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Review

Biotechnological Potential of *Rhodococcus* Biodegradative Pathways

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The genus *Rhodococcus* is a phylogenetically and catabolically diverse group that has been isolated from diverse environments, including polar and alpine regions, for its versatile ability to degrade a wide variety of natural and synthetic organic compounds. Their metabolic capacity and diversity result from their diverse catabolic genes, which are believed to be obtained through frequent recombination events mediated by large catabolic plasmids. Many rhodococci have been used commercially for the biodegradation of environmental pollutants and for the biocatalytic production of high-value chemicals from low-value materials. Recent studies of their physiology, metabolism, and genome have broadened our knowledge regarding the diverse biotechnological applications that exploit their catabolic enzymes and pathways.

Keywords: Biodegradation, degradative pathway, catabolic enzyme, bacteria, *Rhodococcus*

Introduction

The genus *Rhodococcus* is a phylogenetically and physiologically diverse bacterial group within nocardioform actinomycetes, which contain mycolic acid in their cell walls. *Rhodococcus* (family Nocardiaceae) belongs to the phylum Actinobacteria that includes two other well-known families, Mycobacteriaceae and Corynebacteriaceae. *Rhodococcus* members are described as nonmotile and aerobic gram-positive bacteria that generally show pleomorphic morphology, depending on the species or growth conditions, such as different growth substrates. They have been isolated from a wide variety of sources, including soil, rocks, groundwater, marine sediments, animal feces, internal organs of insects, and healthy and diseased animals and plants [1]. Recently, cold-adapted rhodococcal strains have been isolated from naturally cold environments, such as alpine areas, the Arctic, and the Antarctic [2–4].

After approximately a decade since the first comprehensive account of the genus *Rhodococcus* in 1998 (see the special issue of *Antonie Van Leeuwenhoek*, volume 74), an excellent book was published that described the biology of *Rhodococcus*. According to this book, rhodococci are generally found to be harmless, and some are very attractive biotechnological candidates, although there are rare pathogenic species such as *R. equi* and *R. fascians* that cause foal pneumonia and leafy gall disease in plants, respectively. The reasons for their value as promising biotechnological candidates for applications are as follows. The members of *Rhodococcus* show a remarkable ability to degrade and transform a wide variety of natural organic and xenobiotic compounds via diverse catabolic pathways. In addition to the capacity to metabolize a broad spectrum of chemical compounds, cell tolerance to toxic substrates and solvents, a frequent lack of catabolite repression, the production of biosurfactants, and environmental persistence make them excellent candidates for bioremediation and bioconversion. They also have the potential to synthesize useful products such as valuable chemical synthons, amides, and polymers [1, 5].

The metabolic versatility of *Rhodococcus* has been extensively studied in microbial biotechnology fields worldwide. Presently, there are over 1,800 and over 3,200 patents retrieved by even a quick search on Google's patent search engine using "rhodococcus biodegradation" and

"rhodococcus biocatalysis," respectively, as keywords. Several review papers have also described the biotechnological potential of rhodococci cells and their biodegradative enzymes for pollutant removal in environmental biotechnology and for biocatalysis in pharmaceutical and chemical industries [6, 7]. The biodegradation and biocatalysis activities are linked by biotransformation reactions and enzymes that catalyze the reactions. In this review, we will briefly summarize the current knowledge on the biodegradative pathways of *Rhodococcus,* including a brief collation of selected genomic data. We will then describe examples and strategies for utilizing the real and potential value of biodegradative rhodococci.

Rhodococci as Superb Degraders

As summarized in Table 1, rhodococci display a diverse range of metabolic capabilities and degrade a wide variety of natural organic and xenobiotic compounds, including aliphatic, aromatic (halogenated and nitro-substituted), heterocyclic, and polycyclic aromatic compounds [1], alicyclic hydrocarbons [8], nitriles [5], cholesterol [9], and lignins [10]. The wide range of substrates metabolized by rhodococci suggests that members of this genus are comparable to pseudomonads in their biotechnological applications. The biodegradative pathways in rhodococci generally consist of many peripheral (upper) pathways and a few central (lower) pathways. For example, in the case of aromatic hydrocarbons, a wide range of monooxygenases and dioxygenases in the peripheral pathways initiate the oxidative attack of the aromatic ring, producing central intermediates such as catechol, protocatechuate, and gentisate, which are further degraded by the *ortho*-, *meta*-, or modified 3-oxoadipate routes to intermediates of the

Table 1. Members of the genus *Rhodococcus* that possess biocatalytic enzymes and/or biodegradative pathways for representative organic compounds.

Strain name	Isolation source	Degradation substrate	Application field	Key pathway or enzyme	Reference for isolation
R. rhodochrous J1	Soil sample	Benzonitrile	Production of acrylamide	Nitrile hydratase (H_NHase α and β subunits)	[72]
R. opacus 1CP	Contaminated soil in Moscow, Russia	4-Chlorophenol, 2,4-dichlorophenol	Remediation of (chloro)aromatics	Modified *ortho*-cleavage pathway for chlorocatechols	[73]
R. rhodochrous PA-34	Soil sample	Propionitrile	Production of acrylamide	Nitrile hydratase (H_NHase α and β subunits)	[74]
R. erythropolis TA421	Dry wood termites, Japan	Biphenyl	Remediation of organic pollutants	Biphenyl-degrading genes (*bph*)	[75]
R. erythropolis BD2	Trichloroethene- and toluene-contaminated soil	Isopropylbenzene	Remediation of trichloroethene	Isopropylbenzene-degrading genes (*ipb*)	[12]
Rhodococcus sp. AJ270	Air-dried soil from an industrial site, UK	Acetonitrile, heteroaromatic nitriles	Remediation of acrylonitrile	Nitrile hydratase and amidase	[76]
R. jostii RHA1	Hexachlorocyclohexane-contaminated soil, Japan	Polychlorinated biphenyl, alkylbenzenes	Remediation of organic pollutants; vanillin production	Biphenyl- and ethylbenzene-degrading genes (*bph* and *etb*); dye-decolorizing peroxidase (*dypB*)	[77]
Rhodococcus sp. T104	Plant-amended soil	Biphenyl, terpenes	Remediation of polychlorinated biphenyls	Biphenyl-degrading genes (*bph*)	[78]
Rhodococcus sp. B-264-1	Oil-contaminated soil, USA	Toluene	Production of *cis*-(1*S*,2*R*)-indandiol	Toluene dioxygenase (*todC1C2BA*)	[79]
Rhodococcus sp. strain	Terra Nova Bay, Antarctica	*n*-Alkanes, biphenyl	Low-temperature remediation	*n*-Alkane-degrading and biosurfactant-producing genes	[80]
R. opacus PWD4	Topsoil in Hilversum, The Netherlands	Toluene	Production of *trans*-carveol	Limonene-6-hydroxylase	[53]

Table 1. Continued.

Strain name	Isolation source	Degradation substrate	Application field	Key pathway or enzyme	Reference for isolation
Rhodococcus sp. DK17	Crude oil-contaminated soil, Korea	Alkylbenzenes, phthalates, bicyclics	Production of *cis*-dihydrodiols and catechols	*o*-Xylene dioxygenase (*akbA1A2A3*) and *cis*-dihydrodiol dehydrogenase (*akbB*)	[81]
Rhodococcus sp. NO14-1	Petroleum hydrocarbon-contaminated alpine soil, Austria	Hexadecane, phenol	Low-temperature remediation of phenol	Phenol-degrading genes	[82]
R. opacus B4	Soil in Hiroshima, Japan	Benzene, aromatic/aliphatic	Remediation of benzene	Benzene dioxygenase (*bnzA1A2*)	[83]
R. erythropolis PR4	Deep sea of the Pacific Ocean	Alkanes	Remediation of alkanes	Alkane-degrading genes (*alk*)	[84]
R. erythropolis MLT1	Soil surrounding hop plants	β-Myrcene	Production of geraniol	Unknown enzymatic mechanism	[54]
R. ruber TH	Nitrile-contaminated soil	Acrylonitrile	Production of ammonium acrylate	Nitrilase	[85]
R. ruber AKSH-84	Petroleum-contaminated sludge, India	Acetonitrile	Production of acrylic acid	Nitrilase	[42]
Rhodococcus spp. strains	Petroleum hydrocarbon-contaminated North Atlantic Canada	*n*-Hexadecane, diesel	Low-temperature remediation	*n*-Alkane-degrading and biosurfactant-producing genes	[86]
R. erythropolis ATCC 25544	Soil	Cholesterol, aromatic compounds	Production of cholestenone	Cholesterol oxidase	Unknown

citric acid cycle.

Biodegradative rhodococci often harbor large catabolic linear plasmids, many of which increase their catabolic versatility and efficiency by contributing to multiple pathways and gene homologs. Such plasmids encode multiple catabolic enzyme systems. Thus, multiple degradative pathways may be involved in the degradation of various organic compounds. Comparative analyses of rhodococcal genomes have suggested that the linear plasmids are possible determinants of the propagation of diverse degradative genes [11]. It is thought that frequent recombination of catabolic genes among bacteria is associated with large plasmids. The hyper recombination contributes to the large number of homologous genes in large genomes, which consequently leads to their catabolic diversity [1]. As representative examples, the highly similar gene clusters for catabolism of aromatic compounds are found on the following three pairs of plasmids: pTA421 from *R. erythropolis* TA421 and pSP6 from *R. globerulus* P6, pBD2 from *R. erythropolis* BD2 [12] and pRHL1 from *R. jostii* RHA1, and pDK2 from *Rhodococcus* sp. DK17 and pRHL2 from *R. jostii* RHA1. In addition to enzyme and pathway multiplicities, different induction mechanisms, substrate specificities, and activities of the catalytic enzymes are thought to contribute to their extraordinary capabilities to metabolize a wide range of compounds.

Since the introduction of the term "megaplasmid" in 1981 to describe a more than 450 kb plasmid from *Rhizobium meliloti*, there have been many reports on the presence of megaplasmids in different bacteria, which encode the degradation enzymes for various classes of natural and synthetic organic compounds. Table 2 summarizes the brief genome comparisons of biodegradative rhodococci containing catabolic plasmids. These genomes vary in size (5.4–10.1 Mbp) and the number of predicted genes (5,023–9,251). Among the biodegradative rhodococci, *R. jostii* RHA1 is the most extensively studied strain. RHA1 is a versatile polychlorinated biphenyl degrader, which contains a linear chromosome and three linear megaplasmids (1,120-kb pRHL1, 420-kb pRHL2, and 330-kb pRHL3). Most of the genes of the upper biphenyl catabolic pathway are located mainly on the pRHL1 and pRHL2 plasmids, whereas duplicated phthalate-degrading operons are located on pRHL1 and pRHL2 [13]. Oxygenases genes (203) are

Table 2. Brief genome statistics of *Rhodococcus* strains possessing catabolic plasmids.[a]

Strain name	Isolation source (degrading substances)	Genome size, Mb (GC%)	Total gene count	Total protein count	Number of plasmids	NCBI BioProject ID
R. jostii RHA1	Hexachlorocyclohexane-contaminated soil (polychlorinated biphenyl, alkylbenzenes)	9.71 (66.94)	9,251	8,695	3	PRJNA13693
R. erythropolis PR4	Deep sea (alkanes)	6.90 (62.29)	6,428	6,278	3	PRJDA20395
R. opacus B4	Soil (aromatic and aliphatic hydrocarbons)	8.83 (67.63)	8,048	7,780	5	PRJDA34839
Rhodococcus sp. DK17	Crude oil-contaminated soil (alkylbenzenes, bicyclics, phthalates)	9.11 (67.10)	8,609	7,954	3	PRJNA157361
R. aetherivorans BCP1	Aerobic butane-utilizing consortium (alkanes)	6.23 (70.29)	5,644	5,478	2	PRJNA213668
R. pyridinivorans SB3094	Diesel waste site (butanone)	5.59 (67.81)	5,094	4,916	2	PRJNA231235
R. opacus R7	Polycyclic aromatic hydrocarbon-contaminated site (naphthalene, *o*-xylene)	10.12 (67.02)	8,621	8,357	5	PRJNA246296
R. opacus 1CP	Contaminated soil (chloroaromatics)	8.64 (67.03)	7,608	7,420	2	PRJNA253567
Rhodococcus sp. p52	Oil-polluted soil (dioxin)	5.41 (67.85)	5,023	4,801	3	PRJNA255270
Rhodococcus sp. AD45	Freshwater sediment (isoprene)	6.79 (61.74)	6,202	6,048	2	PRJNA272922

[a]Based on the National Center for Biotechnology Information (NCBI) genome information accessed in mid-May 2017.

exceptionally rich in the RHA1 genome, containing 9,251 predicted protein-encoding genes, many of which are involved in numerous pathways predicted to degrade aromatic compounds (30) or steroids (4) [14]. Another well-studied *Rhodococcus* strain, DK17, utilizes various alkylbenzenes and phthalates as growth substrates, and also possesses three linear megaplasmids (380-kb pDK1, 330-kb pDK2, and 750-kb pDK3). The genes for alkylbenzene degradation are present in pDK2, whereas the gene clusters for phthalate degradation are duplicated in both pDK2 and pDK3. Duplicated phthalate-degrading operons are equally functional, allowing DK17 to achieve the maximal degradation of phthalate [15]. In addition, it is noteworthy that the phthalate degradation pathway is completely inhibited by benzoate in DK17 [16].

The genomic features of *R. jostii* RHA1 and *Rhodococcus* sp. DK17 were compared to study basic genetic similarities and differences between the two strains. A comparative protein-protein BLAST analysis (BLASTp) showed that approximately 75% of open-reading frames (ORFs) from the two strains, including ones for the degradation of aromatics, share >90% identity. Almost identical phthalate operons duplicated on two separate catabolic megaplasmids were also present in the RHA1 genome as in DK17. The DK17-specific ORFs are not randomly distributed over the genome, but occur in groups or clusters. Analyses of the DK17-specific ORFs indicated that many encoded proteins are likely to be involved in the transport of organic molecules, such as aromatic hydrocarbons [17]. This is consistent with the recent genomic discovery that biodegradative rhodococci are well-equipped with genes for the transport of many different substrates [18].

Metabolic Pathways for *o*-Xylene Degradation as a Representative Example

Most *o*-xylenes are produced by cracking petroleum and largely used in the production of phthalic anhydride, which is a precursor to many materials, drugs, and other chemicals. Thus, it is a widespread environmental pollutant in groundwater and soil. Although bacteria capable of degrading *m*-xylene or *p*-xylene under aerobic conditions are common, bacteria capable of degrading *o*-xylene are less frequently reported. Thus, *o*-xylene is considered to be difficult to biologically degrade and only a few bacteria have been studied for the ability to degrade *o*-xylene. Five different possible metabolic pathways for *o*-xylene degradation were proposed (Fig. 1), three of which have been experimentally confirmed from *Rhodococcus* spp. until recently. *Rhodococcus* sp. DK17 and *Rhodococcus* sp. C125 (originally *Corynebacterium* sp. C125) metabolize *o*-xylene through an aromatic ring dioxygenation to form *o*-xylene *cis*-3,4-dihydrodiol (1,2-dihydoxy-3,4-dimethylcyclohexa-3,5-diene), which is converted to 3,4-dimethylcatechol by a dehydrogenation reaction. *Rhodococcus* sp. B3 and *R. opacus* R7 degrade *o*-xylene through two successive monooxygenation reactions of the aromatic ring to form 2,3-dimethylphenol and 3,4-dimethylcatechol. In *Rhodococcus* sp. B3, a methyl

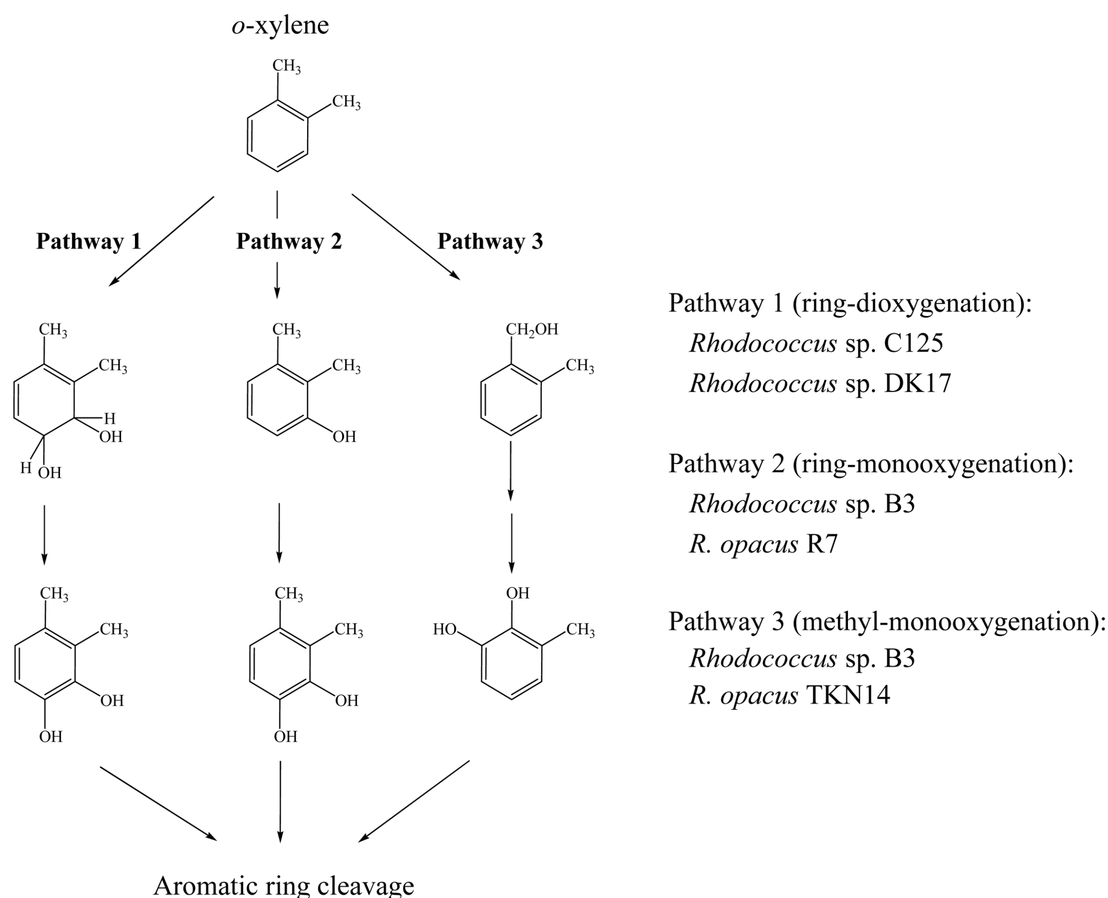

Fig. 1. *o*-Xylene degradation pathways operating in *Rhodococcus* spp. isolates.

monooxygenation reaction operates simultaneously that oxidizes a methyl group of *o*-xylene to form 2-methylbenzyl alcohol, which is metabolized to form 3-methylcatechol via 2-methylbenzoic acid. The methyl monooxygenation pathway functions for *o*-xylene degradation in *R. opacus* TKN14, from which the genes (*nidABEF*) for methyl group hydroxylation have been identified and characterized [19]. The central intermediates (dimethyl or monomethyl catechols) are further degraded by *ortho*- or *meta*-ring cleavage to intermediates of the citric acid cycle.

An approximately 25-kb-size gene cluster for *o*-xylene degradation by *Rhodococcus* sp. DK17 is present on the 330-kb pDK2 (Fig. 2). The cluster contains the genes encoding *o*-xylene dioxygenase: large and small subunits of the oxygenase component, which are present in two copies 6 kb apart from each other ($akbA1_a$- $akbA2_a$ and $akbA1_b$-$akbA2_b$), a gene for the ferredoxin component (*akbA3*), and a gene for the ferredoxin reductase component (*akbA4*). When expressed in *Escherichia coli*, recombinant $AkbA1_a$-$AkbA2_a$-AkbA3 transformed *o*-xylene into *o*-xylene *cis*-3,4-dihydrodiol. Moreover, a gene encoding *cis*-dihydrodiol dehydrogenase (*akbB*) and *akbCDEF* genes for a complete *meta*-cleavage pathway (*meta*-cleavage dioxygenase (AkbC), hydrolase (AkbD), hydratase (AkbE), and aldolase (AkbF)) are present in the cluster. AkbBCDEF are thought to completely metabolize the intermediate *cis*-3,4-dihydrodiol produced by *o*-xylene dioxygenase [20]. Two-component regulatory genes (*akbS* and *akbT*) are detected near the *o*-xylene-degrading operon, which are required to induce the expression of *o*-xylene dioxygenase [21].

Application of Targeted Biodegradative Pathways

Biodegradation

Many alkyl- and hydroxyl-substituted aromatic hydrocarbons and nitriles are released into the environment by industrial activity, whereas some are natural products. Most aromatic hydrocarbons and nitriles are highly toxic, mutagenic, and carcinogenic. Microorganisms and their degradative pathways have been examined and used in an

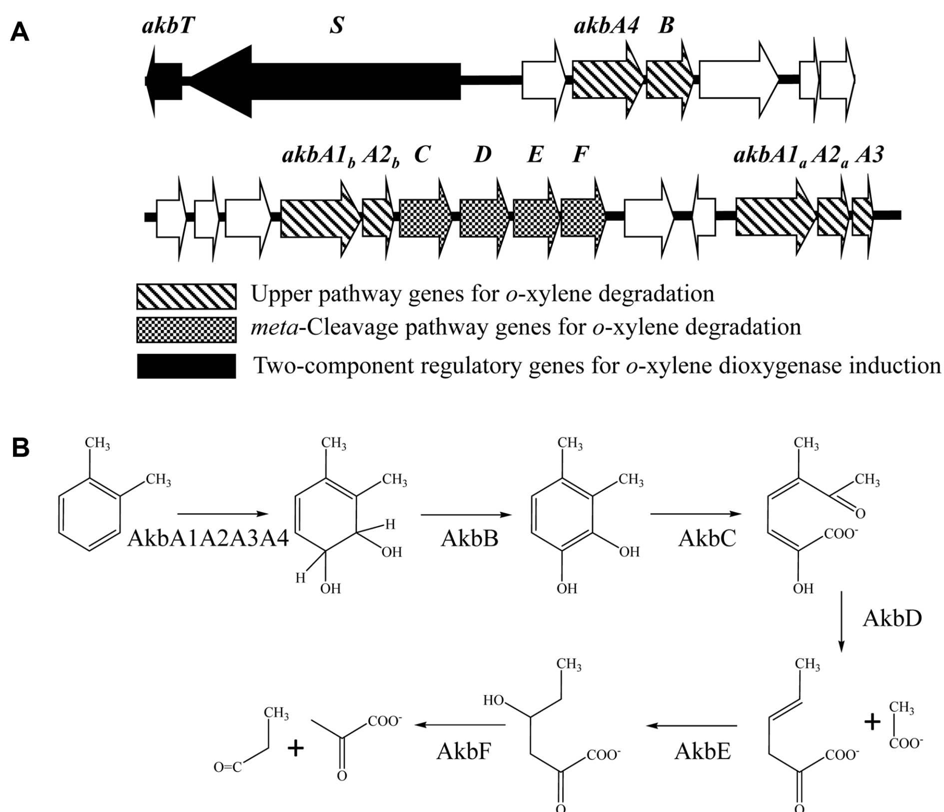

Fig. 2. Gene organization on the 330-kb pDK2 in *Rhodococcus* sp. DK17 (**A**), and proposed metabolic pathway for *o*-xylene degradation by DK17 (**B**).
The direction of transcription is indicated by arrowheads.

ecologically favorable process for the biodegradation of various pollutants at contaminated sites. The degradative capacities of aerobic rhodococci are of particular relevance for the bioremediation of organic pollutants derived from the constituents and halogenated products of petroleum. Indeed, there have been many studies involving the biological removal of aromatic and aliphatic hydrocarbons, such as benzene, chlorobenzene, phenol, *o*-xylene, naphthalene, and *n*-alkanes, using *Rhodococcus* strains that grew in extraordinarily high concentrations of pollutants and/or metabolized them at high degradative rates [22, 23].

As mentioned above, it has been examined if the rhodococcal strains with *o*-xylene degradation ability are able to remove *o*-xylene at a small scale in a laboratory and then be exploited for in situ *o*-xylene removal in natural environments where the strains were originally isolated. A laboratory-scale biofilter inoculated with *o*-xylene degrading *Rhodococcus* sp. BTO62 maintained more than 90% removal efficiency of *o*-xylene (41 $g/m^3/h$) under sterile condition and the efficiency was enhanced to 160 $g/m^3/h$ under nonsterile condition. This indicates the possibility that *o*-xylene and its degradation intermediates are co-metabolized by BTO62 and other contaminant microorganisms in the biofilter [24]. When co-inoculated with *m*- and *p*-xylene-degrading *Pseudomonas* sp. NBM21, BTO62 was able to remove a mixture of *o*-, *m*-, and *p*-xylenes at an elimination capacity of 180 $g/m^3/h$ at 20°C under nonsterile conditions [25]. *Rhodococcus* sp. RHA1 possesses two well-known degradative gene clusters for biphenyl (*bph*) and *o*-xylene (homologous to DK17 *akb*) degradation. RHA1 was used, together with the bacterium *Burkholderia xenovorans* LB400 carrying adequate operons for chlorinated benzoate and biphenyl degradation, for sediment microcosms contaminated with a mixture of structurally similar compounds of polychlorinated biphenyls [26]. Rhodococcal cells immobilized in agar or synthetic polymers were also used for the bioremediation of acrylonitrile and toxic amides. Bioaugmentation application of *Rhodococcus* sp. AJ270 pure culture resulted in the same level of acrylonitrile degradation in the sterilized topsoil as that achieved by whole

indigenous bacterial flora in unsterilized control soil [27].

Plant essential oils are abundant, inexpensive, and usually considered nontoxic to humans. Components in these oils have been reported both to stimulate bacterial cell growth and induce catalytic enzymes in their degradative pathways, resulting in higher degradation rates for biphenyl, polychlorinated biphenyls, and trichloroethylene (TCE). For example, the component limonene in orange peels was able to induce the biphenyl degradation pathway in limonene-degrading *Rhodococcus* sp. T104 [28]. The induction ability of these essential oil components is probably due to the similarity between their chemical structures and the pollutants. Thus, these essential oils or plant materials may be used as a cost-effective bioremediation strategy to stimulate environmental pollutant degradation. When induced by cumin aldehyde and cumene (two components in cumin oil), toluene-degrading *Rhodococcus* sp. L4 was capable of cometabolic degradation of TCE, with the degradation efficiency being similar to toluene grown cells ($57 \pm 5\%$ of the initial TCE concentration of 80 μM) [29]. To overcome the need for the repeated addition of inducing substrates to maintain TCE degradation activity, *Rhodococcus* sp. L4 was immobilized on cumin seeds as plant-immobilizing materials containing cumin aldehyde and cumene. The immobilized organisms tolerated up to 68 μM TCE and continuously degraded TCE, demonstrating that rhodococcal immobilization on plant materials rich in essential oils is a promising method for efficient TCE cometabolic degradation [30].

Even naturally cold environments such as alpine areas and Arctic and Antarctic regions are known to be contaminated with various aromatic and aliphatic compounds. Oil spillage is one of the main concerns, and the petroleum-derived contaminants are generally thought to be persistent in the environment owing to their toxic effects on indigenous microbes and the low temperatures, which limit the rates of microbial degradative processes. However, cold-adapted bacteria play a key role in in situ biodegradation in the cold environments, and their degradative activities and community composition shifts have been extensively studied [31]. Thus, the psychrotolerant and/or psychrophilic bacteria have been characterized as useful tools for low-temperature bioremediation of the environmental contaminants [32, 33]. *Rhodococcus* members from hydrocarbon-contaminated sites are commonly recognized for their high ability in petroleum hydrocarbon degradation. Several studies have reported the use of indigenous rhodococci to degrade phenolic pollutants in cold environments. Cold-adapted *R. erythropolis* BZ4 from alpine soil was able to degrade high amounts of phenol (0.7 g/l) at as low a temperature as 15°C [34]. *Rhodococcus* sp. AQ5-07 from Antarctic soil was capable of completely degrading 0.5 g/l phenol within 120 h at 10°C at the laboratory scale [35].

Biocatalysis

The generation of high-value chemicals from abundant and low-value renewable organic materials using microbial and/or enzymatic biocatalysts is an area of great interest to microbiologists and organic chemists. Biocatalysts are usually more effective than the use of traditional chemical methods, because they metabolize under mild conditions, with high specificity and stereoselectivity, which are needed in the (agro)chemical and the pharmaceutical industries. As shown in a representative review for the biocatalytic application of rhodococcal enzymes, *Rhodococcus erythropolis* contains a large number of enzymes that are involved in many diverse reactions in bioconversion and degradation processes, such as oxidations, dehydrogenations, epoxidations, hydrolysis, hydroxylations, dehalogenations, and desulfurizations [36]. Several representative bioconversion reactions by catalytic enzymes from *Rhodococcus* spp. strains are depicted in Fig. 3 and described in detail in the subsections below.

Aromatic compounds. Oxygenases are defined as enzymes that catalyze the specific introduction of one (monooxygenase) or two (dioxygenase) oxygen atoms into the substrate. Aromatic dioxygenases have attracted interest for their potential application as biocatalysts for the regioselective and enantioselective synthesis of vicinal *cis*-dihydrodiols and their dehydrogenation product catechols [37]. As a result of the demand for chiral intermediates, dehydrogenases have also been used for the production of chiral compounds, such as substituted catechols. For example, the naphthalene *cis*-dihydrodiol dehydrogenase enzyme has been used for the production of catechol-like metabolites in biotransformation studies.

The *o*-xylene dioxygenase from *Rhodococcus* sp. DK17 possesses the unique ability to perform specific regioselective hydroxylations, depending on the size and position of the substituent groups on the aromatic ring [38]. Molecular modeling studies predicted that the substrate-binding pocket of the DK17 *o*-xylene dioxygenase is large enough to accommodate bicyclics, and can be divided into three regions (distal, central, and proximal), where hydrophobic interactions in the distal position may be important in substrate binding. Subsequent site-directed mutagenesis studies combined with metabolite analyses confirmed these

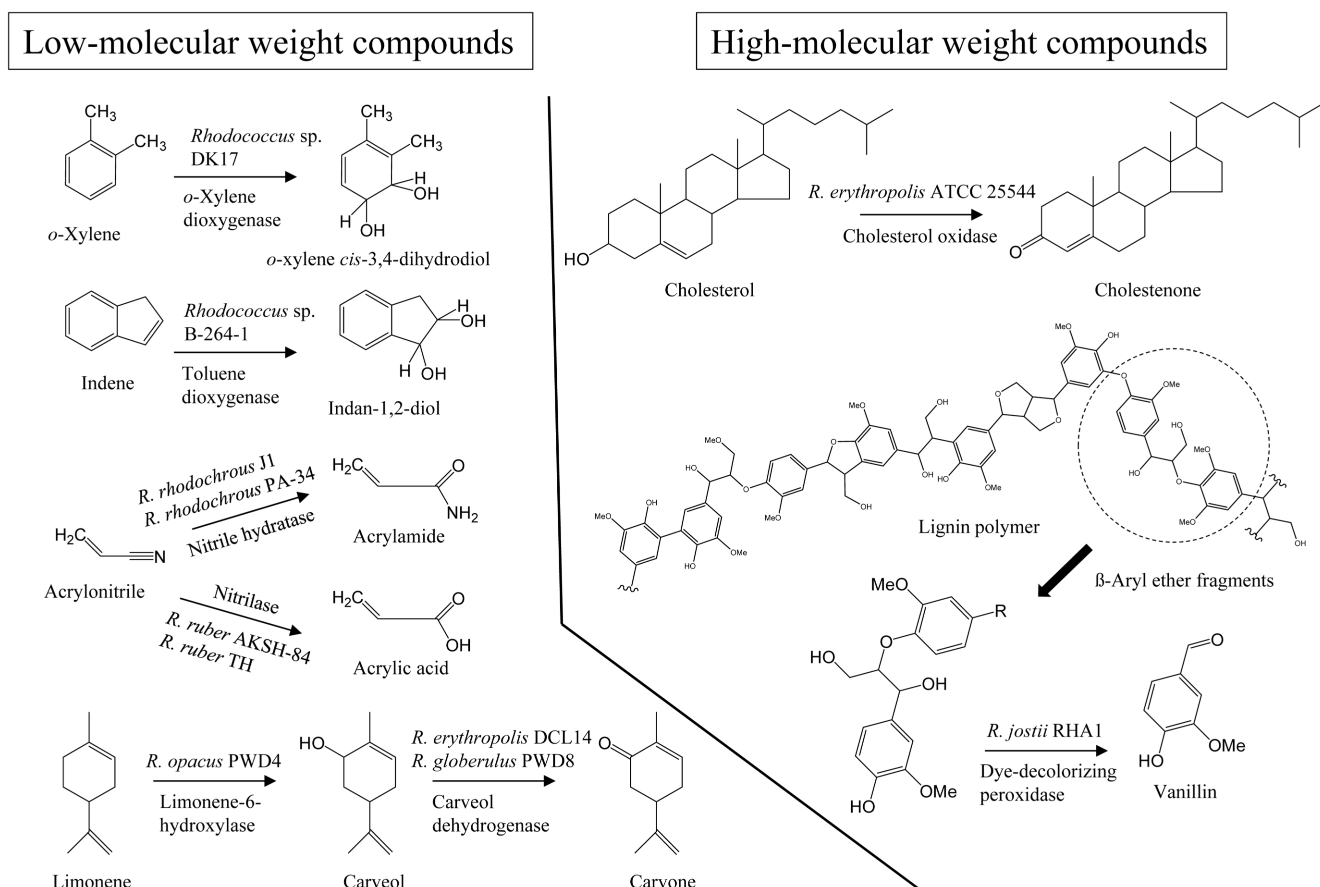

Fig. 3. Representative bioconversion reactions by catalytic enzymes from *Rhodococcus* spp. strains.

predictions, and further showed that different positioning of substrates in the active site of the enzyme resulted in the generation of different products. For example, DK17 transformed toluene and ethylbenzene into two different *cis*-dihydrodiols that resulted from an oxidation at the 2,3 and the 3,4 positions on the aromatic ring, whereas it transformed all of the larger substrates tested (biphenyl, naphthalene, indan, tetralin, indene, and 1,2-dihydronaphthalene) exclusively into each corresponding *cis*-dihydrodiol [8]. Moreover, when expressed in *Escherichia coli*, the *o*-xylene dioxygenase (AkbA1A2A3) was able to transform tetralin, indene, and 1,2-dihydronaphthalene into each corresponding *cis*-dihydrodiol (tetralin *cis*-dihydrodiol, indan-1,2-diol, and *cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene, respectively) [8]. These bioconversion studies suggested that the DK17 cells or recombinant *o*-xylene dioxygenase, with the capability for aromatic and alicyclic hydroxylation of indan derivatives, could be used as a promising biocatalyst for the development of new chemicals (Fig. 4).

Indan and its derivatives are frequently used as starting materials for the preparation of synthons for pharmaceutical and fine chemical synthesis. *Rhodococcus* sp. I24, possessing a toluene dioxygenase, was capable of transforming indene to *cis*-(1*S*,2*R*)-indandiol, a potential precursor to (−)-*cis*-(1*S*,2*R*)-1-aminoindan-2-ol, a key chiral synthon for indinavir, an HIV protease inhibitor [39]. When scaled up in a 14-L bioreactor, *Rhodococcus* sp. B-264-1 produced 2,000 mg/l of *cis*-(1*S*,2*R*)-indandiol (an enantiometric excess > 99%) and yielded up to 4,000 mg/l in process development studies. In addition to the oxidation of indene to *cis*-(1*S*,2*R*)-indandiol, indan and tetralin were transformed to a variety of indandiols for chiral drugs by enantioselective benzylic microbial hydroxylation [38]. Research has also been performed to obtain patentable melatonin receptor ligands, including synthesis of tricyclic indan derivatives for the development of new melatonin receptor agonists [40].

Nitriles. Nitriles are organic compounds that have a −C≡N functional group. They are common environmental pollutants. Rhodococci have been shown to degrade and

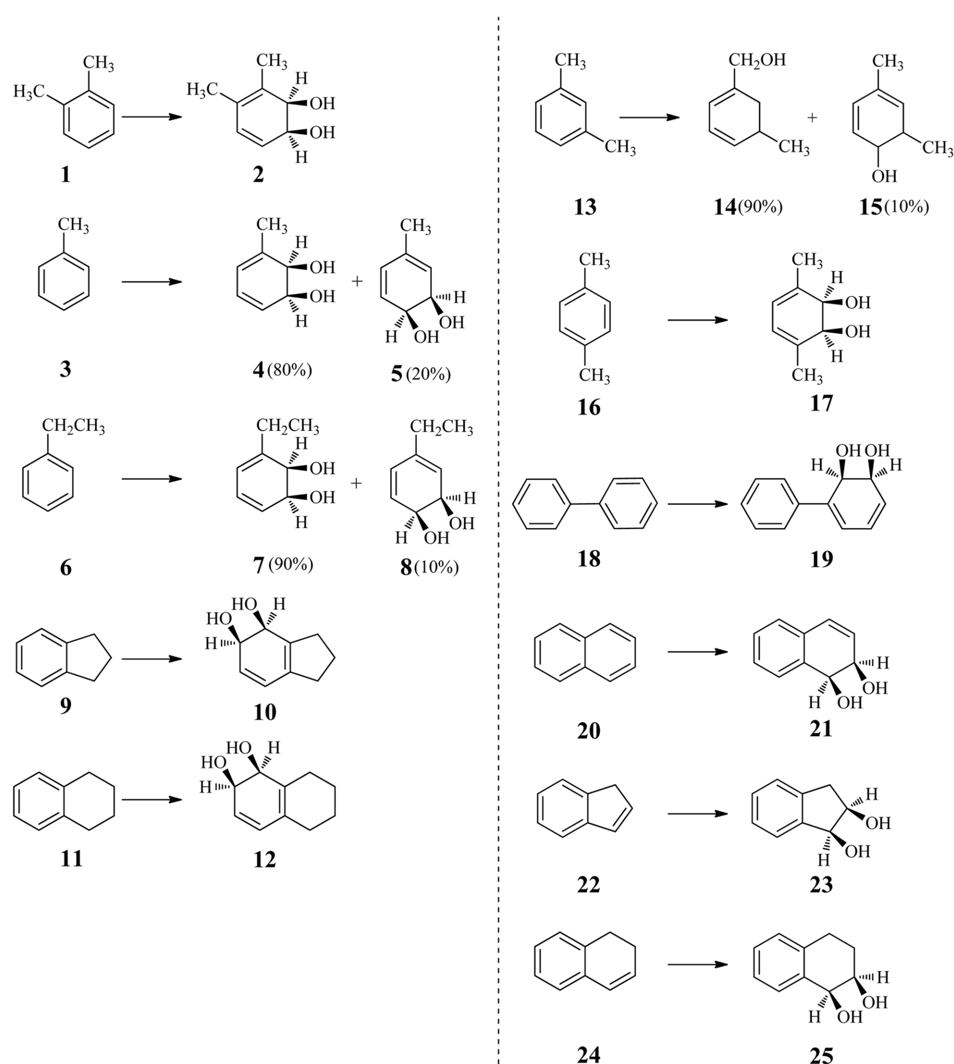

Fig. 4. Regioselective oxidation of monocyclics and bicyclics by *Rhodococcus* sp. DK17 *o*-xylene dioxygenase.

The oxidation product(s) from each substrate are as follows: **1**, *o*-xylene; **2**, 1,2-dihydroxy-3,4-dimethylcyclohexa-3,5-diene; **3**, toluene; **4**, *cis*-2,3-toluene dihydrodiol; **5**, *cis*-3,4-toluene dihydrodiol; **6**, ethylbenzene; **7**, *cis*-2,3-ethylbenzene dihydrodiol; **8**, *cis*-3,4-ethylbenzene dihydrodiol; **9**, indan; **10**, *cis*-indan-4,5-dihydrodiol; **11**, tetralin; **12**, tetralin *cis*-dihydrodiol; **13**, *m*-xylene; **14**, 3-methylbenzyl alcohol; **15**, 2,4-dimethylphenol; **16**, *p*-xylene; **17**, *cis*-*p*-xylene dihydrodiol; **18**, biphenyl; **19**, *cis*-2,3-bipheyl dihydrodiol; **20**, naphthalene; **21**, *cis*-1,2-naphthalene dihydrodiol; **22**, indene; **23**, indan-1,2-diol; **24**, 1,2-dihydronaphthalene; **25**, *cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene. The left and right panels show growth and non-growth substrates of DK17, respectively.

bioconvert a wide range of nitriles. The hydrolysis of nitrile to the corresponding carboxylic acid is catalyzed by a nitrilase or by the successive actions of a nitrile hydratase (NHase) and an amidase, which metabolizes the intermediate amide to carboxylic acid. Thus, the nitrile-converting enzymes, nitrilase and NHase, are responsible for the bioconversion of a wide variety of structurally different nitriles. The bioconversion of acrylonitrile has become attractive because of its industrial applicability in acrylamide production. Acrylamide can be prepared by the hydrolysis of acrylonitrile by NHase. This is beneficial in acrylamide production because nitrile-hydrating whole-cell biocatalysts generally exhibit low or no amidase activity for acrylamide.

Because of progress in understanding microbial nitrile metabolism, NHases from *Rhodococcus* sp. N-774 and *R. rhodochrous* J1 have been used in the kiloton-scale production of acrylamide, which is known as one of the most commercially successful microbial biotransformations. The polyacrylamide-entrapped resting cells of *R. rhodochrous*

PA-34 produced a total of 1,217 g/l acrylamide in a partitioned fed-batch reactor recycling the entrapped cells [41]. Rhodococci with nitrilase activity was also used to convert acrylonitrile into acrylic acid, which is traditionally used for the production of diverse acrylic esters in many industries. A versatile acrylonitrile-bioconverting *R. ruber* AKSH-84 was studied for optimization of medium and biotransformation conditions for acrylic acid production [42]. *R. ruber* TH, expressing NHase, amidase, and nitrilase, was transformed to a NHase-amidase double-knockout mutant, and the resultant mutant, which was still able to express nitrilase at extremely low level, was subsequently engineered to overexpress a heterologous nitrilase from *R. rhodochrous* tg1-A6 with a high activity of nitrilase. When the engineered cells expressing two nitrilases were used as a catalyst at room temperature, the amount and productivity of ammonium acrylate increased to 741.0 g/l and 344.9 g/l/h, respectively, which were the highest values reported to date [43].

Lignin polymer. Large amounts of small molecular aromatic compounds originate from decaying plant material containing (hemi) cellulose and lignin (a complex aromatic heteropolymer). In addition to natural lignin, large quantities of kraft lignin are formed in the pulp industry. Thus, lignin degradation products are of considerable interest as a renewable source of high-value aromatic compounds and other lignin-derived products [44]. Lignin is composed of phenylpropanoid aryl-C_3 units (guaiacyl, syringyl, and *p*-hydroxyphenyl), linked together via a variety of ether and C-C bonds. The most common chemical linkage found in lignin is the β-aryl ether, accounting for 45–50% and 60% of the units in softwoods and hardwoods, respectively [45]. When these linkages are broken, a wide range of lignin-derived low-molecular phenolic compounds are produced [46].

R. jostii RHA1 was intensively characterized for its degradation properties of aromatic compounds (especially, polychlorinated biphenyl and alkylbenzenes) using its catalytic enzymes and pathways. RHA1 was later shown to break down lignin and lignocellulose. In RHA1, lignin degradation is proposed to be initiated by an extracellular dye-decolorizing peroxidase B (DypB), resulting in vanillin as a main intermediate. Vanillin is further catabolized to vanillate and then protocatechuate by the activities of vanillin dehydrogenase (Vdh) and vanillate *O*-demethylase (VanAB), respectively, which is subjected to intradiol oxidative ring cleavage. Recombinant DypB was shown to recognize the β-aryl ether structure and cleave the C_α-C_β bond in β-aryl ether lignin model compounds, producing vanillin, a valuable chemical for food flavoring. DypB also showed linkage cleavage activity for kraft lignins and wheat straw lignocellulose [47]. Interestingly, when encapsulated with encapsulin protein from RHA1, the assembled complex DypB showed an 8-fold enhanced lignin degradation activity, when compared with DypB alone [48]. A *vdh* gene deletion mutant of RHA1, when grown on minimal medium containing 2.5% wheat straw lignocellulose and 0.05% glucose, accumulated vanillin (96 mg/l after 6 days) [10]. Natural vanillin production is of intense interest to the flavor and fragrance industry.

Terpenes and terpenoids. Terpenes, composed of isoprene units, are a large and diverse class of organic compounds produced by a variety of plants. They are used in the food, cosmetics, pharmaceutical, and biotechnology industries. Terpenoids (isoprenoids) are known as modified terpenes involving methyl groups that have been moved or removed, or added oxygen atoms, thus containing additional functional groups. Terpenes and terpenoids are the primary constituents of plant and flower essential oils, and are widely used as fragrances and flavors in the food, pharmaceutical, and cosmetic industries. Synthetic variations and derivatives of natural terpenes and terpenoids also greatly expand the variety of aromas used in perfumery and flavors used as food additives. Monoterpenes are branched-chain C_{10} hydrocarbons with two isoprene units that are widely distributed in nature, with more than 400 different monoterpenes identified. Thus, bacteria with the ability to degrade monoterpenes are relatively common, and a degradation pathway via initial oxidation of the benzylic methyl group has been extensively studied at the biochemical and genetic levels. For example, in the cases of *p*-cymene and limonene, the initial oxidation of the benzylic methyl groups of monoterpenes was performed by a monooxygenase, producing corresponding alcohols that were oxidized to aldehydes and then acids by respective dehydrogenases. The further degradation of those acidic intermediates was processed through lower degradation pathways [49].

Limonene, a monocyclic monoterpene, is the main constituent of orange and lemon peel oil (92–96%), which is a byproduct of the fruit juice industry, and is present in a mixture of both enantiomers (4*R*)-limonene and (4*S*)-limonene. In plants, the terpenoid carvone is biosynthesized via ring hydroxylation of limonene to carveol, the hydroxyl group of which is subsequently oxidized to ketone, resulting in carvone. There have been many reports concerning the biotransformation by microorganisms of low-priced limonene to more valuable naturally flavored monoterpenoids such

as carveol and carvone. Carveol and carvone are widely used in the flavor and fragrance industries. Carveol also has chemopreventive potential for mammary carcinogenesis with little or no toxicity. Oils containing carvones are used in aromatherapy and alternative medicine.

R. erythropolis DCL14, which is able to grow on limonene and limonene derivatives such as carveol and carvone, was reported to metabolize limonene via an initial attack at the 1,2 double bond by monooxygenase, leading to limonene-1,2-epoxide. Limonene-1,2-epoxide is converted to limonene-1,2-diol by limonene epoxide hydrolase activity [50]. DCL14 also metabolized all four stereoisomers of carveol via oxidation by carveol dehydrogenase to two carvone enantiomers [51]. In a laboratory-scale aqueous/organic system, using an air-driven column reactor, DCL14 whole cells biotransformed carveol (a mixture of isomers) to carvone (0.68 g carvone/g carveol) [52]. The chemostat-grown cells of toluene-degrading *R. opacus* PWD4 were able to hydroxylate (4*R*)-limonene (D-limonene) to enantiomerically pure *trans*-carveol, with a final yield of 94–97%. Another toluene degrader (*R. globerulus* PWD8) oxidized most of the formed *trans*-carveol to (4*R*)-carvone, which is used as a flavor compound [53]. As another example of the use of rhodococcal microbial or enzymatic catalysis, although the chemical-enzymatic mechanism is presently unclear, the resting cells of *R. erythropolis* MLT1 converted the monoterpene β-myrcene to geraniol, which has a rose-like scent and is commonly used in perfumes. A scalable process for geraniol production is being developed [54].

Cholesterol. Steroids are organic compounds composed of four fused rings (three six-member cyclohexane rings A, B, and C; and one five-member cyclopentane ring D). Steroids vary by the functional groups attached to this four-ring core and by the oxidation state of the rings. As an example, cholesterol, having a hydroxyl group attached at position C-3 in ring A, is an important component of cell membranes and is biosynthesized by all animal cells and even by plants in very small amounts. Cholesterol is a major starting material for the production of steroid drugs and hormones, because of the low cost and ease of transformation [9]. Microorganisms capable of degrading cholesterol as a common carbon source have been used to promote selective removal of steroid side chains, while leaving the steroid rings intact [55].

Degradation of the cholesterol rings is initiated by either an NAD^+-dependent 3β-hydroxysteroid dehydrogenase or cholesterol oxidase, depending on the strain [56]. The side chain degradation is initiated by a cytochrome P450 that catalyzes the oxidation of C26 to the corresponding carboxylic acid, and side chain removal proceeds via a mechanism similar to the β-oxidation of fatty acids. However, it is not clear whether the side chain and ring degradation processes occur simultaneously or independently [9]. In the case of *R. jostii* RHA1, the reaction product of the side chain removal and initial ring oxidation (4-androstene-3,17-dione) is metabolized to 1,4-androstadiene-3,17-dione, which is later subjected to successive B and A ring cleavages by a nonenzymatic reaction and an extradiol dioxygenase (HsaC enzyme), respectively [57]. All the rhodococci genes presently sequenced are predicted to encode this pathway, suggesting that cholesterol degradation is a common characteristic of this genus [9]. Bioconversion of steroids has been applied to resting cells in aqueous/organic solvents in two-phase systems [58]. Because the oxidation of cholesterol to cholestenone (cholest-4-en-3-one) by cholesterol oxidase is the first step of ring oxidation in microbial degradation, cholestenone production by cholesterol oxidase-producing rhodococci cells was investigated as a model system for the bioconversion of steroids. A systematic investigation for efficient biotransformation of cholesterol to cholestenone was performed with cell-linked and extracellular cholesterol oxidases from *R. erythropolis* ATCC 25544 [59].

Concluding Remarks

Over the past decade, bioinformatics has been integrated with biodegradation and biocatalysis to produce new research tools useful in databases [60−62]. With online databases linking genomic information, such as UM-BBD (http://umbbd.msi.umn.edu/) and KEGG (http://www.genome.ad.jp/kegg/), the functions of genes within these genomic sequences can be assigned, and biocatalytic reactions and biodegradation pathways can be predicted [63, 64]. These pathway prediction systems allow prediction of the degradation pathways and identification of the enzymes involved in the degradation pathways.

As of mid-May 2017, a total of 226 rhodococci genome sequences have been submitted to the National Center for Biotechnology Information (USA). These sequences can be used for genome-scale comparisons of the genetic relatedness among rhodococcal strains and metabolic characteristics involving pathways, reactions, catalytic enzymes, and metabolites. Genomic analyses of *Rhodococcus* strains have already provided insights into their biotechnological potentials and ecological roles [65, 66]. As stated by Arora and Bae [67], bioinformatics and biodegradation are two critical scientific fields in applied microbiology and

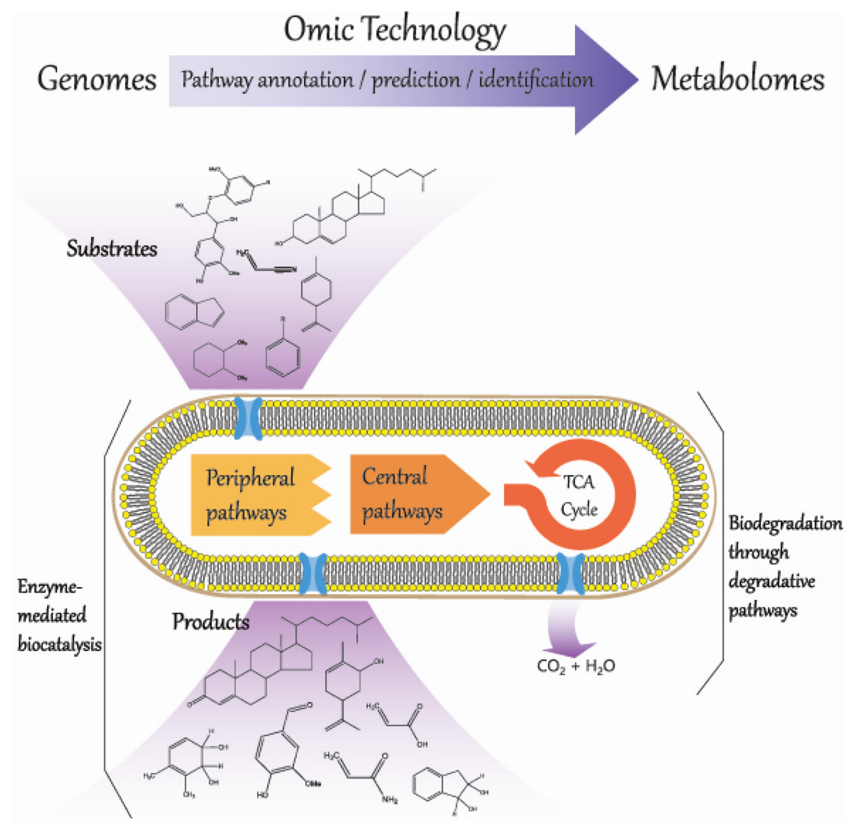

Fig. 5. Schematic illustration of *Rhodococcus* biodegradative pathways and omics technology outline.

biotechnology. In this context, the cutting-edge "omics" technology, combined with genetic engineering tools, can exploit extensively the potential of biodegradative rhodococci to develop new approaches for biodegradation and biocatalysis. The concept of "from genomics to metabolomics" has already been successfully applied to biodegradation and biocatalysis [68, 69]. This approach would also be very useful for developing strategies to harness the real and potential value of biodegradative rhodococci (Fig. 5).

The catabolic enzymes specifically adapted to low temperatures (cold-active enzymes) are thought to be a product from cold adaptation mechanisms used by microorganisms. The cold-active enzymes from cold-adapted microbes show high catalytic activities at low temperatures, which makes them excellent biocatalysts in biotechnological and industrial applications. However, few reports have been published about rhodococcal cold-active enzymes, in spite of their biotechnological potential [70, 71]. Thus, the isolation and characterization of novel cold-active enzymes, such as esterase, protease, lipase, cellulase, and peroxidase, from rhodococci inhabiting different cold environments (polar marine, soil, and glacier) should be an important research subject in the near future.

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Conflict of interest

The authors have no financial conflicts of interest to declare.

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