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Biotechnological Potential of Oxidative Enzymes from Actinobacteria

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Abstract

Oxidative enzymes are often considered for use in industrial processes because of the variety of reactions they are able to catalyse. In the past, most of these oxidative enzymes were obtained from fungi. However, in recent years, it has become evident that these enzymes are also produced by bacteria, including actinobacterial strains, which can therefore be considered as an underexploited resource of oxidative enzymes with potential for application in various industries. This chapter will focus on selected oxidative enzymes found in actinobacteria, their potential for application in industrial processes and how we can access and improve these enzymes to suit the required bio-process conditions.

Keywords: Actinobacteria, oxidative enzymes

1. Introduction

Dating back over 100 years, organisms belonging to the class *Actinobacteria* have been the focus of different studies [1]. This should come as no surprise: the class contains five subclasses and nine orders of which the order *Actinomycetales* have received the most amount of attention, not only because it is the largest order (Figure 1), but also because of the importance of the genera and species represented in this order. For example, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, marine *Micromonospora* and *Salinispora* strains with the ability to produce unique anti-cancer agents, and the genus *Streptomyces*, whose members are known to produce a variety of bio-active compounds and enzymes with potential for industrial application are all members of the order *Actinomycetales* (to name but a few).

Over the past 20 years, there has been an increased interest in the production of certain oxidative enzymes by actinobacteria, especially in the field of lignin degradation, detoxifica-

tion of organic pollutants such as polynuclear aromatic hydrocarbons (PAHs), organophosphate pesticides and azo dyes [2]. Oxidative enzymes or oxidoreductases (EC 1.x.x.x) catalyse oxidation-reduction reactions. These enzymes are further subdivided depending on the target donor molecule and the final electron acceptor, for example, the EC 1.1 grouping refers to enzymes acting on the CH-OH group of donors, while EC 1.1.3 further defines the type of electron acceptor, which in this case is oxygen (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>). Based on the information obtained from BRENDA, the comprehensive enzyme information system (<http://www.brenda-enzymes.org/>), there have been more than 1,500 reports of oxidoreductase production by actinobacteria. This represents a vast number of oxidoreductases, and therefore this chapter will focus only on enzymes grouped within the EC 1.x.3.x, EC 1.11.x.x, EC 1.13.x.x and EC 1.14.x.x classes (where oxygen acts as an electron acceptor or is incorporated during the reaction). Based on the literature reported in BRENDA, the following actinobacterial genera are represented in the EC 1.x.3.x, EC 1.11.x.x, EC 1.13.x.x and EC 1.14.x.x classes: *Acidothermus*, *Actinomyces*, *Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Dietzia*, *Gordonia*, *Kocuria*, *Leifsonia*, *Lechevalieria*, *Microbacterium*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Pimelobacter*, *Prauserella*, *Pseudonocardia*, *Rhodococcus*, *Saccharopolyspora*, *Streptoalloteichus*, *Streptomyces*, *Thermobifida* and *Williamsia*.

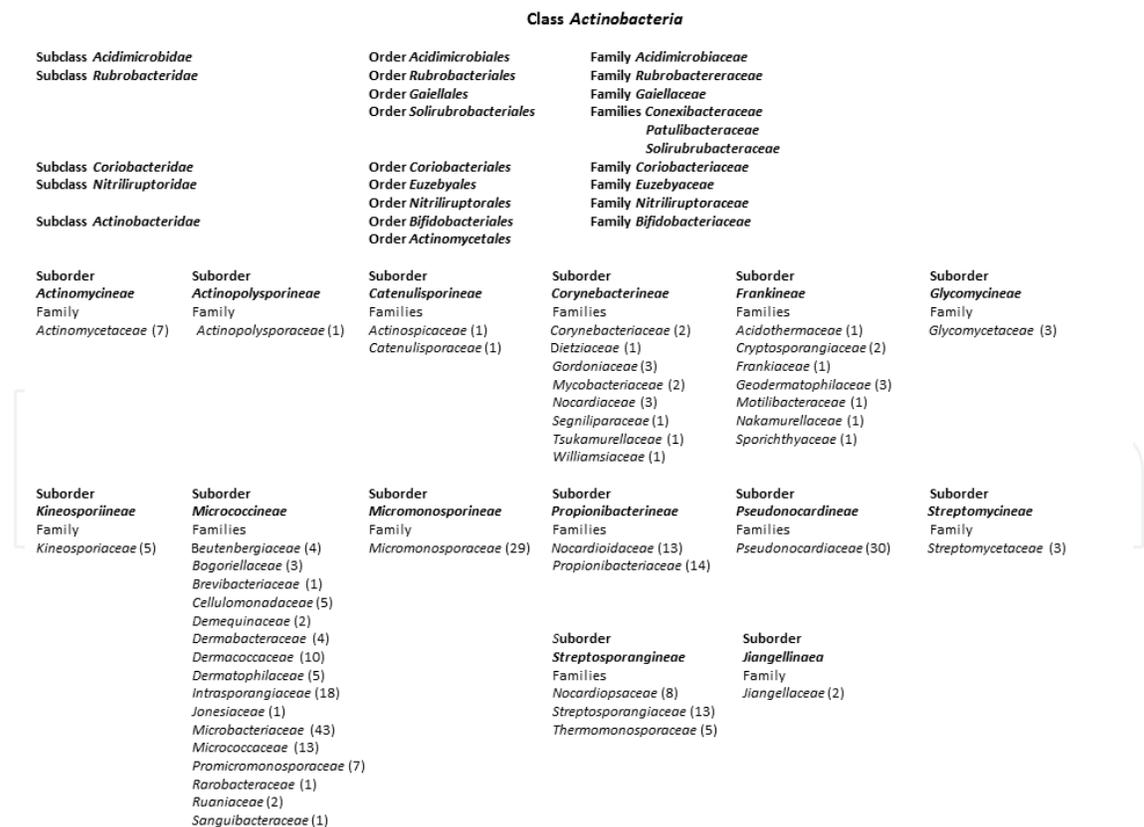


Figure 1. The class *Actinobacteria*, subclasses, orders, suborders and families. Values in brackets represent the number of genera described for each family (adapted from [3]; updated June 2015).

2. Oxidative enzymes produced by actinobacterial strains

The development of practical biocatalytic oxidation/reduction (redox) processes is very important because many chemical and biochemical transformations involve redox processes [4]. It is therefore not surprising that oxidative enzymes have been applied to a wide range of industrial processes: in the beverage industry to remove phenolics (which causes turbidity) in drinks and to remove/diminish the cork smell in wines [5]; in the pulp paper industry to delignify wood for the bleaching process [6]; in the textile industry to decolourise the effluents from dyes [7]; to bleach textiles and to synthesise dyes [8]; in the nanotechnology industry as biosensors [9-11]; for clinical and environmental analysis and for cosmetics, for use in hair dyes and for skin lightening creams [12]. Peroxidases, laccases and tyrosinases are among the oxidative enzymes that have been widely researched and much information on their applications and mechanistic roles has been published, with a particular interest directed towards the fungal producers. In this chapter, we wish to draw attention to selected oxidative enzymes that are produced by the actinobacteria, including the well-known peroxidases, laccases and tyrosinases.

2.1. Cholesterol oxidase (EC 1.1.3.6)

Many actinobacteria carry out useful biotransformations, which allow for the production of a wide range of substances of clinical and commercial interest [13]. Of these compounds, steroids are among the most important pharmaceutical products used for the treatment of various diseases [14]. In eukaryotic organisms, cholesterol is an essential component for the maintenance of cell membrane structure and the synthesis of a number of compounds. Cholesterol oxidase (CO) is a prokaryotic enzyme that has been very useful for biotechnological applications, where it has been applied in the detection and conversion of cholesterol [15-17].

CO is a flavoenzyme that catalyses the oxidation and isomerisation of cholesterol to cholest-4-en-3-one, with the reduction of oxygen at C₃ to hydrogen peroxide (Figure 2) [18-19]. Despite having a broad substrate range, the presence of a 3 β -hydroxyl group is essential for the CO activity. Whilst most microorganisms produce cell-bound CO, the actinobacteria are prolific producers of high levels of extracellular CO [18].

Actinobacterial COs have been isolated from *Corynebacterium* spp., *Rhodococcus erythropolis*, *Rhodococcus rhodochrous*, *Mycobacterium* spp., *Brevibacterium* spp. and *Streptomyces* spp. [20]. Ivshina et al. [21] demonstrated the use of CO isolated from *Rhodococcus* strains for the bioconversion of β -sitosterol to 17 β -hydroandrost-4-ene-3-one (testosterone), with the addition of co-oxidant glucose and in the presence of the inhibitor, 2,2'-dipyridyl. CO from actinobacteria is currently in use in analytical practices, such as the measure of cholesterol in biological fluids and the quantitation of dehydroepiandrosterone sulphate (DHEAS) in liquids from cysts of ducts of the human mammary gland [14]. CO from *Streptomyces* spp. may also be used as a source of insecticidal proteins. CO, however, is not only beneficial but also been implicated in the causation of disease in humans. For example, *Rhodococcus equi*, a primary pathogen for horses, requires CO for opportunistic infection in immunosuppressed humans by causing membrane damage [22]. Brzostek et al. [23] demonstrated that CO also plays a key

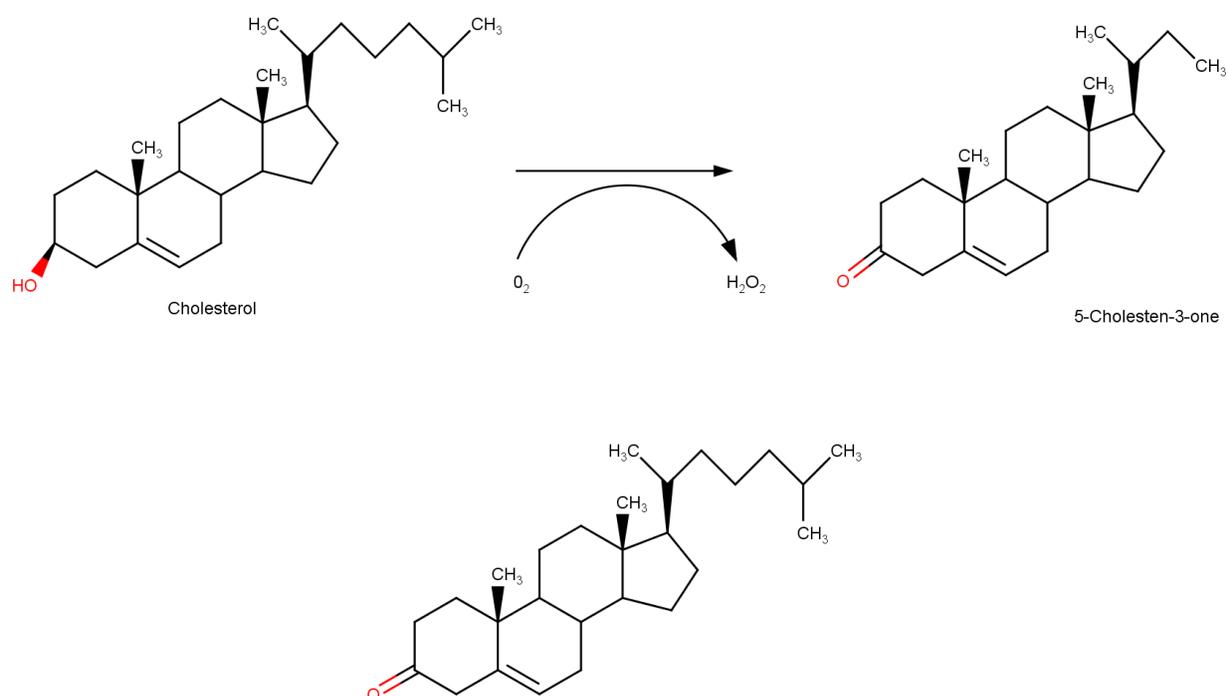


Figure 2. Mechanism of action of cholesterol oxidase on cholesterol (adapted from [20]).

role in the pathogenesis of *M. tuberculosis*. Therefore, by understanding how these COs act as virulence factors, it is possible to develop alternate means of treatment of these opportunistic infections.

2.2. Laccases (EC 1.10.3.2)

Laccases are multicopper oxidases that catalyse the one electron oxidation of four reducing substrate molecules, whilst simultaneously reducing molecular oxygen to water [24] (Figure 3). These enzymes have been extensively studied and are ubiquitous in nature, found in higher plants, fungi, insects and prokaryotes, including the actinobacteria [24, 25]. For decades, laccases of fungal origin have been at the centre of research efforts. Bacterial laccases, or laccase-like enzymes, are becoming increasingly prominent. As of 2014, less than 10 bacterial laccases from actinobacteria have been fully characterised, all of which belong to the genus *Streptomyces* [26]. As with other bacterial laccases, however, they possess characteristics that make them suitable for industrial applications, including increased thermostability, a broad pH range, stability under denaturing conditions, and for some actinobacterial laccases an atypical structure [27-29].

Whilst typical laccases consist of three cupredoxin domains, several laccases of actinobacterial origin have only two domains, which were first elucidated for the structure of the small laccase (SLAC) from *Streptomyces coelicolor* [31]. Several other two domain laccases have since been isolated, including the SilA from *Streptomyces ipomoea*, Ssl1 from *Streptomyces sviveus* and SCLAC from *Streptomyces* sp. C1 [32-34].

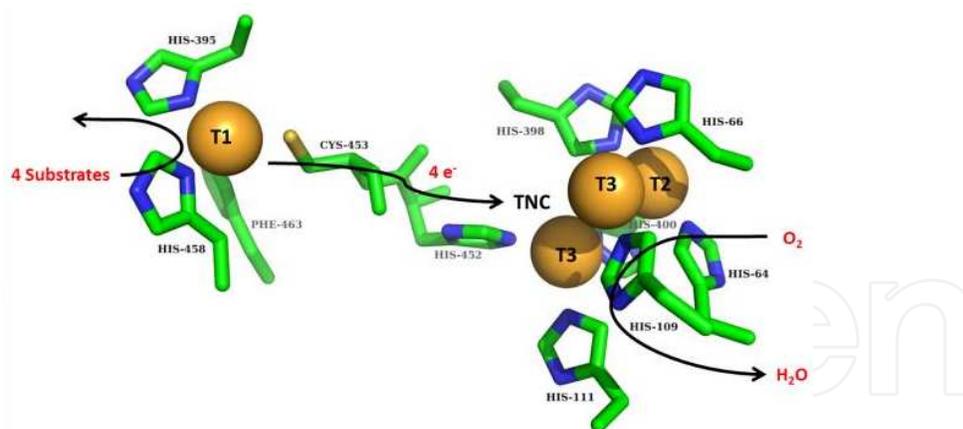


Figure 3. The typical laccase reaction mechanism [30].

Given the broad substrate range that laccases exhibit they can be applied in a wide variety of industries. Laccases have been employed in the following industrial applications: degradation of dyes in the textile industries, in the food industry where the consumption of oxygen in the packaged food is removed to avoid spoilage, in the paper and pulp industries for decolourisation of ink and the breakdown of lignocellulosic compounds, bioremediation for the treatment of toxic environmental pollutants including PAHs, pesticides, dyes from the textile industries and endocrine-disrupting chemicals [12, 35-40].

2.3. Peroxidases (EC 1.11.1.X)

Peroxidases are a large group of oxidoreductases that catalyse the oxidation of substrate molecules using hydrogen peroxide (H_2O_2) as the electron acceptor, with the majority of peroxidases using haem as a cofactor [41]. Haem peroxidases are typically grouped into two superfamilies: the first consists of bacterial, fungal and plant peroxidases and the second contains peroxidases from animals, fungi and bacteria [42]. The production of peroxidases from actinobacteria have been well described and are a good potential source of novel industrially relevant peroxidases, especially in a market that is largely dominated by the plant horseradish peroxidase (HRP) [42-44].

Antonopoulos et al. [45] investigated the biotechnological potential of the extracellular peroxidase from *Streptomyces albus* in the biobleaching of kraft pulps. It was found that the enzyme exhibited sufficiently high peroxidase activity so that it could be applied directly to the alkaline kraft pulp (alkalotolerant). The peroxidase was stable at high concentrations of H_2O_2 and no expensive co-mediators were necessary, when compared to fungal manganese-dependent and lignin peroxidases. Van Bloois et al. [41] isolated a DyP-type peroxidase from the thermophilic actinomycete, *Thermobifida fusca*, which showed high reactivity towards anthraquinone dyes, but moderate activity towards standard peroxidase substrates, aromatic sulphides and azo dyes. In 2014, Jaouadi et al. isolated a highly thermostable humic acid-biodegrading peroxidase from *Streptomyces albidoflavus*. This peroxidase exhibited a catalytic efficiency that is higher even than that of HRP [46].

2.4. Catechol 1,2-dioxygenase (EC 1.13.11.1) / Catechol 2,3-dioxygenase (EC 1.13.11.2)

Aromatic compounds are typically broken down by bacteria. However, recalcitrant varieties, such as polycyclic aromatic compounds do exist [47]. Bacterial populations often possess genes which code for enzymes that are able to degrade such toxins into protocatechuate and catechol [48]. The catechol in question is further broken down by one of two processes: (1) the action of catechol 1,2-dioxygenase (C12O) via an *ortho*-pathway or (2) via a *meta*-pathway which occurs when catechol 2,3-dioxygenase (C23O) cleaves catechol adjacent to the hydroxyl groups (Figure 4) [49-50].

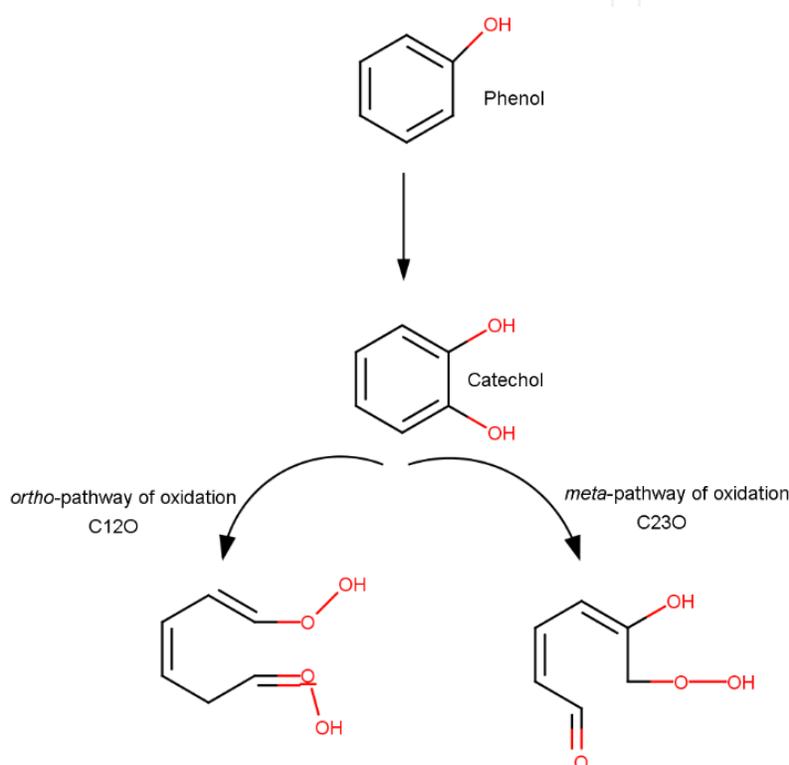


Figure 4. The breakdown of catechol through the action of (1) catechol 1,2-dioxygenase and (2) catechol 2,3-dioxygenase.

These dioxygenases are widely distributed among actinobacteria. Molecular analysis of catechol-degrading bacteria has shown that the *catA* gene which encodes for C12O is detected among several actinobacterial genera, including *Rhodococcus*, *Gordonia*, *Streptomyces*, *Corynebacterium* and *Mycobacterium* [48-51].

Many studies have demonstrated the use of C12O and C23O in the degradation of environmental contaminants using actinobacterial strains. Sutherland et al. [52] demonstrated C12O activity in four thermophilic *Streptomyces* strains when inducing the cultures with substituted benzoic acids. In addition, C12O activity was also induced during the culture of *Rhodococcus* sp. NCIM 2891 when using a medium supplemented with phenol [53]. An et al. [54] cloned a thermophilic C12O from the total DNA of *Streptomyces setonii* and heterologously expressed

it in *Escherichia coli*. This unique C12O exhibited remarkable thermostability, up to 65°C. Whilst C12O and C23O play a major role in (1) the breakdown of environmental contaminants, including aniline and its derivatives in agricultural soils [55] and (2) the degradation of biodiesel, diesel, chlorinated benzenes and some PAHs such as dibenzothiophene [56-57], the use of free enzymes are currently not a viable option since the enzymes are typically unstable under certain environmental conditions. Silva et al. [57] isolated C12O and C23O from *Gordonia polyisoprenivorans* and tested both the cell-free extracts and immobilised extracts. Activity was observed over a range of environmental conditions. Higher activity was observed for the immobilised C12O and C23O, thereby stabilising the enzymes and increasing the potential for greater industrial application of these enzymes.

2.5. Baeyer-Villiger monoxygenases (EC 1.14.13.X)

The interest in using Baeyer-Villiger monoxygenases (BVMOs) as biocatalysts has increased over recent years. BVMOs are flavin-dependent enzymes that are used to efficiently perform not only regio-, chemo- and/or enantioselective Baeyer-Villiger oxygenation reactions (Figure 5) using stoichiometric quantities of O₂ as an oxidant and NADPH as an electron donor, but also selected sulphoxidations and epoxidations [58-62].

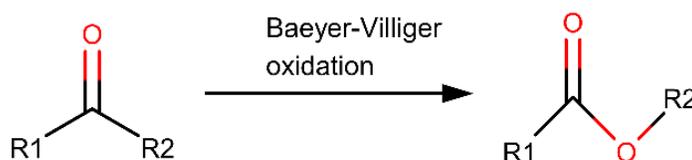


Figure 5. The Baeyer-Villiger oxidation reaction in which BVMOs convert ketones to their corresponding esters [63].

BMVOs are widely distributed among bacteria, being especially prevalent among the actinobacteria, with an average of one BVMO per genome [64]. In contrast, no Type 1 BVMOs (based on sequence similarity) have been found in plant, human or animal genomes [63]. As with many oxidases, the presence of a highly conserved protein sequence motif can be used to identify BVMOs [65]. Using this motif, Fraaije et al. [66] identified a putative BVMO from the genome of the thermophilic actinomycete, *T. fusca*. Jiang et al. [67] mined the genome of *Streptomyces avermitilis* and recombinantly expressed *PtIE*, which in the presence of NADPH and catalytic FAD exhibited Baeyer-Villiger activity. When the isolated enzyme was incubated with 1-deoxy-11-oxopentalenic acid, it gave rise to an unknown derivative of pentalenolactone, a sesquiterpenoid antibiotic, giving further insight into the pentalenolactone metabolic pathway [67].

In *Gordonia* sp. strain TY-5, BVMOs were shown to be implicated in the metabolism of acetone that is derived from propane oxidation and provides further knowledge on the poor understanding of acetone oxidation in microbes [68]. BVMOs can also be used for the synthesis of β -amino acids, compounds of considerable industrial importance due to their function as essential components in the preparation of β -peptides, terpenoids and β -lactam antibiotics [69].

2.6. Cytochrome P450 monooxygenase (EC 1.14.14.1)

Cytochrome P450 monooxygenases (CYPs) are perhaps one of the most widely studied enzymes. CYPs are haem *b* containing monooxygenases. Haem is a prosthetic group which contains an iron ion that is coordinated to four nitrogen atoms of porphyrin [70]. Similar to the BVMOs, the CYPs are remarkable in the amount of reactions they are able to catalyse, including but not limited to hydroxylation, epoxidation, peroxidation, deamination, dehalogenation, alcohol and aldehyde oxidation and C-C bond cleavage. As such, they are exploited for their potential applications in the production of drugs, vitamins, fragrances and pesticides [71-72]. CYPs play significant roles in various organisms: from carbon-source degradation and the production of metabolites in prokaryotic cells to the breakdown of toxic environmental xenobiotics found in mammals and insects [73].

CYPs, with their ubiquitous nature, have been observed in a number of actinobacteria. An environmental *Mycobacterium* strain (RP1) was isolated from contaminated activated sludge. This strain was able to use morpholine and other heterocyclic compounds as the sole carbon source for growth. Poupin et al. [74] deduced that a soluble CYP was involved in the breakdown of morpholine through the cleavage of the C-N in morpholine. Lamb et al. [75] studied the entire complement of CYP (the CYPome) in *S. coelicolor* and found that many of the CYPs are involved in the biosynthesis of antibiotics. Shresta et al. [76] subsequently cloned a CYP from *Streptomyces peuceticus* and showed the hydroxylation of the macrolide, oleandomycin. This showed that CYP have flexibility towards unnatural substrates and can be used in the generation of a variety of biological synthetic compounds of clinical value.

2.7. Tyrosinases (EC 1.14.18.1)

Tyrosinases (polyphenol oxidases) are copper-dependent oxidases that catalyse the *ortho*-hydroxylation of monophenols to diphenols (cresolase activity), and subsequently oxidising the resultant catechols to *o*-quinones (Figure 6) [77, 78]. To date, only three tyrosinase crystal structures have been elucidated, of which one was isolated from the actinomycete, *Streptomyces castaneoglobisporus* [79].

These enzymes are ubiquitous in nature, and serve a multitude of biological functions [80]. Most notably, tyrosinases play a key role in the production of melanin. For example, in plants tyrosinases are responsible for the browning of open surfaces in fruits [77], while in microbes melanin plays a key role in the defence of DNA against radiation and reactive oxygen species (ROS) and binds to toxic heavy metals [81-82]. Biologically active melanin has been shown to have many advantages, including anti-tumour activity and providing protection against UV radiation [83-86].

The most commonly used tyrosinase for commercial purposes is that of the fungus, *Agaricus bisporus* [87]. However, actinobacteria are well-known to produce tyrosinases, especially since many *Streptomyces* species produce a melanin-like pigment [88], and as such, actinobacterial tyrosinases have become increasingly prevalent [79, 89].

The cresolase and catecholase activities of tyrosinase are advantageous for many industrial processes, including the production of pharmaceutically important compounds such as the *o*-

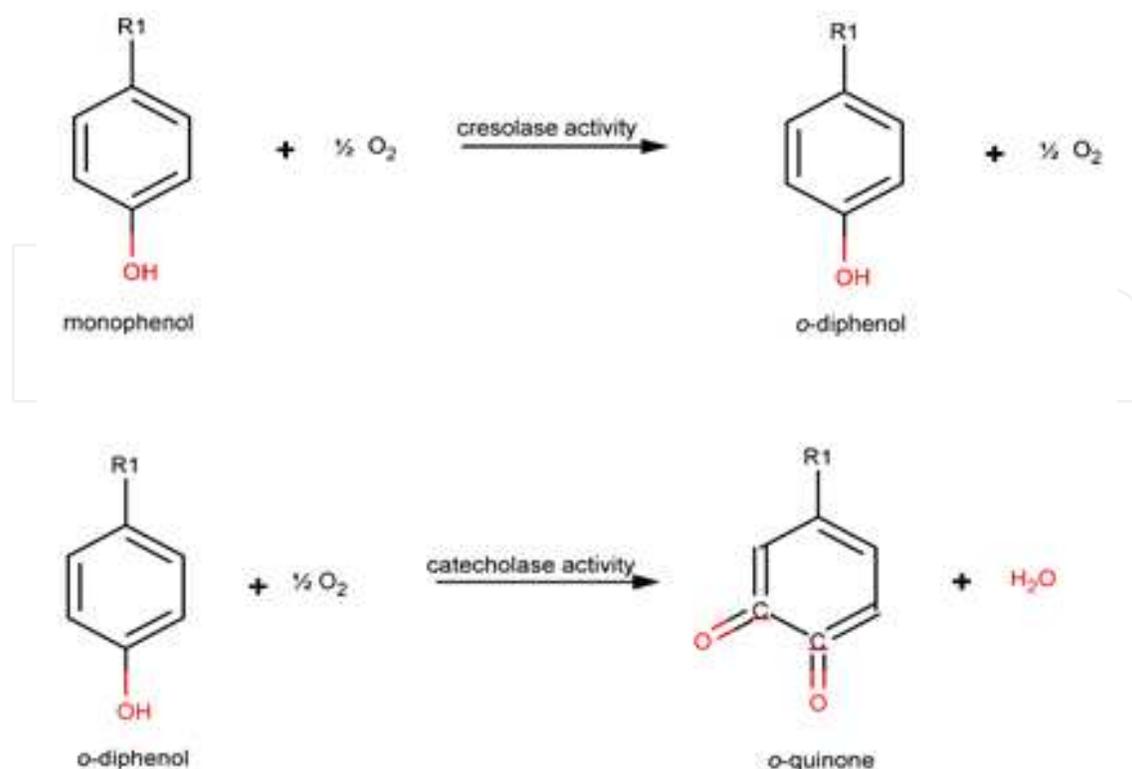


Figure 6. Enzymatic activity of tyrosinase on a monophenol (adapted from [78]).

diphenols, L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine [78]. For example, in humans, melanin plays an important role and melanin deficiencies can cause severe abnormalities and diseases. Parkinson's disease is one of these adverse conditions and is caused by a reduction of melanin in neurons [90-91]. The use of water-soluble melanin, such as that produced by several *Streptomyces* species, could therefore be useful in the treatment of Parkinson's disease. Madhusudhan et al. [92] investigated the production and cytotoxicity of extracellular insoluble and soluble melanin that were produced by *Streptomyces lusitanus* DMZ-3. Whilst their study showed that both the soluble and insoluble melanin were highly cytotoxic, it was observed that the soluble melanin was more biologically active than the insoluble melanin [92]. There has also been an increase in using safe, biologically produced compounds in the food industry, for example, the use of silver nanoparticles to control food pathogens [93]. Kiran et al. [94] further demonstrated the efficacy of silver nanoparticles as biocontrol agents by using response surface methodology to optimise the production of melanin from *Nocardia alba* MSA10. The melanin produced showed the rapid reduction and stabilisation of nanostructures and the produced structures had a broad spectrum of antimicrobial activity against common pathogens, including *Bacillus cereus*, *E. coli*, *Vibrio parahaemolyticus* and *Salmonella typhi*. Tyrosinases are also useful for the remediation of phenol-contaminated waters. Roy et al. [95] isolated and immobilised a tyrosinase from a marine actinomycete, *Streptomyces espinosus* strain LK4, which was able to effectively remove phenol from aqueous solutions.

2.8. Other oxidases

2.8.1. L-amino acid oxidases (EC 1.4.3.2)

L-amino acid oxidases (L-AAO) are oxidoreductases that catalyse the oxidative deamination of L-amino acids to yield keto-acids, ammonia and hydrogen peroxide [96]. These enzymes exhibit broad substrate ranges, and as such are commonly applied for the resolution of racemic mixtures. For example, an L-AAO isolated from *Rhodococcus opacus* DSM 43250 exhibited a broad substrate range, which includes the amino acids L-phenylalanine, L-leucine, L-alanine and L-lysine. It was able to resolve a racemic mixture of D,L-leucine and D,L-phenylalanine [96].

2.8.2. Putrescine oxidase (EC 1.4.3.10)

Putrescine is a low molecular weight diamine that belongs to a group of compounds that are termed biogenic amines. The accumulation of biogenic amines in foods, such as putrescine, can be used as a marker for food spoilage caused by microbes such as *Enterobacteriaceae* and *Clostridium* spp. [97-98]. Putrescine oxidases (PuOs) catalyse the oxidative deamination of putrescine to 4-aminobutanal, ammonia and hydrogen peroxide [99]. PuOs have been isolated from a number of actinobacteria, most notably from *R. erythropolis* and *Kocuria rosea* (*Micrococcus rubens*) [100-101]. Standard analytical methods for the detection of biogenic amines are thin-layer chromatography, gas chromatography and ultra-performance liquid chromatography [102-103]. Newer, more rapid methods of detection, such as biosensors, have been developed. PuOs from *K. rosea* was immobilized onto multi-walled carbon nanotubes for application as a biosensor, which allowed for the rapid detection of putrescine in mammalian plasma, with little interference from other biological species such as cadaverine or histamine, and without the need for prior purification of sample plasma [104]. Additionally, Bóka et al. [103] also immobilized PuO onto the surface of a spectroscopic graphite electrode and employed it for the detection of putrescine in beer samples. The biosensor measurements were compared to measurements performed through the use of high-performance liquid chromatography (HPLC), and higher sensitivity was exhibited when using the biosensor, demonstrating a rapid, efficient method for the detection of putrescine.

2.8.3. L-glutamate oxidases (EC 1.4.3.11)

In contrast to the broad substrate range of L-AAOs, there are strict substrate-specific amino acid oxidases, such as the L-glutamate oxidases [105], the first of which was isolated from the actinobacterium, *Streptomyces violascens* [106]. Glutamate oxidases have been shown to play a key role in the synthesis of pharmaceutically relevant chiral intermediates, specifically, the conversion of glutamate to α -ketoglutarate [107-108].

2.8.4. Sarcosine oxidases (EC 1.5.3.1)

Sarcosine oxidases (SOs) catalyse the hydrolysis of sarcosine and formaldehyde, while simultaneously yielding hydrogen peroxide [109]. It is predominantly being exploited in

clinical assays for the determination of creatinine in serum. The SO from *Corynebacterium* sp. U-96 is perhaps the most extensively studied SO to date. Whilst the *Corynebacterium* SO remains the most industrially relevant, SOs have been cloned and characterized from various *Athrobacter* spp. [110-112]. In addition, a *Streptomyces* sp. SO has also been cloned and expressed in a *Streptomyces* expression system [109-113]. Furthermore, recent genome studies have identified the presence of SO genes in the genomes of many actinobacterial species, which could potentially serve as a source of novel SOs [114-116].

3. Industrial relevance of actinobacterial oxidases: how to access them and improve their functionality

With oxidising enzymes, as with most other enzyme groups, the emphasis in the discovery and development of new enzymes for industrial processes is increasingly focused on the properties of the new enzymes that need to match the stringent conditions imposed by the industrial setting [117]. Thus, while the existence of large numbers of novel enzymes is demonstrated constantly, via a host of modern gene discovery technologies, this evidence of their existence is not sufficient to guarantee our capacity to provide the enzymes that industry demands. A further consideration in the development and application of new oxidizing biocatalysts is the requirement to demonstrate the novelty which will provide market advantage [118]. Thus, as new oxidizing enzymes are discovered, they need to be characterised in terms of substrate selectivity, product scope and stability in the presence of process constraints (e.g. the presence of organic solvents, temperature conditions, pH conditions).

The majority of oxidising enzymes are co-factor-dependent, which leads to requirements for co-factor recycle or replacement in the industrial processes utilising these enzymes. While this can be overcome by application of whole-cell biocatalysts, the search for non-co-factor-dependent oxidases which can catalyse equivalent reactions is a useful goal [117-118]. The three most commonly used screening methods to screen for novel enzyme activity include: (1) screening environmental samples for organisms whose enzymes have the ability to catalyse certain reactions; (2) the use of protein engineering to manipulate an existing biocatalyst and (3) looking for novel functionality/substrate specificity in existing biocatalysts [119]. These processes along with others can further be grouped into molecular-based (*in silico* screening of genome sequences; metagenomics and PCR-based screening; reverse genetics) and non-molecular-based screening techniques (dye decolourisation; high-throughput screening with liquid-based enzyme assays; selective isolation directly from an environmental sample) (Figure 7).

3.1. Isolated strains

Based on information obtained from BRENDA, the majority of the reports on oxidative enzymes from actinobacterial strains have originated from studies based on isolated strains and yet only a fraction of the genera within the order *Actinomycetales* is represented. This could be due to various reasons, including the fact that many of the strains that have been the focus

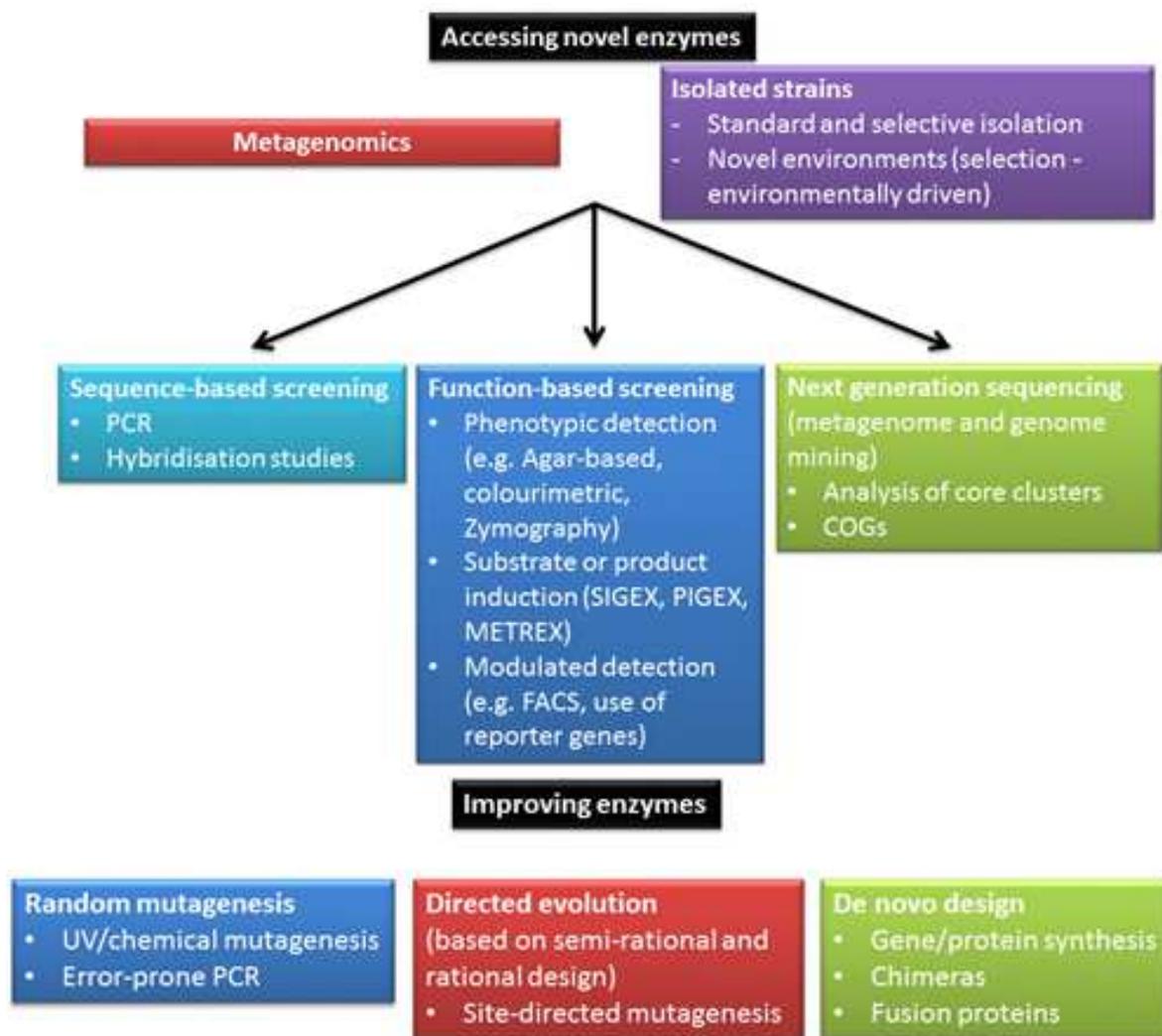


Figure 7. Summary of standard methods employed to access novel enzymes and to improve enzymes.

of studies are either pathogens (human and/or animal, e.g. *Mycobacterium* and *Rhodococcus* spp.) or are known producers of bio-active compounds (e.g. *Streptomyces* spp.). In addition, standard isolation techniques only allow for the detection of a small fraction of actinobacterial populations in environmental samples, often missing out on the isolation of the 'rare' actinobacteria (those not readily isolated) [120]. To access these strains and their genetic diversity, researchers have designed selective isolation techniques, many of which are based on the properties of the targeted organisms (e.g. motility or heat resistance) and/or the properties of the environment the sample was collected from [121]. Kurtböke [120] reiterated that the successful isolation of 'rare' actinobacterial strains or strains producing bio-active compounds/enzymes from any given environment would be dependent on our understanding of the function of the strains within the environment of interest. For example, Le Roes-Hill et al. [122] identified various oxidase-producing actinomycetes that were isolated from the hindguts of a higher termite where it is hypothesised that the oxidase-producing actinobacteria may be involved in lignin degradation. An alternative to the isolation of actinobacterial strains from

environmental samples, the genetic diversity of 'rare' actinobacteria can also be accessed through metagenomics-based studies.

3.2. Metagenomics - Accessing the genetic information of the 'unculturables'

Over the past two decades, there has been a dramatic increase in the number of metagenomics-based studies. The belief that the majority of bacteria in the environment (>99%) are unculturable or that culture techniques for their isolation have not yet been developed have necessitated the analysis of the metagenome [123]. With the advent of metagenomics in the 1990s, numerous novel genes have been discovered [124-126]. Metagenomics allows for the cloning and expression or screening of multiple genomic DNA extracts from any given sample [127]. Accessing the gene or enzyme of interest is, however, dependent on various factors, but typically involve sequence-based screening and/or function-based screening, some of which are also applicable to isolated strains.

3.2.1. Sequence-based screening

Sequence-based screening relies heavily on prior knowledge of the enzyme of interest, e.g. the polymerase chain reaction (PCR) primers and hybridisation probes need to be designed based on conserved regions in sequences that are currently available in databases. This approach is therefore not suited for the discovery of novel protein classes [128]. In two metagenomic studies, a sequence-based screening approach allowed researchers to identify the presence of actinobacterial two-domain laccases in lignin-rich environments. Ausec et al. [129] made use of previously published primer sets, while Lu et al. [130] designed their own primers that were designed based on sequence alignments of known two-domain laccases. Both of these studies showed the vast biodiversity of these laccases in the respective environments analysed: drained peat soils [129] and compost prepared from agricultural waste [130].

Similarly, sequence-based screening has been shown to be a valuable tool for determining the diversity of *Streptomyces* genes in isolated strains. Due to the biased codon usage of actinobacterial strains [131], it is possible to design primers based on short consensus sequences. Decker et al. [132] showed with a comparison of the sequences of known dNDP-glucose 4,6-dehydratases from *Streptomyces griseus* N2-3-1, *Streptomyces violaceoruber* Tii22 and *Saccharopolyspora erythraea* DSM 5908 that several conserved regions are present. Consequently, PCR primers were designed and used to amplify genes encoding for dNDP-glucose 4,6-dehydratase from eight different actinomycetes [132].

3.2.1.1. In silico screening of genome sequences

Of the nearly 6,400 bacterial genomes available on the NCBI 'Microbial genomes' page, (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html; accessed 24 June 2015), 871 represent the class *Actinobacteria*. Even though this represents a vast resource for the discovery of novel oxidative enzymes [133], *in silico* screening is often hampered by the misannotation of sequences [123]. Various researchers have, however, successfully identified, amplified, cloned and expressed actinobacterial oxidases discovered by genome mining, e.g.

the phenylacetone monooxygenase (EC 1.14.13.92; BVMO) from *T. fusca* [134]; laccase (EC 1.10.3.4) from *S. sviveus* [33]; cuprous oxidase from *Corynebacterium glutamicum* [135]; laccase (EC 1.10.3.4) from *S. coelicolor* [31] and cholesterol oxidase (EC 1.1.3.8) from *Mycobacterium neoaurum* [136], to name a few.

In addition, access to various genomes allows for comparative genomic analysis of the diversity of specific enzyme groups/classes among the genomes analysed. For example, Li et al. [137] performed comparative genomic analyses on 18 *Nocardiosis* genomes. Functional analysis of homologous gene clusters allowed for the identification of genes encoding for cytochrome P450 monooxygenases, thereby broadening our knowledge-base on the distribution of these enzymes in actinobacteria.

3.2.1.2. Reverse genetics

Recently, a strategy known as 'reverse genetics' has been developed for the identification of numerous gene clusters [138]. The first step in this strategy entails the deduction of a biosynthesis hypothesis for the corresponding substance. On the basis of the chemical structures, radiolabelling studies and other available data about the substances, the enzyme systems required for backbone-synthesis and tailoring enzymes can be predicted. In the second step, characteristic target genes and enzymes are selected and compared in multiple sequence alignments with homologous genes and gene products retrieved from public and in-house databases. Subsequently, conserved protein motifs can be determined and used for primer design. In the last step, internal fragments of the selected biosynthesis genes can be amplified by PCR and used as probes to identify these genes in genome libraries [138]. This method has been successfully used in the identification of genes from *Streptomyces lavendulae* coding cytochrome P450 monooxygenases involved in the biosynthesis of the antibiotic, complestatin [139]. Pirae and Vining [140] also used the same method to identify genes encoding for a halogenase, which is involved in the biosynthesis of chloramphenicol in *Streptomyces venezuelae*.

3.2.2. Function-based screening

Function-based screening is often preferred to sequence-based screening because it allows for access to a wider range of enzymes or biological activities [128, 141]. In metagenomics, the success of function-based screening is dependent on various factors: (1) abundance of the target gene; (2) the size of the gene; (3) the presence of a full-length sequence; (4) the expression host; (5) the expression system and (6) the assay or means of detection of the enzyme activity [123]. In the majority of metagenomic studies, *E. coli* is used as the expression host. *E. coli*, however, can only express 40% of environmental genes, but for the high G+C% actinobacteria, it is predicted to drop as low as 7% [141]. Functional metagenomics may therefore underrepresent the potential of actinobacterial strains. A solution to this is the use of more than one expression host: various *Streptomyces* spp. and *Rhodococcus* spp. have been used for the expression of actinobacterial genes. McMahon et al. [141] demonstrated the importance of using more than one host. In their study, the vectors from 12 functionally active clones were transformed into *Streptomyces lividans* and *E. coli*, and activity was only observed in the *S. lividans* host.

There are three main approaches in function-based screening: the detection of a phenotypic trait (most commonly used), production of the enzyme or compound due to substrate/product/metabolite-induced gene expression (SIGEX/PIGEX/METREX) and modulated detection where the expressed product is linked to a reporter gene allowing for detection by fluorescence or luminescence [142]. In the next few paragraphs, methods used for the detection of oxidases in actinobacteria are described. For more detail on the different function-based screening approaches mentioned above, see Ekkers et al. [142] for a complete review.

3.2.2.1. *Selective isolation of oxidase-producing strains directly from an environmental sample*

Kiiskinen *et al.* [143], described a method where the substrate for the oxidase of interest have been incorporated into the isolation media for new isolates, e.g. for the detection of laccase activity, guaiacol was incorporated - a laccase-producing strain would cause a change of colour in the agar from a dark red-brown to red-orange colour. Similarly, Bordeleau and Bartha [144] described a method whereby newly isolated strains on agar plates were sprayed with liquid *p*-anisidine-H₂O₂. Strains positive for the production of peroxidase developed a dark halo around the colony.

Isolated strains can also be screened for enzyme activity on solid media containing dyes. McMullan *et al.* [145] gives a short review on published work regarding the degradation of dyes by filamentous actinomycetes. The extracellular enzymes that are involved in the degradation of dyes are typically the enzymes involved in lignin degradation: lignin peroxidase, laccase and manganese-dependent peroxidase [146-147]. In filamentous actinomycetes, however, it was found that there is no correlation between the degradation of the polymeric dye, Poly R478, and that the enzymatic process involved is still unexplained [145]. The degradation of the azo dye, Remazol Brilliant Blue R (RBBR), was, however, found to be linked to the action of peroxidases which are similar in structure and function to the fungal manganese-dependent peroxidases [145, 147].

3.2.2.2. *High-throughput screening with liquid-based enzyme assays*

Oxidoreductases produced by actinobacteria can also be accessed by using various high-throughput assays. With the increased interest in finding novel enzymes for use in biocatalysis, the number of assays currently available is quite vast and most are based on the use of chromogenic or fluorogenic substrates [148]. Most of these assays employ the use of a standard UV/visible spectrophotometer or microtiter plate readers, but for certain assays, the screening process involves the use of mass spectrometry, nuclear magnetic resonance (NMR), Fourier-transform infrared (FT-IR), thin layer chromatography (TLC), capillary array electrophoresis and enzyme-linked immunosorbent assays (ELISAs) to detect the changes in the catalysed reaction [148-149]. Enzyme fingerprinting and the use of microarrays are increasingly becoming powerful tools in the high-throughput screening of enzymes [148]. In addition, fluorescence-activated cell sorting (FACS) is a powerful high-throughput screening method that allows for the screening of large (10⁹) clone libraries in a relatively short time period [123]. Zhu and Fang [150] recently also reviewed the potential of droplet microfluidics as a high-throughput technique for the screening of enzyme activities.

3.2.2.3. Protein engineering

Protein engineering is an alternative approach to obtain an enzyme with novel activities and biochemical properties. Random mutagenesis through the use of UV radiation or chemical mutagenesis is often favoured when the enzyme structure or sequence is not known. Even though this approach is typically used to generate an enzyme with improved properties, it is often limited to a change in a single property [123]. Random mutagenesis, however, was successfully used by Fujii et al. [151] to improve the vitamin D₃ hydroxylase activity of *Pseudonocardia autotrophica*. The mutated enzyme (four mutations) was expressed in *R. erythropolis* and exhibited 21.6 times higher activity, while the isolated mutated enzyme showed a six times higher activity than the wild-type enzyme [151]. Dudek et al. [152] generated multiple mutations in a single step through the use of the OmniChange method (allowed for random mutation of up to five sites), resulting in a quadruple mutated BMVO with an expanded substrate specificity. In addition, Yao et al. [136] successfully applied two mutated cholesterol oxidases (mutated by UV mutagenesis) in the production of steroids to determine whether cholesterol oxidase plays a role in the transformation of sterols. These examples emphasise the fact that random mutagenesis is a powerful tool for the development of enzymes with enhanced biochemical properties and still has its place in protein engineering.

For more complicated changes, such as changes to specific amino acids, knowledge is required on sequence-structure information, allowing for a more rational or semi-rational design. Liu et al. [153] made use of a combination of site-directed mutagenesis and error-prone PCR to generate 7,800 variants of a cytochrome P450 monooxygenase from *Rhodococcus ruber* DSM44319. The best variant showed 240 times increased de-ethylation activity towards 7-ethoxycoumarin and 10 times increased demethylation activity towards 7-methoxycoumarin. Site-directed mutagenesis have also been applied to the phenylacetone monooxygenase from *T. fusca* [134], the cyclohexylamine oxidase from *Brevibacterium oxydans* [154], the small laccase of *S. sviveus* [155], the small laccase of *S. coelicolor* [156-158] and the tyrosinase of *Streptomyces kathirae* [159].

The de novo synthesis of enzymes allows for the design of enzymes with specific properties. This, however, requires a deep understanding of sequence-structure-function [160]. A similar approach is the production of chimeric proteins, where different enzymes are combined so that the properties from the different enzymes can be harnessed in one reaction setup. These fusion enzymes are designed to enhance the biocatalytic activity/function of the enzymes. They can consist of enzymes that act synergistically (e.g. enzymes involved in lignin degradation) [161] or as in the case of the BVMO from *T. fusca*, the enzyme was fused with a phosphite dehydrogenase (an NADPH regeneration enzyme), thereby supplying the BVMO with the co-factor required for function [162].

4. In conclusion

Members of the order *Actinomycetales* clearly represent a vast untapped resource for oxidative enzymes with potential for biotechnological application. Only selected genera from selected

families are currently represented in literature and databases, leaving a great scope for further exploration. Specialised research in the area of actinobacterial genetics has allowed for the development of expression systems that allow access to environmental actinobacterial genetic material. In addition, the 871 genome sequences currently available on the NCBI database (a number that would surely be doubling over the next year), as well as the multitude of type strains in culture collections, provide numerous opportunities for the discovery of new and interesting oxidative enzymes. The recent move towards the sequencing of metagenomes will also present a vast resource from which sequence information of oxidative enzymes can be accessed. Even though we would be limited to accessing known enzyme classes, the potential for the discovery of novel enzymes would be great. Researchers in the field of actinobacterial oxidative enzymes are therefore encouraged to (1) develop effective screening programs; (2) make use of a full suite of biochemical properties to determine the potential of the enzyme for industrial application; (3) demonstrate the biotechnological potential of the enzyme; (4) determine the protein sequence of isolated enzymes so that the information on characterised enzymes can be expanded and used for directed protein engineering approaches; (5) look towards novel environments for new and interesting actinobacterial strains or genetic information and (6) make use of sequence resources currently available (e.g. genome sequences) in order to expand our knowledge base on oxidative enzymes from actinobacteria.

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