bed bioreactor	Complete degradation of CN^- (0.05×10^{-3}–7.48×10^{-3} g/L) continuously over a 110-day steady-state operation period.	[100]
Treatment of groundwater contaminated with N-nitroso- dimethylamine	Whole cells of *R. ruber* ENV425, self-immobilized	A propane-fed membrane bioreactor	Degradation of > 99.95 % N-nitroso- dimethylamine (7.4×10^{-6}–77.0×10^{-6} g/L) continuously over a 135-day steady-state operation period.	[102]
Treatment of wastewater containing crude oil	Whole cells of *R. opacus* IEGM 249 and *R. ruber* IEGM 615 co-immobilized on modified sawdust, acclimated	A column fluidized-bed bioreactor	Degradation of 70–90% alkanes and PAHs of crude oil (2–3%) in 21 days; 4–6 cycles of re-using.	[76,88,130]
Treatment of oilfield wastewater	Whole cells of *R. opacus* IEGM 249 and *R. ruber* IEGM 615 entrapped into cryo-PVA	A column bioreactor	Degradation of 80% crude oil (0.45 g/L) at strong (194 g/L) mineralization in 21 days.	[88,133]
Bioremediation of crude oil-contaminated soil	Whole cells of *R. erythropolis* IEGM 275 and *R. ruber* IEGM 231 entrapped into cryo-PVA	Model soil	Degradation of 45% crude oil (5%) in 14 months under dry conditions.	[93]
Bioremediation of crude oil-contaminated soil	Pure identified strains from the IEGM Collection, SC or adsorbed on hydrophobized sawdust	*In situ*; *ex situ* in a slurry bioreactor	Degradation of 80–90% crude oil (5–30%) in cold and temperate climate regions in a summer season.	[134–137]
Waste gas treatment	Whole cells of *P. putida* F1 and *R. erythropolis* PWD1 adsorbed on polypropylene disks	A rotating biological contactor	Degradation of 30–90 % toluene vapors (3–5 g/m^3) under steady-state operation conditions for 400 days.	[131,132]
Biofuel production	Whole cells of *R. jostii* RHA1 pJAM2/RO00075 or pTip-QC2/RO00075, SC; *R. opacus* PD630 pJAM2/RO00075 or pTip-QC2/RO00075, SC	Batch	Lipid production 35–50% CDW [2]	[127]
Biofuel production	Whole cells of *R. jostii* XYLAB or *R. opacus* XYLAB, SC	Batch	Lipid production 53–68% CDW	[126]

[1] SC—suspended cells. [2] CDW—cell dried weight.

As seen from Table 1, environmental applications of the developed *Rhodococcus* biocatalysts include (1) wastewater treatment to remove phenol and its chloro- and nitroderivatives, individual hydrocarbons, crude oil, formaldehyde, amides, amines, azo-dyes, cyanides, pharma pollutants (drotaverine hydrochloride), and components of pulp and paper wastes (dehydroabietic acid); (2) bioremediation of open ecosystems (groundwater, soil, and marine environments); (3) biofuel production; and (4) clean-up of polluted air. Most of the biocatalysts discussed here are laboratory prototypes awaiting future implementations in environmental biotechnologies. However, there is an oleophilic biopreparation consisting of suspended or adsorbed *Rhodococcus* cells in the complex with *Rhodococcus* biosurfactants, which was developed for bioremediation of crude oil-contaminated soils and field-tested under temperate and cold climate conditions in the Russian Federation (Urals, Western Siberia, etc) [134,135]. The *Rhodococcus* cultures used in this biopreparation were nonpathogenic and thoroughly studied strains from the Regional Specialized Collection of Alkanotrophic Microorganisms (acronym IEGM, WDCM # 768, www.iegmcol.ru). The vast majority of the Collection strains were isolated from the local environments over the territories remediated.

Importantly, *Rhodococcus* biocatalysts are suitable for various bioremediation conditions, such as single introduction (bioaugmentation or batch processes) [38,70,85,93,96,103,136], semicontinuous pollutant supplementation (with repeated and gradual adding of a substrate, up to 40 times in total) [91], and continuous bioreactors [13,67,70,83,85,100,102,130,136]. Different-type bioreactors are used that are equipped with diverse constructions, from slurry and single-column fluidized bed bioreactors to three-phase airlift and 2–5-column ones. Catalytic activities of *Rhodococcus* bacteria in bioreactors can differ from those in batch systems [70,85], while a biocatalyst behavior in laboratory and pilot bioreactors is generally similar to the industrial scale reactor conditions.

Functional activities of *Rhodococcus* biocatalysts varied between 30% and 100% of the target compound removal during biodegradation processes, and between 35% and 68% of cell dried weight for the target compound synthesized. However, it is hard to compare the activities of biocatalytic systems since they depend on many factors, such as substrate (its bioavailability and toxicity), strain(s), operating regimes, and environmental conditions (pH, temperature, humidity, salt concentration, chemical composition, and presence of other microorganisms). For example, the degrading activity of *R. ruber* cells immobilized into biocer [70] against phenol was 4 times lower than that of *R. wratislavensis* cells entrapped into the cryogel [91]. This could be related to differences in intrinsic phenol-degrading abilities of the strains used, operating conditions (batch and flow dynamic vs. semicontinious), or to positive effects of the cell acclimation and a gradual increase of phenol concentration [91]. In laboratory experiments, *Rhodococcus* strains immobilized into cryo-PVA degraded only 45% of crude oil in model soil at the initial contamination level of 5% (w/w) within 14 months [93], whereas in open soil ecosystems, rhodococci as a component of an oleophilic biopreparation provided a removal of 80%–90% of crude oil at the initial contamination level of 30% (w/w) within 3 months (summer season) [134–136]. This example reflects common differences in laboratory and field bioremediation results, indicating that higher biodegradation rates under natural conditions are probably due to enhanced self-restoring abilities of natural soil ecosystems.

The literature and our experimental data show that *Rhodococcus* biocatalysts compete successfully with the biocatalysts based on other microorganisms, particularly in terms of biodegradation of phenol and petroleum hydrocarbons. For example, rhodococci completely degraded phenol at concentrations of 0.1–20.0 g/L within a duration range from 8 min to 8 days and removed 45%–100% of petroleum hydrocarbons at concentrations of 2%–30% (w/w) within 5 days to 14 months (see Table 1). Other referenced biocatalytic systems (based on *Alcaligenes faecalis*, *Alcanivorax borkumensis*, *Bacillus cereus*, *Bacillus firmus*, *Halomonas hamiltonii*, *P. putida*, *Shewanalla chilikensis*, and microbial fuel cells) provided complete phenol degradation at concentrations of 0.1–6.0 g/L within the time periods from 12 h to 8 days [138–140] and a 24%–90% removal of petroleum hydrocarbons at their concentrations of 1%–16% (w/w) in 3–66 days [141–143].

Concerning other technological characteristics, *Rhodococcus* biocatalysts can be re-used from four times up to 35 cycles, and can operate continuously up to 400 days and stored for 12 months without the catalytic activity lost. Sustainability of immobilized *Rhodococcus* biocatalysts in long-term bioremediation processes was clearly demonstrated in soil microcosms [93]. In this study, a high number of viable *R. erythropolis* IEGM 275 and *R. ruber* IEGM 231 cells entrapped into cryo-PVA beads was maintained over 14 months in crude oil-contaminated dry (10–20% relative humidity) soil, thus impacting the efficient oil biodegradation.

7. Conclusions

Rhodococcus-based biocatalysts meet modern biotechnology requirements, such as broad substrate range, high enzymatic activity, robustness, economic feasibility and environmental safety. Immobilized *Rhodococcus* cells can be easily recovered from the fermentation broth, regenerated and repeatedly used without significant cell losses. However, *Rhodococcus* biocatalysts do not pretend to be a "catalytic panacea" since each catalytic system is unique and fits particular bioprocess conditions. A present review is aimed at revealing the possible abilities of immobilized *Rhodococcus* cells to degrade the priority organic pollutants, mostly hydrocarbon-derived ones, thus suggesting their potential for advanced ecobiotechnological solutions. Considering the main challenges of *Rhodococcus* applications, such as complex growth cycle and cell pleomorphism, relatively slow growth and ecological k-strategy (persistence under non-optimal environmental conditions with low metabolic activity), there is a need for the development of knowledge-based approaches to mitigate these problems. Therefore, future developments could focus on strain improvements using genetic modifications, namely over-expression of important catabolic genes, and search for molecular inducers of target degradation pathways. Other promising approaches include acclimation of *Rhodococcus* to high pollutant concentrations and development of co-immobilized bacterial consortia containing rhodococci and based on synergetic metabolic interactions revealed with ecological modeling tools.

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