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Evaluation of new biorecognition elements for environmental monitoring

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Tutor:

Eng. Luca Marmo, Ph.D

Dr. Francesca Bosco, Ph.D

Dr. Luca Varani, Ph.D

Candidate:

Dr. Alessandro Chiadò

Coordinatore:

Prof. Franco Ferraris

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Abstract

To date, environmental monitoring is mainly focused on traditional chemical techniques, or on the assessment of specific biomarkers. However, these analyses are affected by several limitations: mainly, they are expensive, spot-sampling and time-consuming. In order to overcome these drawbacks, new biological monitoring methods, such as biosensors and biological early warning system (BEWS) are under development. These kinds of devices, built around whole cells, enzymes and antibodies, are well-suited to cooperatively and continuously monitor the environmental conditions. The key-factor of this very promising approach is the biological sensing element. Whole cell systems and enzymes are well suited for environmental monitoring: they are able to determine the bioavailable and toxic concentration of xenobiotics, especially if the source and nature of the compound cannot be predicted. Microorganisms usually detect a broad spectrum of chemicals, and represent a good opportunity for low cost, long shelf-life, and wide range of conditions in which they can be applied. Besides, enzymes are effective when a particular kind of pollutant would be detected because is possible to fine tune their metabolic behaviour by means of protein engineering.

In this work, three biological sensing elements, related to three different index of toxicity were evaluated, in order to develop new biosensors for environmental monitoring: a broad toxicity index associated to the decrease of light emission (EC_{50} or half effective concentration) of a bioluminescent bacterium, *Vibrio fischeri*, a metal toxicity connected to the metal-regulated production of a siderophore (pyoverdine) by the soil and water microorganism *Pseudomonas fluorescens*, and finally an index of toxicity given by PAHs, was related to the metabolization of these compounds by laccase of *Trametes versicolor*.

One of the first step during the assessment of a new biological sensing element is the study of the effect of physical-chemical parameters. The tested physical-chemical parameters (temperature, pH, inoculum percentage (v/v) and carbon source) influenced both microbial sensible elements (*V. fischeri and P. fluorescens*), therefore, these sensible elements can be used in a whole-cell biosensor for *in-situ* application, even if the response is affected by the environmental variables. Furthermore, the light emission of *V. fischeri* was highly variable, although a more stable bioluminescence was obtained by means of a glucose fed-batch: this is one step towards the direct application of this system, usually tailored for laboratory assays, to estimate the broad acute toxicity directly *in situ* in a portable device.

Regarding the interaction between *P. fluorescens* and Fe^{3+}, Cu^{2+}, and Zn^{2+}, the minimum inhibitory concentration (MIC) and the pyoverdine critical concentration (PCC) obtained values were compared to those indicated in the WHO Guidelines for drinking water quality and in European directive 98/83/EC: MICs of Fe^{3+}, Cu^{2+} and Zn^{2+} are always above the threshold specified, whilst PCCs are very near to the recommended thresholds for iron and copper. The PCC was not determined for zinc in the tested range of concentration and conditions. These results highlighted that this sensible element should be further investigated for the development of a biosensor able to monitor metals in the environment.

The last and most promising sensing element assessed in this work was the lccβ laccase of *T. versicolor*. A combination of computational docking (SwissDock) and molecular biology techniques was used to generate rationally engineered laccases with increased ability to process large and persistent PAHs. These mutated isoforms were produced by heterologous expression in *P. pastoris*, successfully purified, and characterized by means of biochemical assays. The activity of the enzymes was initially tested and characterized with phenolic and non phenolic substrates at different pH (3.0-8.0): the best mutated enzyme F162A/L164A (M1) showed an increased specific activity (UI/mg) in

comparison with the wild type, in every tested condition. This result was in agreement with those obtained by computational docking simulations (estimated free binding energy), validating the rational design approach.

Moreover, decolourization assays of large aromatic dyes, used as model compounds, have shown that the mutated enzymes are reactive towards molecules with chemical structure resembling that of aromatic organic pollutants. By means of example, enzyme mutants with a larger binding pocket (e.g. M1) showed higher activity against triphenylmethane dyes (e.g. Methyl Green), especially without a mediator of the reaction (HBT), and high stability under a variety of temperature conditions (4, 22 °C, room temperature). Therefore, the best enzyme should be integrated on an appropriate transducer (e.g. electrode), and coupled to a wireless platform generating a BEWS for environmental monitoring.

1 – Chemicals in the environment

Chemicals are essential to our daily lives: everyone take advantage form the use of many synthesized compound at work or at home. Without any doubts, they clearly bring us important benefits, but many of them are hazardous, raising concerns for human health and the environment: the estimated annual production of hazardous chemicals in Europe, related to the year 2008, was about $2*10^8$ t [1].
The impact of hazardous substances on air, water and land is well documented by various sources, and detrimental effects have been observed at molecular, cellular, tissue, organ and ecosystem level [1]. Therefore, human health is continuously exposed to many compounds implicated in a considerable number of chronic diseases, including cancer as well as reproductive and developmental impairment. For these reasons, an accurate awareness of the presence, fate ad toxicity of the xenobiotics is mandatory: monitoring of contaminants is a step forward for the knowledge and management of risks posed by chemicals to human health and the environment [2].
It is widely known that xenobiotics could be dispersed into the environment form a broad range of land and marine sources during their life cycle, from production and use, as well as our own homes, to their eventual disposal. These industrial and household chemicals, including metals, pesticides and pharmaceuticals, could be released directly or indirectly to the environment through diffuse and point source pathways. Moreover, certain types of naturally occurring chemicals, such as metals, could be hazardous only in certain concentration or condition [3]. In some circumstances xenobiotics are toxic at very low concentrations. This happen with particular kind of compounds, such as antibiotics, hormones and pharmaceuticals, or pesticides and endocrine disrupting chemicals (EDCs), which are harmful albeit they usually occur only as trace amounts [4]. For instance, different EDCs have been shown to impair reproduction in fish and shellfish in different European locations at very low concentration, raising concerns for fertility and population survival [1].
The large number of modalities of pollutants dispersion entails a large number of possible interactions of these chemicals with man or the environment. Human exposure to toxic chemicals can occur via inhalation, ingestion and direct contact with skin, depending also on the physical-chemical properties of the pollutant. Furthermore, concerns about exposure to mixtures of several chemicals are growing, especially in the more polluted landscapes all around the World. For instance, aquatic life should be damaged by mixtures of substances even if these are present, individually, at concentrations below which any adverse effects can be expected. Laboratory studies have shown that this could be due to synergic effects of chemicals [1]. A new hope for the future is that problems rising from complex chemical mixtures in European water bodies seems to be effectively addressed by biological effects-directed measurements [1].
The environmental pollution is, then, a complex issue, and climate change is enhancing this complexity [1]. More intense rainfall and floods increase urban storm flows from the cities and the flushing to water of agrochemical pollutants, such as pesticides. At the same time, the draining of river flows due to hotter and drier summers concentrates the xenobiotics, leading to elevated concentrations of hazardous substances, especially in the sediment. Furthermore, industrial pollution and acid rain, as well as ocean acidification driven by increasing atmospheric CO_2 concentration, change speciation of metals, increasing their bioavailability and toxicity.

Environmental sustainability and governments' regulations

The descript scenario is forcing people and governments to promote a more sustainable consumption and production of chemicals. A lot of countries and international organizations have introduced regulations to control the use of chemicals [4]. Much of this legislation has been implemented in the last decade, including the Registration, Evaluation and Authorisation of

Chemicals (REACH, EC Regulation 1907/2006) and the Water Frame Directive (WFD, 2000/60/EC) while others, such as the well-known Integrated Pollution Prevention and Control Directive (IPPC, 2008/1/EC) are more established [1]. The limits are usually defined both in terms of annual average and maximum allowable concentrations: the former protect against long-term chronic pollution problems, while the latter against short-term acute pollution.

All these rules and guidelines are based on the principle that to realize an environmental-friendly economy, a mix of regulation, economic incentives and information-based instruments are strongly necessary. This kind of strategy wouldn't only benefit Europe's environment but also reduce the deleterious effects originating in other parts of the world as a result of the growing amount of goods imported to Europe. The economic point of view must be taken into account, but not forgetting that the economic system is only a part of our society, and men are only one over thousands of species present in the World. This was one of the guiding principles under the IPPC Directive that forces the use of 'Best Available Techniques' (BAT), designed to balance the cost of compliance to the operator against the benefits to the environment [5]. An extensive implementation of 'green chemistry' is required to achieve a more sustainable production of chemicals. This approach implies that new processes and technologies which maintain the quality of a product but reducing or eliminating the use or generation of hazardous substances must be continuously elaborated. This was one of the objectives of the REACH Regulation, which aims to protect the environment and the people, but maintaining the competitiveness and enhancing innovation within the chemicals industry, by introducing the concept of chemical substitution [5]. Chemicals evaluated as of most concern (e.g. category I and II carcinogens) are subjected to authorization, and, wherever possible, should be replaced with an alternative compound or with a process which poses a lower risk to the environment. Positives outcomes have been created by the implementation of these directives and laws related to environmental pollution, especially by a combined effort in regulation and monitoring. For example, thanks to the IPPC Directive, that have fixed abatement measures, a dramatic drop in metal emission to water and air has been recorded, whilst legislation relating to the production, use and disposal of polychlorinated biphenyls (PCBs) has resulted in declines in concentrations found in marine biota [1].

Other regulations, such as the WFD, are more focused on monitoring [6]. This directive, which introduces a new integrated approach to the protection of Europe's water resources, requires also that 'good chemical and ecological status' must be obtained for all surface waters, by establishing quality standards for chemical substances at European level. This 'good status' should be reached by 2015 [5]. The WFD assumes that the water body should not be polluted at all, because it represents a potential source of water for drinking: the discharge of any potential pollutant to groundwater is prohibited, and monitoring is essential to determine input of chemicals from indirect sources and take remedial action where necessary [5].

Main environmental pollutants

Chemical pollutants are classified as inorganic (e.g. metals, amines, thiols) or organic (e.g. alkanes, aromatic compounds) [7].

Inorganic contaminants are commonly present in the environment, as natural constituents of soils and rocks. These elements or compounds, such as heavy metals, metalloids, radionuclides, and oxyanions (e.g. nitrate) exhibit a broad spectrum of characteristics (e.g., oxidation state, speciation) that allow them to react with inorganic or organic geosorbent components, or both [8].

The alkanes are soluble in non-polar solvents and almost insoluble in water: all alkanes are less dense than water, therefore they will float and spread as thin films on water, and their boiling points and melting points increase as the number of carbon atoms rises [7]. Aromatic compounds are benzene

and compounds that resemble benzene: the fundamental feature required is the aromatic ring, in which all carbon atoms share equally the pool of electrons which constitute the double bonds. Benzene and alkylbenzenes possess low polarity with similar physical properties to hydrocarbons. Single or multiple hydrogen or even carbon atoms can be substituted in these compounds to form a host of derivatives (e.g. alkyl halides) containing various functional groups, e.g. saturated and unsaturated aliphatic chains, amino, carboxylic acidic, halogens, nitro, and sulphonic acid groups [7]. These substitutions generally vary the physical-chemical properties of the parental molecule, leading, for instance, to higher boiling points because of the increased molecular weight. Furthermore, these substitutions significantly alter the toxicity: for example, substitution involving a chlorine atom in an aromatic hydrocarbon leads to an increase in the potential carcinogen effects, while reducing its chemical or biological oxidation, and hence, increasing its persistence in the environment [7].
A particular class of aromatic compounds which finds an extensive industrial application is that of polycyclic aromatic compounds (PAHs). PAHs refer to a wide and assorted group of a few hundreds of molecules with two or more fused benzene rings, generally lipid-soluble and persistent [10]. The chemical structures of the 15 PAHs enclosed in the EPA priority pollutant list are illustrated in Figure 1.1. It has been estimated that $2.3*10^5$ t of PAHs enter the global environment annually: they can be derived from endogenous and anthropogenic sources, but mostly from incomplete combustion of organic matter [11]. Some of them are considered fairly innocuous from an environmental and healthy point of view, but the large majority of them are known to cause cancers and genetic damages [10].

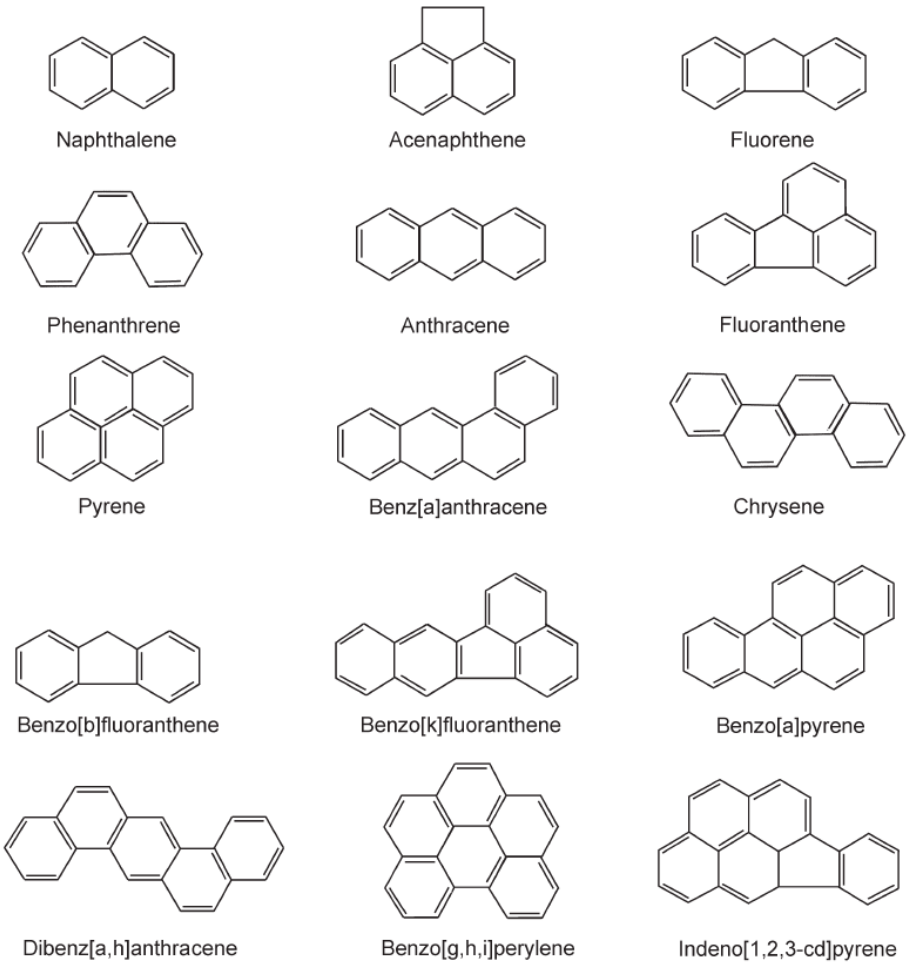

Figure 1.1: Structures of the 15 PAHs enclosed in the EPA priority pollutant list.

Many PAHs are included in the group of Persistent Organic Pollutants (POPs): they are semi-volatile, low-water-soluble hydrocarbon chemicals that hardly break down under environmental conditions, and exhibit harmful effects in man and the environment [12]. These compounds are typically lipophilic, and then, they accumulate in the food-chain: high concentrations have been detected in animals and humans [12]. Formally, these compounds refers to twenty-one (originally twelve) chemicals addressed in the Stockholm Convention on POPs, a global treaty negotiated by the United Nations Environment Programme (UNEP) in order to eliminate the production and use of these chemicals [13]. Some POPs, e.g. pesticides (aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin), polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs), have been deliberately produced by the industry for a lot of applications. Some of them, such as brominated flame retardants (BFRs) (e.g. polybrominated biphenyls (PBBs)), are still produced in large quantities for use in electric devices, plastics, and building materials. Others are accidentally formed and eventually released as a by-product from industrial processes (e.g. polychlorinated-dibenzo-p-dioxins (PCDDs), polychlorinated-dibenzofurans (PCDFs)) [10].

The toxicity of POPs is mainly based on the interaction of these molecules with the aryl hydrocarbon receptor (AhR). This receptor is a ligand-activated nuclear transcription factor that mediates responses to toxic halogenated aromatic molecules such as PCDDs and PCDFs, but also to PAHs, combustion products, and numerous phytochemicals such as flavonoids and indole-3-carbinol (I3C) [14]. All these compounds able to bind the AhR are called dioxin-like compounds (DLCs) because their biochemical behaviour and effect is very similar to that of dioxins and furans. They are: coplanar PCBs (which can assume a planar orientation), PXDDs/PXDFs (X=chlorine, bromine, or fluorine), alkyl-substituted R-PCDDs/PCDFs, PCNs, polychlorinated dibenzothiophenes (PCDTs), and polychlorinated thianthrens (PCTAs), and some PAHs, such as hexachlorobenzenes (HCBs) [10], as reported in Figure 1.2. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has become the prototype agonist for the study of AhR biology, because of its potency and environmental persistence [15]. As a result, the toxicity of a sample is generally measured as toxic equivalency factor (TEF): a number expressing its toxic potency relative to TCDD toxicity is given to each analyzed compound [10].

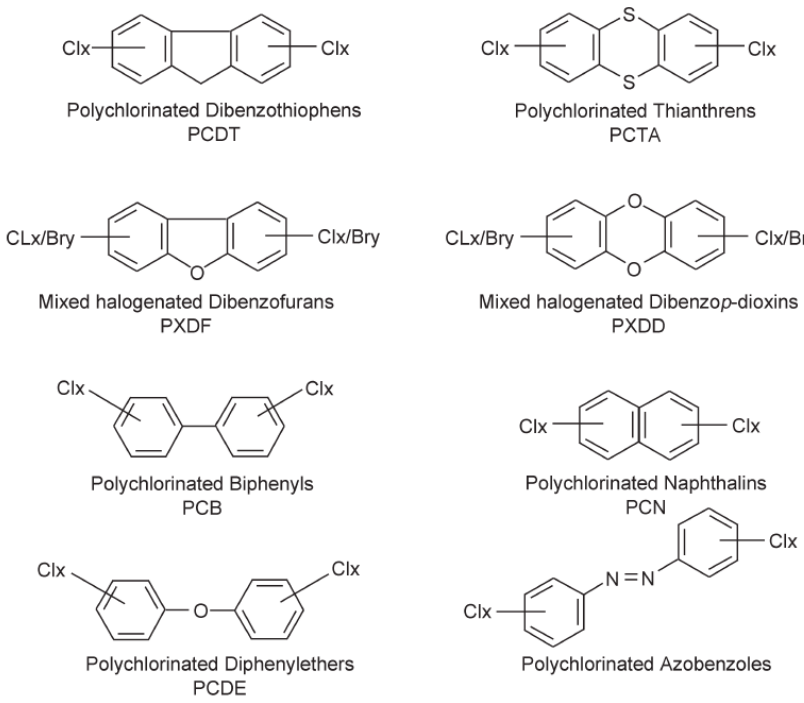

Figure 1.2: Structures of POPs.

In the list of known DLCs, various emerging contaminants are reported (e.g. BFRs). Another class of emerging environmental pollutants is that of endocrine disrupting chemicals (EDCs). An endocrine-disrupting compound was defined by the U.S. Environmental Protection Agency (EPA) as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" [16]. At the beginning, development of endocrine disruption research was focused almost exclusively on so-called oestrogen mimics, chemicals acting as the natural oestrogens (e.g. 17β-estradiol). Since oestrogens act by binding to specific receptors in target tissues (e.g. estrogen receptor (ER)), the ability of a molecule to bind to the same receptors was taken as sufficient evidence to define it as an oestrogen mimic [17]. But EDCs often act via more than one mechanism [18]. TCDD, for example, reduces the ability of the oestrogen-ER complex to bind to the DNA oestrogen responsive element, reducing gene transcription [19], whereas tributyltin inhibits the conversion of androgens to oestrogens (in neogastropods) by inhibiting aromatase or by inhibiting testosterone metabolism and excretion [20]. The set of molecules recognized as endocrine disruptors is extremely heterogeneous and includes synthetic chemicals used as industrial solvents/lubricants and their by-products (e.g. PCBs, BFRs, dioxins), plastics (e.g. phthalates), plasticizers (bisphenol A (BPA)), pesticides (e.g. DDT), and pharmaceutical agents (diethylstilbestrol (DES)) [18]. Many of them are POPs, but others, such as BPA, may not be as persistent but are so widespread in their use that there is prevalent human exposure [18]. At the same, also natural chemicals found in human and animal food (e.g., phytoestrogens, including genistein and coumestrol) can also act as endocrine disruptors [18], and, although they have relatively low binding affinity to ER, are widely consumed. The urinary concentrations of the phytoestrogens genistein and daidzein were about 500-fold higher in infants fed soy milk compared with those fed cow's milk formula [21]. Therefore, the potential for endocrine disruption of a large number of chemicals needs to be considered: even heavy metals and metalloids may have estrogenic activity, suggesting that these compounds are EDCs [18]. As far as it is concerned, all EDCs have been associated to different increasing disease and pathologies: breast cancer [22], testicular cancer [23], sperm impaired quality and count fall [24].

Even if the known xenobiotics are already a great number, the list of emerging pollutants is still expanding. In the last few years a 'new-generation' of pesticides with a wide range of polar structures has been recognized [25].

Different xenobiotics, different fate: source and dispersion of main pollutants

Pollutants are regularly classified into groups according to their chemical structure but can also be divided into groups according to their toxicity, such as cytotoxicity, carcinogenicity, mutagenicity, genotoxicity, or endocrine disruption [5]. Both classifications are built around the assumption that the potential harm of chemicals depends on physical-chemical and toxic properties. To be honest, toxicity is correlated to the amount of xenobiotics that enters the environment, to the emission source, to its distribution between different environmental compartments (air, water and soil). All these variables connected to the source and fate of pollutants, are strictly related to their physical-chemical properties [4]. Hence, by knowing the physical-chemical properties of pollutants it is possible to give an inkling of their fate and toxicity in the environment [7]. By means of example, the physical-chemical properties of POPs are the basis of long-range transport, bioaccumulation and toxicity of the substances: e.g. progressive chlorination decreases water solubility, whilst increases toxicity and bioaccumulation [12].

The main physicochemical principles that influence the fate of pollutants into the environment are: vapour pressure, solubility and miscibility, liquid-vapour and solid-liquid phase changes, density differences of gases, vapours, and liquids, effects of particle size [7].

Hazardous substances are emitted into the environment from a various and broad range of sources that are usually divided in diffuse and point sources [1]. Usually point sources, such as wastewater treatment plants, or industrial smokestack are well-documented, whereas, diffuse sources, such as vehicle emissions or surface run-off and leaching from agricultural soils, are more spread over the landscape. Combustion, and especially, fossil fuel combustion, is an important source of xenobiotics. Fuels are hydrocarbons and their products of combustion are complex and depend upon the nature of the fuel, the amount of oxygen present, and the temperature. Furthermore, if nitrogen or sulphur are present combustion products may include oxides (NO_X and SO_X), whereas, in anoxic conditions incomplete oxidation occurs to produce partially oxidized carbon compounds that could be more toxic and persistent than the parental compounds [7].

Many industrial and chemical facilities have been historically located adjacent to rivers and water courses, due to the ease of extraction of water for industrial processes, or simply to discharge waste effluent to the receiving water, resulting often in extensive damage to the water quality and to aquatic organisms [1]. In some instances, effluents pass through a sewage treatment plants before entry into water: this can potentially cause harm to the treatment plant itself, or to the receiving water body, for inadequate degradation of chemicals [5]. Discharges of municipal and industrial wastewater are a major route of EDCs entering the aquatic environment [1]. Furthermore, all the chemicals that pass through sewage treatment plants could be a source of soil pollution. At present, about 40 % of the sewage sludge generated in Europe is used as fertilizer in agriculture, which is considered to be one of the most acceptable and encouraged disposal options [26]. Unfortunately, the range of organic xenobiotics occurring in sewage sludge is extremely wide, and largely corresponds to EDCs and PAHs [26].

Other kinds of sources of pollutants are agriculture and land usage (e.g. mine workings). There is evidence that agricultural sources can make a significant contribution to freshwater metals loads [27, 28]. Metal emissions from agriculture include zinc, an essential trace element added to animal feed, and cadmium, found naturally in the phosphate fertiliser [1]. A significant proportion of zinc is not absorbed by livestock, and is excreted in manure that may then be flushed into rural streams. The same phenomenon was recorded for copper found in feedstuffs and hence in manure [1].

All these considerations imply that the toxicant could be released directly or indirectly into one of the three environmental compartments, air, soil, and water [7]. These three compartments are each other strictly interconnected as a consequence of many different physical-chemical phenomena, for instance wet and dry deposition, vaporization, adsorption or solubilisation. By means of example, a particular hazardous compound, such as an unburned hydrocarbon, could be released to air from the smokestack of an industrial facility, adsorbed on a fine carbonaceous particle. These kind of contaminants (the partially combusted hydrocarbon, or the particulate), called ‘primary pollutant’ may be involved in reactions in the atmosphere producing ‘secondary pollutants’ [7]. In the air, the compound could be modified by photochemical reactions giving rise to more or less toxic molecule, and subsequently, the particle or the compound alone, could be deposited to soil or water. Furthermore, the pollutants bioavailability, defined as the amount of contaminant present that can be readily taken up by microorganisms and degraded [9], depends on many environmental factors, such as pH, temperature, or adsorption [8]. There are six hypothesized mechanisms able to reduce the availability of xenobiotics for biotic uptake and metabolism in soils, sediments, and waters [8]. These are: insolubility of the contaminants, sorption reactions to solid phase, partitioning reactions

that place contaminants in non-aqueous phase liquids (NAPLs), or in natural organic matter, spatial separation in micro/nano-pores, and complexation to humic substances.
Some kind of pollutants are more mobile than others, and, as example, deposition may occur close to the point of emission, or very far [1]. For instance, mercury, a typically toxicant present in the smoke of thermal plants fed with charcoal, is subjected to long-range trans-boundary atmospheric transport, and hence its harmful effects can affect aquatic environments in remote regions of the World, such as the Arctic [1]. The world-wide impact of this kind of xenobiotic, known since a connection was drawn between emissions in Europe and effects on Scandinavian lakes [12], requires regulations at the regional and the global scale [1]. Although this kind of purpose was one of the main aims of the Stockholm Convention on POPs, some POPs are still used in some countries. DDT, widely used during World War II to protect people from disease spread by insects, continues to be applied to control to control malaria in several countries. This pesticide has a great stability and persistence (as much as 50 % can remain in the soil 10-15 years after application), and, due to its long-range atmospheric transport, its residues can be found everywhere [13].
These considerations are the result of an extreme complexity and heterogeneity of air, water and soil, which are impossible to fully describe or quantify by simple chemical or physical models.

Relevant pollutants of air, water and soil

Pollutants could be dispersed through many sources, to the environment: the major air, water and soil contaminants are listed in Table 1.1 [1, 5, 7].

Table 1.1: Air, water and soil pollutants.

Contaminant	Anthropogenic source	Effects
Air pollutants		
SO_X	Combustion fossil fuels, thermal power plants, metal smelting, petroleum refining, paper-making	Vegetation damage, acid rain, corrosion, discoloration of buildings, irritant
Hydrogen sulphide	Various chemical processes, oil wells and refineries, sewage treatment	Crop damage/reduced yields, odours, toxic, acid rain
Carbon monoxide, carbon dioxide, NO_X	Atmospheric reaction between nitrogen and oxygen, combustion processes, by-product from manufacture of fertilizer and concrete	'Greenhouse' effects, reduced visibility, adverse health effects, sensory irritants, crop damage
Ammonia	Waste treatment	Odour, irritant
Fluorides	aluminium smelting, production of ceramics and fertilizer	Crop damage, adverse health effects
Lead	Lead smelting, combustion of leaded petrol, solder, lead-containing paint	Adverse health and environmental effects (e.g. bioaccumulation)
Mercury	Manufacture of chemicals, paper, paintings, and pesticides	Adverse health and environmental effects (e.g. bioaccumulation)
Volatile hydrocarbons	Chemical industry, solvent processes and motor vehicles	Vegetation damage, adverse health effects
Particulates	Chemical processes, thermal power plants, combustions of fossil fuels and wastes, buildings	Deposition on buildings, reduced visibility, adverse health effects
Water pollutants		
Alkali solutions	Chemical industry, by-product from manufacture of fertilizer, waste treatment, paper-making	Odour, irritant, adverse health and environmental effects, crop damage/reduced yields

Biocides (mainly pesticides)	by-product from manufacture of agrochemicals, agriculture	Vegetation damage, adverse health and environmental effects
Biodegradable waste	Food industry, agriculture and livestock, sewage treatment plants	Eutrophication and dissolved oxygen depletion
Chloride and Chlorine	Chemical industry, paper-making, by-product from manufacture of agrochemicals and pesticides	Vegetation damage, sensory and respiratory irritation, corrosion, adverse health and environmental effects, crop damage/reduced yields
Cyanide	Chemical industry, by-product from manufacture of agrochemicals and pesticides	Adverse health and environmental effects (e.g. carcinogenic nitrosamines)
Fluoride and fluorine	aluminium smelting, production of ceramics and fertilizer, electronic components	Crop damage, adverse health and environmental effects
Metals	Metals smelting and production of fertilizer, chemical industry, electronic components,	Adverse health and environmental effects (e.g. bioaccumulation or pH changes)
NO_X	Agriculture and livestock, sewage treatment plants, chemical industry, solubilisation from air	Eutrophication and dissolved oxygen depletion, adverse health and environmental effects (e.g. carcinogenic nitrosamines)
Oil, grease, wax, immiscible organics (PAHs)	Chemical industry, oil spillage transport and refineries, combustions, agriculture, paints, adhesives, solvents and synthetic materials	Adverse health and environmental effects (e.g. bioaccumulation)
PCBs, PCDDs, PCDFs, chlorinated PAHs, phenols and related compounds	Transformers and electronic components, fluorescent lights, metals smelting, production of fertilizer and pesticides, chemical industry, paints, car manufacture	Adverse health and environmental effects (e.g. bioaccumulation)
Pharmaceuticals and EDC	Agriculture and livestock, sewage treatment plants, chemical industry, plastics and plasticizers	Adverse environmental and health effects (e.g. imposex in amphibians and fishes)
Phosphate	Detergents, chemical industry, fertilizer, sewage treatment plants	Eutrophication and dissolved oxygen depletion
SO_X	solubilisation from air, metals smelting and production of fertilizer, chemical industry, thermal plants, paper-making, petroleum refining	Vegetation damage, acid rain, corrosion, discoloration of buildings, irritant
Suspended particles	Deposition from air, metals smelting, chemical industry	Adverse environmental and health effects
Soil pollutants		
All the pollutants reported above, unless the most volatile ones		

The most important source of air pollutants is related to combustion processes and particulate (e.g. aerosols, dust, fumes) [7, 29]. The fate of compounds have been previously reported above, whereas, the behaviour and toxicity of an airborne particle depend upon its size, shape and density. For instance, particles in the respirable size range (about 0.5–7 μm), once dispersed, remain in air for extended periods [7].

The chemical quality of Europe's surface waters is primarily addressed by the Environmental Quality Standards Directive (2008/105/EC), a 'WFD daughter', which defines concentration limits for 33 pollutants of EU-wide relevance, known as 'priority substances'. These are included in Table 1.1.

Mostly, water contaminants are related to wastewater treatment and agriculture. Pesticides, used in agriculture, are widely detected in freshwater: they are often transported by diffuse pathways via leaching and surface run-off [1], or by point sources of contamination, such as disposal sites and landfills where industrial or agricultural wastes are illegally buried [25]. Landfill sites can also be important sources of pollution to the aquatic environment (e.g. through percolate) [1].

Although household and industrial wastewater plants has been progressively improved, the process does not remove all hazardous substances (e.g. pharmaceuticals and EDCs), being detected in treated effluent that is subsequently discharged to surface waters [1].

The toxicity in water is very changeable, and depends on many factors. By mean of example, most of these hazardous substances are hydrophobic and tend to accumulate in sediment and biota, with the result that their concentration in these matrices is high. The toxicity to benthic biota of metals and pesticides accumulated in sediments is well documented [1]. During periods of higher river flow, or through dredging, however, bed sediments and their associated pollutants can be resuspended into the water column and transported downstream, causing harmful effects.

All the pollutants reported in Table 1.1 are representative of soil pollution, and the sources of these xenobiotics are various. Contaminants can be deposited from airborne pollutants, or simply originated by leakage from landfill sites. It is known that these airborne contaminants produced by industrial activity significantly contribute to the amount of aromatic compound in soils [30, 31]. The main sources of these aromatic compounds, mostly adsorbed on particles, are thermal power plants [29].

2 – Environmental monitoring

In the previous chapter (Chapter 1) an overview of environmental pollution is reported.
The descript scenario is quite concerning, especially for human health. In the Hazardous Chemicals Handbook [7] the toxicity of a substance is defined as its capacity to cause injury once inside the body. The main modes of entry into the body by chemicals are inhalation, ingestion and absorption through the skin, and may involve solids, liquids, or airborne matter contaminants, in every combination. These considerations make hard to quantify exposure, and consequently toxicity. Among different approaches, environmental biomonitoring have been successfully used to assess, prevent and reduce human exposure to xenobiotics. These methods are summarized in this Chapter.

Traditional chemical analyses

In order to improve water quality and people health, many countries in the World increased the policies related to environmental protection. These regulations, such as the WFD and the IPPC, are focused on monitoring. For example, a group of experts, the Analysis and Monitoring of Priority Substances (AMPS) have been created to evaluate existing analytical methodologies for their use in the implementation of the WFD [2].
Traditionally, the impact of pollutants released into the environment by human activities has been assessed evaluating physical parameters, or using chemical analyses, mainly based on High performance liquid chromatography (HPLC) or on Gas chromatography mass spectrometry (GCMS) [32]. For instance, physical-chemical parameters are used to assess the overall water status. Other investigated parameters are microbiological indicators (e.g., cyanobacteria, pathogens, and parasites), organic and inorganic chemicals. These indicators are usually classified into two groups: those which have a direct toxic effects on the biota (e.g. heavy metals, salinity, pesticides, and temperature), and those which affect ecosystems indirectly (e.g. nutrients or turbidity). Others, such as low pH can adversely affect aquatic biota directly and also can result in release of heavy metals from sediments [33].
To date, environmental monitoring is mainly based on '*spot-sampling*': some of the physicochemical measurements (e.g. pH) can be made *in situ* when collecting samples for other analyses, while other measurements are carried out by laboratory techniques. Metals and organic pollutants (e.g. pesticides) are the major contaminants of water courses. Metals are usually detected by spectrophotometry (UV-vis or atomic absorption), polarography, or anodic stripping voltametry [33]. Instead, organic pollutants are typically quantified by GCMS or HPLC–MS [25].
Many methods are regularly used for air monitoring. Generally, the pollutants are trapped on membrane of active or passive samplers and subsequently analyzed by chromatography (mainly GCMS). The determination of time-weighted average (TWA) concentrations, requested by different regulations (e.g. 2008/50/CE), is usually achieved by means of passive sampling: a reference (or receiving) phase is exposed to the water/air phase, extracting by absorption/adsorption the pollutants [34]. Subsequently, by knowing the rate of mass transfer to the reference phase, the TWA concentration of a pollutant in the water/air phase can be calculated. Some portable devises based on colour indicator tubes have been developed by Drager, especially to detect gas and volatile compounds: tubes are available to detect over 300 substances [7]. The glass tube containing crystals (e.g. silica gel or alumina) impregnated with a reagent which undergoes a colour change by reacting with a specific pollutant or class of pollutant: the tube is generally calibrated and then the length of stain that develops is proportional to the concentration of contaminant.

Soil samples are analyzed in a similar way to the other environmental samples, but following some pre-treatment steps (e.g. solid-phase micro-extraction) [35]. These pre-treatment steps are required because pollutants are adsorbed on clay, sand and organic matter that compose soils.

A lot of studies have revealed several limitations of traditional methods used for environmental monitoring: noteworthy among these, the inability of a detailed evaluation over time, limited by the few samples, high costs, long analysis times, and the requirement of an experienced operator [6]. It is not practical or affordable to sample and analyse at sufficient spatial and temporal resolution for hundreds of individual chemicals within environmental samples, and by taking into account only a few pre-selected priority substances, other relevant pollutants may be omitted [5]. Furthermore, environmental samples are usually characterized by a mixture of components, and traditional analytical methods may be inadequate to get information related to toxicity: conventional techniques are not able to determine the bioavailable fraction of pollutants, the one that is really able to give toxic effects [32].

In the very last years, biosensors and biological assays are more and more applied in environmental monitoring thanks to their ability to get over these limitations. These biological tools play an important role in the detection and screening of the toxic effects of a substance or a mixture of substances by quantifying that effect on living organisms or on their biochemical components [36]. Moreover, biomonitoring is able to estimate the amount of bioavailable fraction of a pollutant, evaluating overall effects (e.g. synergic or antagonistic effects) of chemicals [25]. In fact, this alternative approach is also used to assess the whole toxicity of an effluent, by the so called Direct Toxicity Assessment (DTA), that establish if the effluent can be released in a water course [5]. This kind of analysis is suited to establish cause/effect relationships between concentration of available pollutants and consequent environmental damages, and is very important in hazard and risk assessment, especially at the screening level [32]. These cause/effect relations are only supposed on the basis of results obtained by traditional analytical methods.

Hence, the main aim of biological analysis applied to environmental monitoring is to measure global biological effects, such as toxicity or genotoxicity, to evaluate global parameters, such as biological oxygen demand (BOD), or to determine the amount of a specific chemical in the ecosystems. All these assays and techniques are based on ecotoxicology.

Ecotoxicological methods and biomarkers

The main goal of ecotoxicology is to understand how chemicals cause detrimental effects in some organisms, which species will be affected, and what effects this will have at the population and ecosystem levels [36]. Ecotoxicology makes use of biological based assays: these are built on the evaluation of the status of living organisms or microorganisms posed in close contact with xenobiotics for a certain time [36]. Usually, data from multispecies toxicity tests are used, because these would better represent the complex interactions that occur *in situ* [33]. Moreover, organisms at the same or at different trophic level respond differently to a range of toxicants and it is important to develop multiple toxicity bioassays using various organisms [32].

Acute toxicity was the first biological tool applied in the environmental field. It uses assays based on mammals, birds, or fish as test species [37]. Unfortunately, these methods are quite expensive, time-consuming, and show a lot of standardization problems. Due to these reasons, new biological methods which use invertebrates, plants, algae or bacteria as test species were developed: a large number are based on microorganisms. Mainly, these assays rely on mortality and citotoxicity induced by the toxic chemicals [6]. A widely accepted microbial toxicity bioassay is based on the measurement of the decrease in light emission by the wild-type luminescent bacterium *Vibrio fischeri*, the most sensitive across a wide range of chemicals, following a short-term exposure to the

sample [38]. These methods give a rapid response, and in many cases allow processing a lot of samples at the same time and at a relatively low-cost. Some of these methods are still under development, but they provide a good alternative to the classical ones, especially at the screening level [36]. By means of example, algae are particularly susceptible to herbicides, and then, by measuring their growth inhibition, or chlorophyll fluorescence changes, the presence of these pollutants in water samples is easily detected [6, 39].

Ecotoxicology is also based on biomarkers. A biomarker is defined as a variation of a biological response at molecular or cellular level, as well as at physiological level, which can be related to exposure to toxic environmental pollutants [40]. According to the World Health Organisation, they can be classified into three categories: exposure, effect and susceptibility.

Molecular biomarkers of exposure cover the detection of a xenobiotic by measuring target molecules involved in cell protection against potential toxic damage, and are mainly composed of proteins: heat shock proteins (HSP), enzymes capable of metabolising xenobiotics (e.g. cytochrome P450), membrane transporters [6]. As an example, the P450 family of enzymes indicates contamination with polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and pesticides [41, 42], while metallothionein are usually involved in essential heavy metal homeostasis [43].

Biomarkers of effect include measurable alterations (e.g. biochemical or physiological) within tissues or body fluids of an organism that are known to be associated with an impairment due to pollution. This kind of biomarker is very often correlated to concentrations or exposure time to a cytotoxic compound and some examples correspond to peroxidation of lipids, intracellular redox state, integrity of DNA, or volume, size and number of lysosomes present in the cell [6]. Some of them are very compound-specific: a decreased activity of antioxidant enzymes (e.g. superoxide dismutase) can be an index of the effect of PAHs, PCBs, and organochlorine pesticides on aquatic organisms [44, 45, 46], while the induction of vitellogenin in male species of test organisms indicate the presence of EDCs in water [47, 48, 49].

Biomarkers of susceptibility correspond to the inherent or acquired ability of an organism to respond to a specific xenobiotic compound by changing its susceptibility to the exposure. For example, one of the first environmentally relevant genes discovered as biomarkers of susceptibility was the paraoxonase gene (PON1), a liver and plasma enzyme involved in lipids oxidation and related to organophosphates exposure [50].

To properly use molecular biomarkers as a measure of toxicity, an understanding of signal transduction and protection mechanisms to pollutants is mandatory. The main advantage obtained from their use is that they act as early alert signals, since they are sensitive to concentrations below those causing cytotoxicity: toxic compounds have an impact at molecular and subcellular levels before their effects are observed at the whole-organism level [6].

Biosensors

Ecotoxicological methods and biomarkers opened new prospective for biomonitoring, creating the starting point for the development of many other biological based techniques and devices, such as biosensors. Some of them were directly derived from specific biomarkers: for instance the inhibition of acetylcholinesterase (AChE), an over-exploited biomarker [51], or the AhR, an index of PAHs exposure, were used to develop different biosensors.

The International Union of Pure and Applied Chemistry (IUPAC) defined a biosensor as ‘a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element, which is retained in direct spatial contact with a transduction element’ [52]. A general scheme of a biosensor is reported in Figure 2.1.

The main advantage of using biological molecules to develop sensors is that these are extremely highly specific [53]. At the same time, global parameters, such as bioavailability, toxicity and genotoxicity cannot be probed with molecular recognition or chemical analysis. In these situations, whole cells could be a better choice [53]. Other advantages offered by biosensors over conventional analytical techniques are the ease of portability, miniaturisation and working on-site, as well as the ability to measure pollutants in complex matrices with minimal sample preparation [2, 36]. Although many of the systems developed cannot compete with conventional analytical methods in terms of accuracy and reproducibility, they can be used for routine testing and screening of samples [54]: they are low cost and short times analysis.

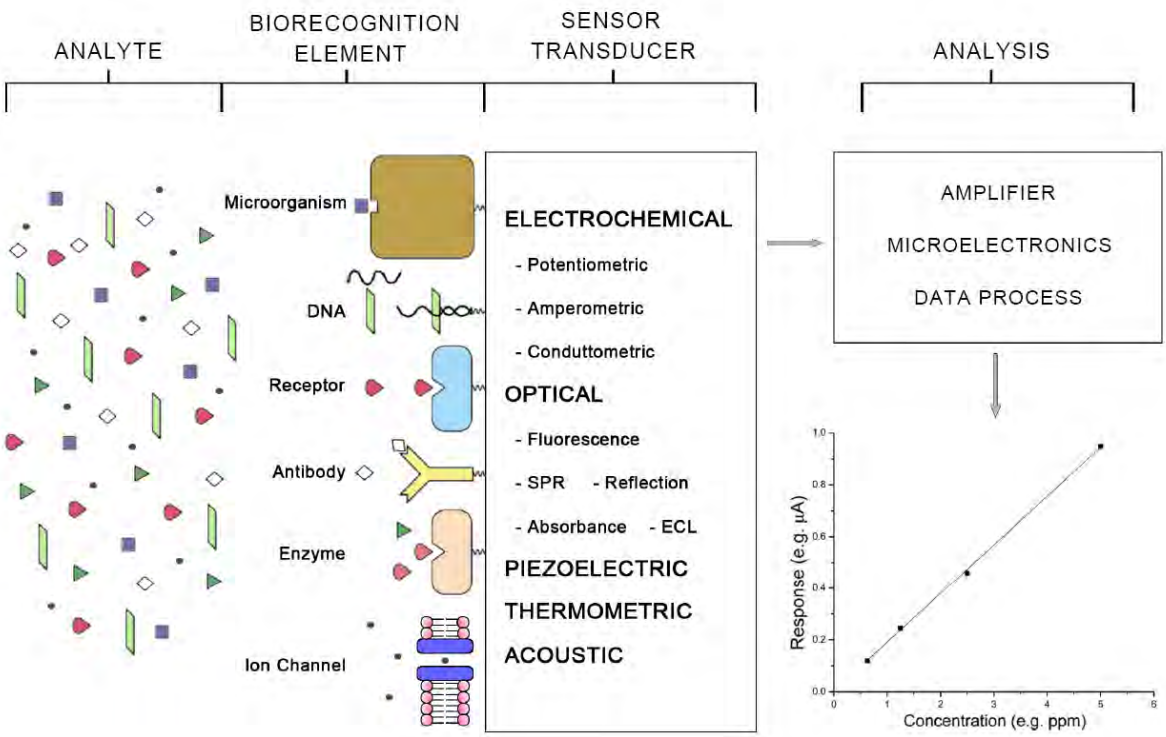

Figure 2.1: General scheme of a biosensor.

The immobilization of the biorecognition element is one of the most important steps, after its choice. Normally, physical adsorption, chemical cross-linking, and entrapment by means of membrane or polymer are the largest used methods of immobilization [55]. Advantages and drawbacks have been extensively reported [56].

Many biosensors have been developed to meet the demands of the biomedical market (97 % of the global market) [57]. Some other relevant application fields are the food industry and environmental monitoring. Surface, drinking and groundwater have been the foremost targets of environmental biosensors: little or no required preparation of water samples is one of the main reasons. Air samples can be analysed directly with biosensors although a very few devices have been developed and applied. Soils and sediment samples require an extraction step (e.g. solid-phase microextraction) to draw out contaminants into solution [2].

Some of these developed bioassays and biosensors have been effectively commercialized. Surface plasmon resonance (SPR) biosensors (e.g. based on BIACORE AB technology), constitute the most successful type of commercial instruments for environmental monitoring [58]. However, despite the great number of recently developed biosensors, most of the literature papers overlook the real-world application and many reports are related to tests in laboratory conditions [58]. Furthermore, a

lot of relevant environmental pollutants (e.g. EDCs and pharmaceuticals) are still not detected by portable biosensors.
Biosensors are usually classified according to the transduction element or to the sensing element. Many different transduction elements have been applied: electrochemical, useful to measure many different enzymatic reactions, optical, based on emissions/absorption of light, piezoelectric, especially if a receptor is involved, and finally thermometric and acoustic. On the basis of the biorecognition element is possible to sort whole cell, enzyme, antibodies, and receptor biosensors.

Whole cell biosensors

Whole cell biosensors, as highlighted by their name, make use of complete cell systems: microorganisms such as bacteria, algae, and yeast, or more complex eukaryotic cells [2]. The cells used as sensing elements can potentially be interfaced with a wide range of transducers, including optical, electrochemical, and piezoelectric ones [59], and are able to respond to the presence of various organic compounds, metals, radiation or pH changes, in all fields of environmental monitoring (soil, water and air) [60].
One of the most important advantages in using whole-cell systems, compared to traditional analytical methods, is to get an indication of broad toxicity of samples [2, 6, 61]. It may be, for example, the case of metal ions: only the fraction that is not complexed to chelating agents or adsorbed on organic matter is really bioavailable and toxic. This broad response of whole cell biosensors implies that the specificity of these devises is lower than the one obtained by the other classes of biosensors [56]. At the same time, these systems, suitable in any case the source and nature of the pollutants cannot be predicted, can work as a biological early warning system or BEWS, which will subsequently supported by more specific actions and analysis [60]. For *in-situ* analysis, it is recommended to use organisms or microorganisms as compatible as possible with the field and the matrix chosen; for this reason, if it's required to investigate the health of a watercourse, it must be essential to rely on an aquatic organism, usually native.
Microorganisms represent a good opportunity for low cost, long life use, wide range of pH and temperature in which they can be applied, in addition to the ability to detect a broad spectrum of chemicals, due also to the involvement of genetic engineering techniques [62]. Many devices, built around a specific cellular response that varies in the presence of the analyte, have been developed by using wild type or recombinant organisms, genetically modified by fusion of a regulatory element (promoter) with a easy detectable responsive element (reporter gene) [59, 60]. These systems are divided in constitutive and inducible [32]. The former are based on measurable response (e.g. the expression of a protein, or bioluminescence) that is regularly present (constitutive) and linked to the metabolic status of cell: if a stress (e. g. a pollutant) interferes with this mechanism, the measurable response considerably varies, giving a signal proportional to the toxicity of a sample. Instead, inducible systems are under the control of a regulatory element that act as a button that switch on the measurable response only if a particular stress or toxic molecule is present. Hence, constitutive systems, usually wild type, give a broad response to xenobiotcs, while inducible systems are more specific for a compound or a class of compounds [60]. Bioluminescence, the emission of light activated by the enzyme luciferase, and fluorescence, the emission of light operated by fluorescent proteins (e.g. green fluorescent protein or GFP) are by far the most used reporters for both constitutive and inducible devices [56].
Wild type bioluminescent bacteria (e.g. *Vibrio fischeri*) are typically applied for ecotoxicological assays, as reported above. Besides, many examples of bacteria engineered with luciferase genes to detect organic compounds (e.g. alkanes and aromatic hydrocarbons), heavy metals or antibiotics are reported [2, 59, 63, 64]. All these biosensors are optical biosensors, however other cleaver but less

exploited methods have been invented: as example, electrochemical biosensors for metals detection built around the response of the β-galactosidase gene (lacZ) have been reported [65, 66]. These systems are generally associated to amperometric or potentiometric transducers based on an ions selective electrode that measure the consumption/generation of ions or gas [56].
Particular kind of whole cell biosensors are those used for biological oxygen demand (BOD) assessment. They measure the oxygen consumption, or the current generated, by pure or mixed microbial cultures fed with sample containing organic compounds. This analysis is very useful for the evaluation of wastewater and effluents [24].
The main limitations of whole cell biosensors are the response time, that could range between minutes (e.g. bioluminescence inhibition) and hours (e.g. BOD biosensors), and the survival of the cell in an active and responsive status [59].

Enzyme biosensors

Enzymes were the first molecular recognition elements used in a biosensor: based on glucose oxidase (GO), this biosensor was built in 1962 [67], and is still the widely used and the economic drive of this sector.
Biosensors that use an enzyme as a sensing element can be interfaced with different transducers, but mostly they are amperometric: the current variations due to enzymatic activity are proportional to the concentration of the analyte [68]. These devices can belong to three different classes. In the first generation of biosensors, reagents or reaction products diffuse to the transducer generating the signal (e.g. the Clark electrode). In the second generation, mediators are used as electronic shuttle between the enzymatic reaction and the transducer to increase sensitivity. Finally, in the third generation, the direct electron transfer (DET) between the active site of the enzyme and the transducer is reached [69].
Enzyme biosensors have several advantages, including the possibility of a change of catalytic properties (e.g. substrate specificity), through protein engineering, easy miniaturization, excellent detection limits, also with small analyte volumes, and ability to be used in turbid fluids [70, 71]. Some of them are also easy available in nature, and well-adapted to environmental conditions (e.g. extracellular fungal enzymes). Moreover, they are quite economic, due to their close link to developments in low-cost production of microelectronic circuits and their easy interface with normal electronic read-out and processing [68]. At the same time, limitations include the lack of selectivity between similar molecules, the inhibition carried out by different compounds (e.g. heavy metals) that contaminate the environment [59], as well as the need of cofactors/coenzymes or secondary systems for generating a detectable signal [56]. The enzyme's inhibition was largely applied to measure the presence of organophosphorus and carbamate pesticides by means of acetylcholinesterase (AChE) and choline oxidase (ChO): despite high sensitivity they aren't selective [2], and the inhibition is not reversible, then the biosensors is not easily reusable.

Immunosensors

Biosensors based on antibodies (immunosensors) exploit the specificity of these molecules to bind exclusively to individual compounds or groups of structurally related compounds. This type of biosensors can contribute to environmental monitoring for high sensitivity and affinity (often the sample does not require a pre-concentration) and fast response [6]. The high affinity for target analytes of antibodies as biorecognition elements in many applications lead to very low detection limits, but also to irreversible binding of the recognized antigen. The regeneration of the surface of the biosensor (e.g. with pepsin), or its replacement after measurements are approaches used to solve this problem [59]. There are, however, other limitations in using antibody-based biosensors,

including the complexity of the test and the number of specific reagents (e.g. antibodies, or antigens) that must be developed and characterized for each compound. Furthermore, the large number of different compounds in environmental samples calls for the development of an array of specific antibodies [59]. At the same time, because of cross-reactions between similar molecules, every positive result still requires further analytical tests based on different principles of detection [6].
An excellent commercial example of antibody-based biosensor is the river analyzer (RIANA): this system is an optical multi-analyte biosensor that uses antibodies coupled to attenuated total internal fluorescence to simultaneously detect EDCs such as atrazine, bisphenol A and estrone [72].
Shimomura and co-workers developed a device for the recognition of PCBs and dioxins based on antibodies: the binding of the analyte is recorded by surface plasmon resonance (SPR) [73].
However, this kind of devices, usually built on the BIACORE AB technology (89% of publications in SPR biosensors in 2007) [74], represented more likely a bioassay to be used in the laboratory, because of the not controlled open field conditions.

Other biosensors

There are different other kind of biorecognition elements used to develop biosensors: among them, DNA, receptors, ionic channels and molecular-imprinted polymers (MIPs).
Thanks to their different physical-chemical and biological activities, nucleic acids have been incorporated in a wide range of biosensors and bioanalytical assays. The reliability of the measurement of DNA damage in electrochemical biosensors has been demonstrated directly, using the measurement of oxidation/reduction of bases, or indirectly with electrochemical probes [59]. Fluorescent dyes have been used for real-time evaluations of specific double-stranded DNA sequences, correlated to DNA damage induced by radiation and mutagens such as glutaraldehyde and benzo(a)pyrene [75]. Synthetic DNA analogs can also be used as biorecognition elements: peptide nucleic acid (PNA) has demonstrated remarkable hybridization properties for biosensing [76]. Other examples include biosensors in which DNA is used to detect microbial pathogens in water samples [59].
Receptor-based biosensors have been presented. These devices are built around a receptor able to interact with the analyte and the response is recorded through cantilevers or by using the SPR phenomenon [2]. These methods have been validated, showing that they are really complementary to chemical methods, at least for endocrine effects measurements (i.e. xenoestrogens) [57]. Biosensors able to reveal endocrine-disrupting effect by measuring vitellogenin, a well-known biomarker, have been reported [77]. Commercial systems have also been accessible: the VeriScan 3000 from Protiveris that is a multiplexed, label-free cantilever sensor, or the Biocom from Kaline with 1,000 micro-cantilevers on a chip that can test 100–200 different compounds [78].
Other biorecognition elements used in biosensors are those based on MIPs, artificial receptor that allows specific recognition of compounds following the same principle of antibodies, but with a selectivity created at the moment of the polymerization in the presence of a templating molecule (the target analyte) [6]. Moreover, they show a greater stability if compared to antibodies.
Finally, starting from the studies of Cornell and co-workers, that published their innovative results on Nature in 1997, ionic channels are another biological component used for biosensing: the conductance of a population of ion channels is switched by the recognition event [79]. These kind of sensible elements are still very interesting for sensing, and they are also used for DNA sequencing: a nanopore-based device provides single-nucleotide detection and great analytical capabilities [80, 81].

3 – Aim of the work

The concern about the state of the environment is increasing in the society, as a consequence of the scenario described in previous chapters. The international policies, aiming to preserve the ecosystems, have a fundamental requirement for the implementation of these programs: environmental monitoring.

Nowadays, environmental monitoring is mainly focused on expensive and spot-sampling-based chemical analyses, as highlighted in Chapter 2. However, several limitations of this approach in determining the concentration of environmental pollutants have been pointed out. In order to overcome these drawbacks, new biological monitoring methods are under development [6]: these include the more and more applied biosensors and biological assays, regarded today as a complementary tool for environmental monitoring.

To date, different kind of biosensing elements have been effectively tested in ecotoxicology and biomonitoring (see Chapter 2). Whole cell systems and enzymes are suitable for environmental monitoring, where the source and nature of pollutants cannot be predicted [60]. Furthermore, such devices give an indication of the mere bioavailable fraction, which often coincides with the actual concentration of xenobiotics able of giving a toxic effect [2]. Microorganisms represent a good opportunity for low cost, long life use, and wide range of pH and temperature in which they can be applied. Moreover, they have the capability to detect a broad spectrum of chemicals, thanks also to genetic engineering techniques [56]. Meanwhile, when a particular kind of pollutant would be detected, enzymes are useful because is feasible to fine tune their metabolic behaviour by means of protein engineering. For these reasons, these biorecognition elements can be used to build up biosensors that act as a biological early warning system or BEWS when implemented on a wireless sensor network. A BEWS is then a grid (wireless network) consisting of spatially distributed stand-alone devices using biosensors to cooperatively and continuously monitor the environmental conditions, such as the presence of pollutants, at different locations (Figure 3.1). If high toxicity levels are detected by the BEWS, this will produce an alarm that could be subsequently supported by more specific actions and analysis [82, 83]. This approach could be in the near future one of the most promising applications of environmental biosensors, just by combining this approach to an appropriate biological sensing element [69]. The aim of this thesis is, then, to study three biological sensing elements, associated to different kind of pollutants (Figure 3.2), in order to develop a new BEWS for environmental monitoring.

The most relevant environmental inorganic and organic pollutants, as reported in Chapter 2, are metals and PAHs. PAHs are also strictly connected to an emerging class of xenobiotics, the EDCs. Moreover, an index of broad acute toxicity could be very useful, in order to develop a biosensor able to detect the largest number of pollutants in the environment, especially if accidentally or illegally discharged. Initially, these three indexes of toxicity were chosen and associated with a proper biorecognition element. First of all, the broad toxicity was associated to the decrease of light emission (EC_{50} or half effective concentration) of a bioluminescent bacterium, *Vibrio fischeri*. Then, metal toxicity was connected to the siderophore production of the soil and water microorganism *Pseudomonas fluorescens* in presence of different concentration of Fe^{3+}, Cu^{2+} and Zn^{2+}. At last, EDCs toxicity, or generally, an index of toxicity given by PAHs, was related to the metabolization of these compounds by laccase of *Trametes versicolor*. A more accurate introduction to these sensible elements is reported in the following part of this chapter.

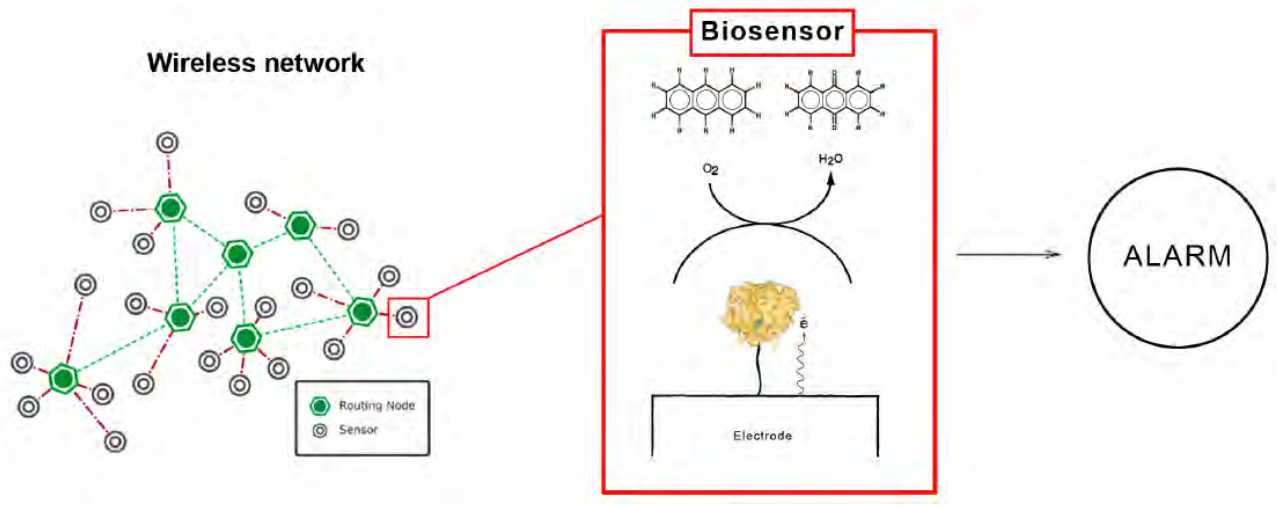

Figure 3.1: General scheme of a BEWS based on an enzyme biosensor implemented into a wireless sensor network.

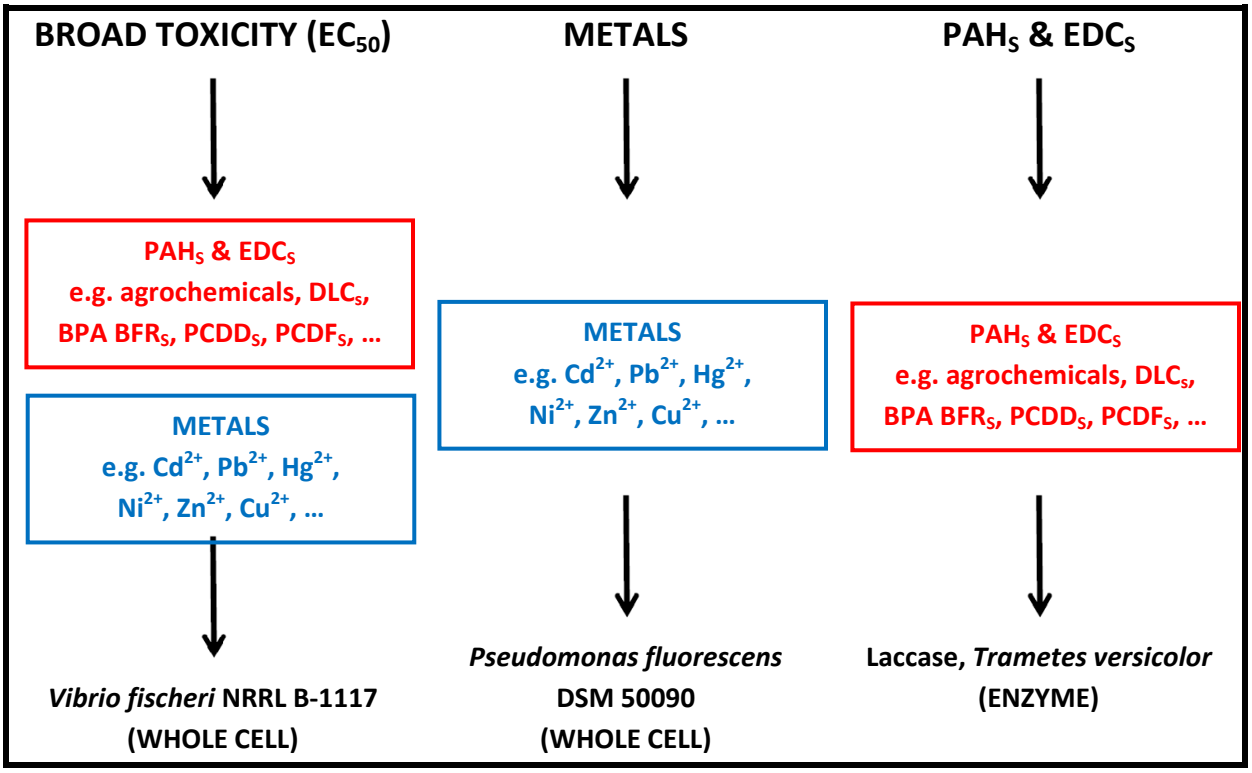

Figure 3.2: Connection between the chosen indexes of toxicity and the tested sensible elements.

Vibrio fischeri: bioluminescent bacteria and broad acute toxicity

The first biorecognition element investigated in this thesis is a luminescent microorganism, *Vibrio fischeri* (Figure 3.3). Bioluminescent bacteria belong to a group of symbiont microorganisms able to emit light under certain conditions. Since the 1960s, when these bacteria were found by plating seawater samples bioluminescence promoted interest among microbiologists and biochemists: in flask cultures the bioluminescence does not increase until mid-logarithmic phase [84]. Initially, this phenomenon was attributed to transcriptional regulation and was called auto-induction [84].

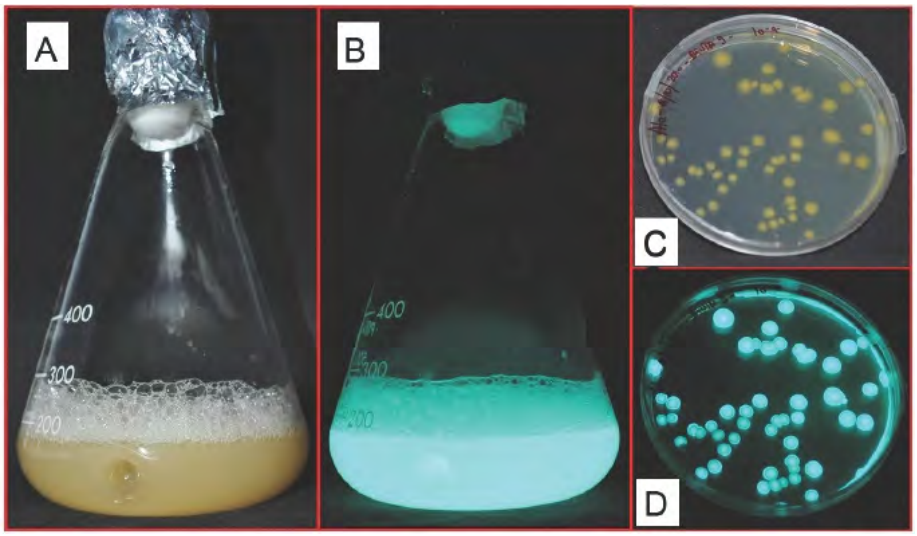

Figure 3.3: V. fischeri bioluminescence: in light (A and C), in the dark (B and D).

At the beginning, the main theory was that this effect was due to an inhibitor in the complex medium [85]. Subsequently, Eberhard was responsible for the first determination of the structure of an auto-inducer, an acylhomoserine lactone in *V. fischeri* [86]. This finding disclosed that the luciferase gene is not transcribed at low cell densities because the auto-inducer cannot accumulate to the level needed: this was the basis of all studies on quorum sensing, the microbial way of communication. Since the isolation and characterization of bacteria from light organs of fish [87] the function of the auto-induction was clear: bacteria in these light organs are tightly packed, accumulating the auto-inducer and emitting a very bright light, which the fish uses for its own purpose [88]. The bacteria that overflow from such organs can survive long periods in seawater [84].
The biochemistry of bacterial bioluminescence has been fully reviewed [89]. The light-emitting reaction of luminous bacteria involves a luciferase-catalyzed oxidation of a long-chain aliphatic aldehyde, probably tetradecanal, by molecular oxygen, with the concomitant oxidation of a reduced flavin mononucleotide ($FMNH_2$) [90] (Figure 3.4). Luminescence accounts for as much as 20 % of the total oxygen consumption, and may represent a significant energetic drain for the cell [91]. The affinity of the system for oxygen is very high: at very low oxygen concentration, where cytochrome electron flow may be completely blocked, luminescence can occur without appreciable diminution [92], but, since the emission of light requires molecular oxygen, during strict anaerobiosis the bioluminescence ceases, and upon readmission of oxygen an excess flash of luminescence occurs.
There are six documented marine luminous species of bacteria, all gram-negative motile rods and facultative anaerobes capable of growth at 25 °C that share a lot of morphological, physiological, and biochemical features with the *Enterobacteriaceae* and the *Vibrionaceae*. *Vibrio fischeri* NRRL B-1117

was selected because it has a well-characterized sensitivity to a wide range of toxicants, and to date is used as biological ecotoxicological biomarker [32, 94].

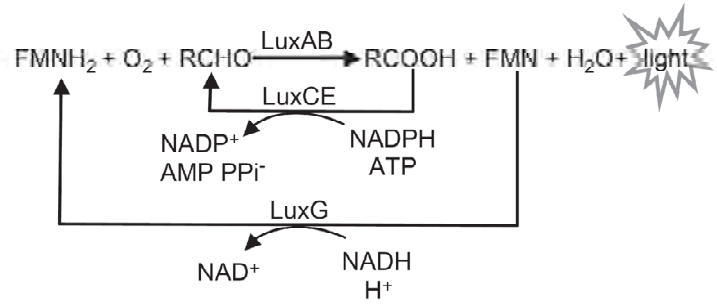

Figure 3.4: Biochemistry of luciferase-mediated bioluminescence in V. fischeri [93].

Many organisms from different trophic levels have been used for ecotoxicological tests (e.g. bacteria, nematodes, fishes, algae, plants, cell lines), but among them bioluminescent bacteria can offer the further advantage of a first screening method in a test battery, based on speed and cost considerations [32]. Therefore, expensive chemical analysis became mandatory only if the bioluminescent test gives an alarm. *V. fischeri* have been reported as the most sensitive, across a wide range of chemicals, compared to other bacterial assays, showing a good correlation to toxicity bioassays based on algae, crustacean, or fishes [95].

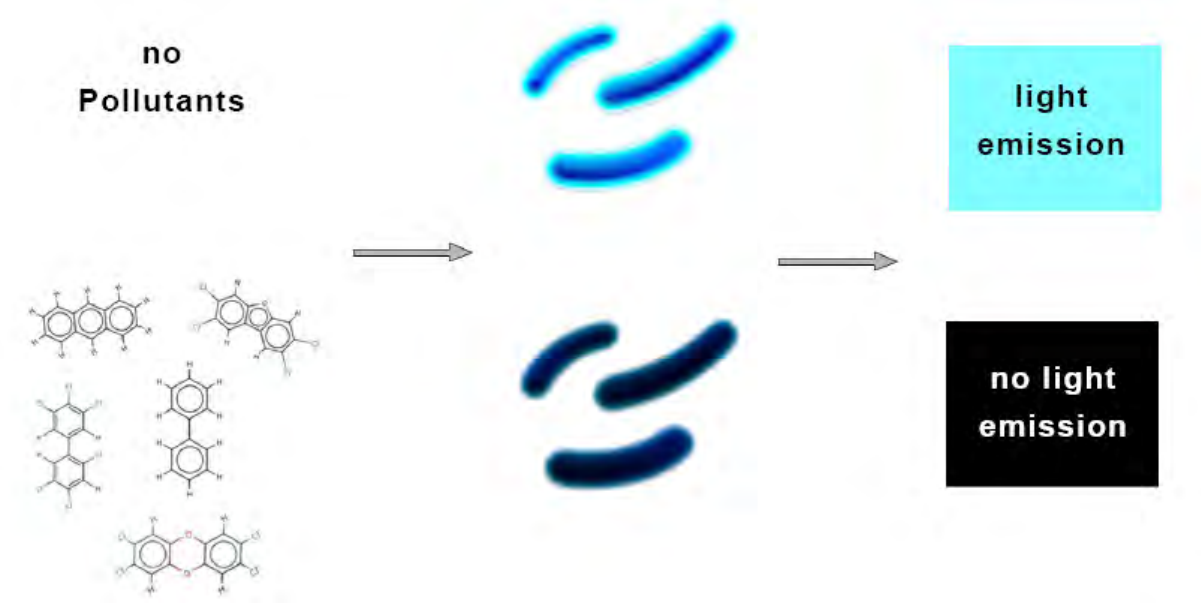

Figure 3.5: Scheme of a biosensor for environmental monitoring based on V. fischeri.

The bioluminescence is a sensitive indicator of xenobiotics toxicity, because it is directly coupled to respiration, via the electron transport chain, and thus reflects the overall metabolic status of the cell: the light emission is proportionally decreased by the concentration of toxicant substances. This is the typical mechanism exploited in those bioassays that measure changes in physiology and metabolism of living organisms resulting from stresses induced by toxic compounds.

The very fast and sensitive variation of the bioluminescent system is, then, useful to reveal any unusual condition in the ecosystem, and it can be regarded as a potential BEWS. Naturally light-emitting bacteria were first proposed for analytical purposes over 30 years ago, and some commercial tests (e.g. Microtox®, Lumistox® or ToxAlert® tests) and normative (UNI-EN-ISO11348) have been arranged for laboratory tests [94]. Furthermore, the lux genes, coding for the enzymes

involved in the bioluminescent reaction, have been inserted into different bacterial species, and coupled to specific promoters that allow their expression only in presence of the proper analyte [32]. To date, this kind of assays are usually tailored only for acute toxicity and are not available for *in situ* toxicity evaluation of various xenobiotics, such as PAHs, heavy metals and pesticides [32]. The direct application of this system in a portable device is of great interest in order to prevent detrimental effects of pollution on human health and ecosystems, because of the potential in continuous environmental monitoring. To assess the reliability of a biosensor based on *V. fischeri* bioluminescence and able to estimate the broad acute toxicity directly *in situ* (Figure 3.5), the influence of environmental physical-chemical parameters must be investigated. Temperature, inoculum percentage and carbon source were evaluated.

Pseudomonas fluorescens: siderophores and metals toxicity

The second sensible element evaluated in this thesis is pyoverdine, a siderophore produced by the strain *P. fluorescens* (DSMZ 50090 or ATCC 13525). Siderophores are low-molecular-weight chelating agents (200–2,000 Da), characterized by an extremely high affinity for Fe^{3+}, and used for the iron uptake via active transport systems [96, 97]. Iron is an essential element for growth and development of almost all living organisms, by acting as an enzymatic cofactor, by promoting the electron transfer, or by participating in oxygen metabolism. The growth and survival of these organisms depend on their ability to incorporate a sufficient quantity of iron. To date, the only known exception to this rule is represented by lactobacilli, which are devoid of heme proteins and hence have no iron requirement [98]. This metal is the fourth most abundant element in the Earth's crust, but its availability for microbial assimilation in environments such as the soil rhizosphere is extremely limited because it has low solubility [99]. In aqueous solution, iron can exist in either the more soluble ferrous (Fe^{2+}) or less soluble ferric (Fe^{3+}) form. However, in highly oxidized and aerated soils, the predominant form of iron is the ferric form [100], which is soluble in water (pH 7.4) at about 10^{-18} M [101]. This concentration is too low to support the growth of microorganisms, which usually need concentrations approaching 10^{-6} M for standard growth [99]. One of the strategies adopted by microorganisms to cope with this limited bioavailability is to secrete iron-complexing compounds called siderophores that form six coordinate octahedral complexes with ferric iron [102]. The main function of these molecules is iron scavenge, but other interesting properties such as antimicrobial activity have been described [103].

The various siderophores have diverse structures, depending on the producing microorganism [99]. Most known siderophores can be grouped into hydroxamate and phenolate/catecholate structures which show different affinities for ferric iron: the latter structure generally has higher formation constants with ferric iron, with highly pH dependent stability of these complexes [104], while the hydroxamate complex is much more stable [102] and hence potentially more ecologically relevant in the soil rhizosphere.

Pyoverdine (Figure 3.6) is one the main siderophore secreted by wide-spread fluorescent *Pseudomonads* for iron uptake in combination with pyochelin [105]. Pyoverdine (MW approximately 1,000-1,500 Da, [106]) is a conserved dihydroxyquinoline derived chromophore to which a short peptide chain is attached [107], whilst pyochelin (325 Da, [106]) consists of a small thiazoline derivative [2(2-o-hydroxyphenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid)] [108], which binds ferric ion in a 2:1 stoichiometry (pyochelin: Fe^{3+}).

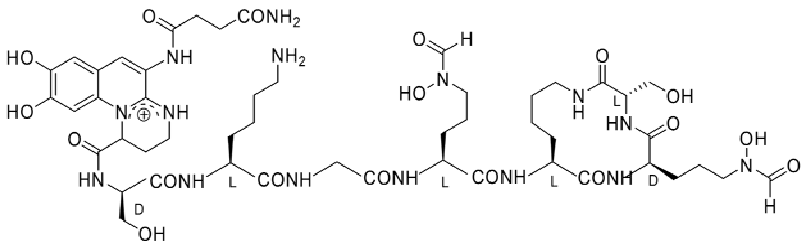

Figure 3.6: Pyoverdine structure [114].

The mechanisms of iron uptake by pyoverdine or pyochelin have been mainly studied in *Pseudomonas aeruginosa* [105, 109, 110, 111] (Figure 3.7). Briefly, an outer membrane transporter (FpvA for pyoverdine and FptA for pyochelin) binds the Fe^{3+}-siderophore complex with an affinity of about 1 nM [112]. This complex is then dissociated in the periplasmic space, most likely by the reduction of ferric iron to ferrous iron [111]. After the dissociation of the complex, pyoverdine is recycled form and exported into the medium [111]. All this mechanism was further confirmed by the lower affinity of the metal-free pyoverdine to the FpvA transporter in comparison with the Fe^{3+}-siderophore complex [113], and by the higher affinity of pyoverdine for iron(III) than iron(II) [114]. As suggested by Hider and Kong [114], there is a logic reason for this selectivity: is easier to be selective for Fe^{3+} than Fe^{2+} because there are not many biologically significant tri-positive cations (only the kinetically inert Co(III)), while there are more biologically important di-positive cations such as calcium(II), magnesium(II), zinc(II), copper(II), nickel(II) and manganese(II). Consequently, a molecule which is selective for tri-positive metals will effectively be selective for iron in biological matrices. Once internalized, the Fe^{3+} is then recovered as Fe^{2+}, as already said.

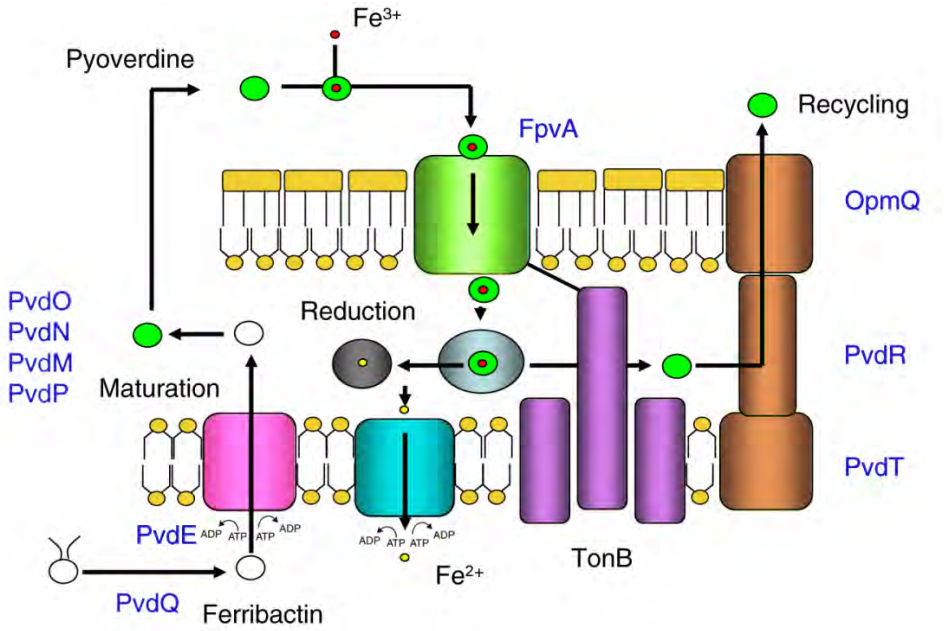

Figure 3.7: Iron uptake in Pseudomonads (adapted from [118]).

Many studies have shown that siderophore biosynthesis in bacteria is regulated by both the amount of iron present in the environment and the amount of iron already assimilated by bacteria [109]. In the presence of iron, repression of both pyoverdine and pyochelin synthesis in *P. aeruginosa* occurs via the cytoplasmic Fur protein [115]. In the absence of iron, the up-regulation of the genes related

to pyoverdine synthesis involves a transmembrane signalling system induced by the binding of the siderophore to the outer membrane transporter FpvA [109, 116, 117, 118].

High concentrations of metals should be present in the environment, due to men activities or to natural occurrence, causing stress conditions for microorganisms, and interfering with the siderophore-iron uptake pathways. Some metals, like cadmium, lead and mercury, are purely toxic for microorganism at each concentration, while others, like zinc and copper, are required in trace concentrations for the regular metabolism of the cell, but becoming dangerous at high intracellular concentrations, through a variety of mechanisms [3] (e.g. by interfering with the function of essential proteins [110]). Bacteria have several mechanisms to avoid heavy metal stress, allowing them to thrive in ecosystems contaminated with toxic levels of heavy metals [119, 120]. Active efflux is a key aspect of microbial resistance, transporting metal cations out of the cytosol and periplasmic space of gram-negative species [120]. High heavy metal concentrations in the environment may also interfere with microorganism siderophore-iron uptake pathways and heavy metal toxicity may be modulated by the presence of siderophores. The binding of heavy metals to siderophore dramatically changes the free metal concentration outside of the cells, affecting the uptake process [110]. This mechanism has been already proposed for *Streptomyces tendae* F4, a Cd-resistant bacterium, which can simultaneously secrete a variety of hydroxamate siderophores that bind Cd^{2+}. The production of these siderophores is up-regulated by Cd^{2+}, in the absence or presence of iron and their presence reduced uptake of Cd^{2+} by the cell while supplying it with iron [121]. Both siderophores of *P. aeruginosa* PAO1 have a protective effect on the toxicity of metals: pyochelin seems to be more efficient than pyoverdine for Ni^{2+}, Pb^{2+} and Tb^{3+}, whereas pyoverdine seems to be more efficient for Cu^{2+} and Zn^{2+} [109]. Furthermore, previous studies in a number of bacteria and fungi show that metals other than iron stimulate siderophore production [3, 109, 110]. The ability of toxic metals to induce or repress siderophore production suggests that these molecules may play a role in bacterial heavy metal resistance [109].

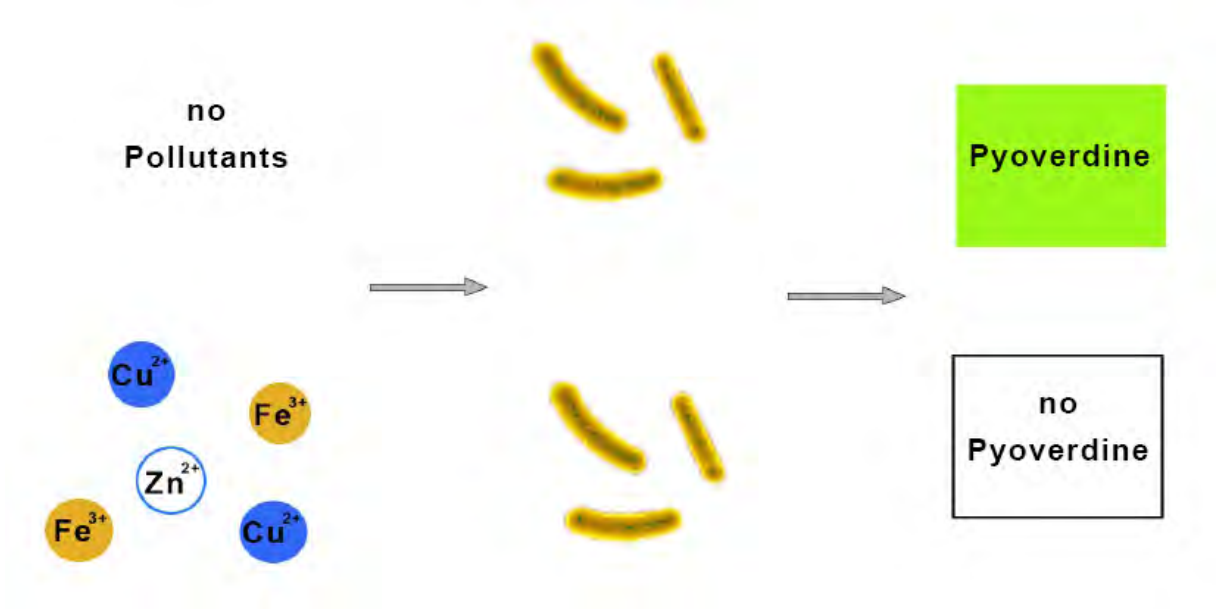

Figure 3.8: Scheme of a biosensor for environmental monitoring based on Pseudomonas response to metals.

Moreover, it is known that these siderophores are produced under iron-limiting conditions, participating in the solubilisation of Fe^{3+} from minerals or organic substances, but these siderophores also chelate other metals with lower affinity [109, 110]. However, the ability of these siderophores to bind a lot of metal ions doesn't mean that these elements are subsequently internalized by the

bacterial cell, indeed the pyoverdine/FpvA and pyochelin/FptA uptake pathways are highly selective and only ferric iron is efficiently transported [110]. None of the other metals screened by Braud et al. [109] was transported, unless Co^{2+}, Ga^{3+} and Ni^{2+} by pyochelin/FptA, and Cu^{2+}, Ga^{3+}, Mn^{2+} and Ni^{2+} by pyoverdine/FpvA, but with lower uptake rates.

All these considerations imply that factors other than iron limitation seem to influence the production of siderophores in *Pseudomonads*. Meyer and Abdallah included also many other environmental factors, among them: temperature, pH, nature of the carbon source and degree of aeration [122]. Temperature, pH and nutrients concentration are the most important variables that regulate microbial growth and culture behaviour. For this reason the effect of these physical-chemical parameters on growth and production of pyoverdine was studied. Moreover, to develop a biosensor able to broadly detect the presence of toxic metal (Figure 3.8), the influence of metals on biomass growth and on pyoverdine regulation was investigated.

T. versicolor laccase protein engineering and PAHs recognition

Many different sensing elements have been used to develop biosensors and especially oxidoreductase enzymes [123] are widely applied for their ability to metabolize aromatic compound, one of the main classes of organic pollutants. The so-called laccase is one such enzyme, biologically well characterized and particularly suitable due to its ability to oxidize a broad range of xenobiotics.

Laccases (EC 1.10.3.1, benzenediol-oxygen oxidoreductase) are glycosylated multicopper oxidase enzymes, constitutively expressed during primary metabolism by various fungi (Ascomycetes, Deuteromycetes and Basidiomycetes), but also by plants [124] as well as in prokaryotes (e.g. bacteria) [125]. This enzyme is mostly extracellular in plants and fungi, whilst is intracellular in bacteria [126, 127], and it is a member of the multicopper protein family, which includes ascorbate oxidase, ceruloplasmin and bilirubin oxidase [128].

Laccases usually contain four copper atoms per monomer, distributed in three copper centres differing in their characteristic electronic paramagnetic resonance (EPR) signals: T1 or blue copper centre, T2 or normal copper and T3 or coupled binuclear copper centres. [129,130]. T1 copper centre shows a strong electronic absorption band near 600 nm (ε = 5000 mol/L*cm), which is responsible for the deep blue colour of multicopper proteins [131]. Some exceptions are represented by laccases which lack this typical absorption at 600 nm: a 'white laccase' (containing 1 Cu, 1 Fe, 2 Zn atoms) in *Pleurotus ostreatus* [132] and a 'yellow laccases' (containing copper but in an altered oxidation state) in *Panus tirinus* [133]. T2 copper shows no absorption in the visible spectrum, whereas T3 copper shows an electron adsorption at 330 nm [128].

The presence of copper makes laccases able to consume O_2 instead of H_2O_2 to oxidize the substrates, differently from peroxidases and other oxidases [134, 135]. The structure and properties of copper centres characterize also the redox potential of this enzyme: low and high redox potential laccase have been classified. Laccases from fungi (especially white-rot Basidiomycetes) show high-redox potential [136], whereas, bacterial and plant laccases are examples of low-redox potential enzymes [137]. The ability of the enzyme to metabolize various phenolic and non-phenolic compounds as well as many environmental pollutants depends on this redox potential: the higher the potential, the greater the oxidation capabilities [138].

Laccases exhibit various functions, including lignification (in plants), delignification, pigmentation, fruiting body and melanin formation (in fungi) as well as endospore coat protein synthesis (in bacteria) [131]. These features depends on the physiological and pathological conditions of the source organism, that produce the enzyme in particular culture conditions, and are broadly divided into three categories: cross-linking of monomers, degradation of polymers, and ring cleavage of aromatic compounds [139]. The first group mainly corresponds to the enzymatic oxidation of anilines

and phenolic compounds that generate radicals that react with each other to form dimers, oligomers or polymers covalently coupled by C–C, C–O and C–N bonds. In soil, the main consequence is that natural and xenobiotic aromatic amines or phenolic compounds are bound to the organic humic matrix, and partial demethylations and dehalogenations may occur with substituted compounds. On this skill, it is based the potential use of laccases to detoxify contaminated soils or wastewaters [140]. At the same time laccase are involved in the degradation of complex natural polymers such as lignin [141] by radical mechanism that lead to the cleavage of covalent bonds and to the release of monomers. The ring-cleavage of aromatic compounds catalyzed by laccase has been reported in several examples, and it is a basic feature for xenobiotics degradation [140, 142]. If the binding site is not accessible because of steric hindrance, the enzymes might not come directly into contact with the polymers, but small molecules such as veratryl alcohol (VA), 3-hydroxy-anthranilic acid (HAA), N-hydroxybenzotriazole (HBT) and manganese, are used to mediate the radical-catalysed depolymerisation [128]. More than 100 possible mediator compounds have been studied for this purpose [131]. Mediators are particularly interesting for increasing the number of laccase applications, especially to oxidize and cleave non-phenolic substrates [143]: as example, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) is converted into a stable radical and acts as a diffusible 'electron shuttle' between laccase and substrate, extending the substrate specificity [144, 145]. This compound, in reduced form, has a very faint green colour, while, in oxidized form, it turns dark green and for this reason it was broadly used to detect laccase activity.

Laccases' substrates are mostly phenols or arylamines, and the low substrate specificity of the binding pocket makes this enzyme able to oxidize a large number of natural and anthropogenic compounds [146]. The reaction mechanism of laccase was strongly investigated because of its structural and functional relevance. Residues involved in copper binding are extremely conserved in plant, fungi and bacteria, and this is probably reflected into the similar redox mechanisms [131]. The enzyme combines the four-electron reduction of O_2 to H_2O with the one-electron oxidation of four reducing substrate molecules: the electrons are extracted from the substrate by the T1 and transferred to the trinuclear T2/T3 centre where molecular oxygen is reduced [146, 147]. The overall reaction that form quinones or polymerization products [148] is reported in Figure 3.9.

A large number of laccase, showing a great variety of characteristic, have been found in different organisms [131, 149], and their features must be properly investigated through a screening for the wanted characteristic, to use this kind of oxidase for a particular application. These features are mostly influenced by substrate specificity, optimal pH, temperature, and degree of glycosylation. The affinity and substrate specificity of laccase change in connection with pH variation [131]. For substrates whose oxidation does not require a proton exchange (e.g. non phenolic), the enzymatic activity decreases as pH increases, while for substrates that involve proton exchange (e.g. phenolic), the optimal pH value depends on source of laccase rather than on substrate [150, 151]. For phenols, fungal and bacterial laccases have an optimal pH ranging between 3.0 and 7.0, while for plant laccases it is higher (9.0) [151, 152]. The difference in optimal pH values is probably linked to the physiological function of the enzyme in different organisms [153]: usually, fungal laccases are secreted and have a lower pH optimum [127] probably because fungi grow well in acidic condition, in comparison to plant laccases, that are intracellular, and have their optimal pH nearer to their basic physiological pH. It has been hypnotized that the acidic optimal pH of fungal laccases is due to the binding at high pH of a hydroxide anion to the T2/T3 coppers of laccase that interrupts the internal electron transfer from T1 to T2/T3 centres [154].

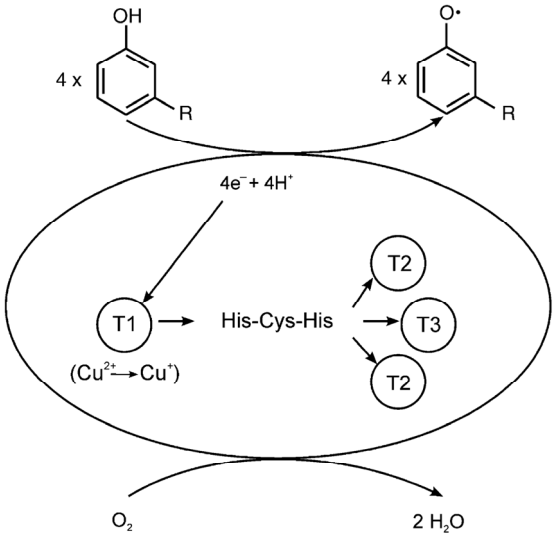

Figure 3.9: Laccase reaction against phenolic compounds [149].

The more acidic laccase iso-enzymes were reported to have also the higher thermostability: indeed their optimal temperature values range from 50 to 70 °C [155]. It seems that laccase thermal stability depends on the temperature range of growth of the producer organism, indeed fungal laccases usually have lower thermal stability than bacterial enzymes [149].

Both plant and fungal laccases are glycosylated enzymes. Usually glycosylations (mainly mannose, N-acetyl glucosamine, and galactose), important for secretion, copper retention and thermal stability are higher in plants (22-45%) than in fungi (10-25%) [131]. Plant laccases have also higher molecular weights than fungal ones because of glycosylations [127]. The degree of glycosylations seems to be involved in enzyme stability: in particular, fungal laccases are more stable outside of the cell because they are highly glycosylated, and the carbohydrate moieties increase their hydrophilicity [127].

Due to high stability and working at broad range of pH from acidic to neutral pH, the fungal laccases find more industrial applications compared to those of bacteria and plants [131]. Furthermore, many authors reported that fungal laccases (laccase of *T. versicolor*) possess oxidation activity 100-fold higher than bacterial ones (e.g. CueO laccase of *E. coli*), maybe for their higher affinity for copper ions [156]. The analysis of crystal structures revealed that, bacterial (1GSK) laccases have larger substrate binding site cavity, as compared to fungal (1KYA) and plants (1AOZ) isoforms [131]. This feature could also explain the differences in the enzymatic activity.

The high catalytic efficiency and broad substrate specificity of laccase make it more advantageous as compared to other conventional chemical or microbial catalysts [131]. The ability of the enzyme to only require molecular oxygen during the substrate metabolization is a further advantage for industrial applications. A great number of application of this enzyme in many agricultural, industrial, and medical areas have been presented: delignification and pulp bleaching, aerobic bioremediation of contaminating environmental pollutants [157], oxidation in the textile and dye industry and wastewater treatment [146], enzymatic conversion of chemical intermediates, development of new cosmetic pigments, such as hair dyeing materials, removal of biogenic amines or the determination of polyphenol in wine [158, 159], as well as laccase-based oxidation, biotransformation, and biosensor technology [127]. For a complete review of laccase applications see [127, 131, 146, 161].

Laccases have shown to be useful for the elimination of toxic compounds through oxidative enzymatic coupling, or through degradation, of pollutants. In particular, laccases of *T. versicolor* have been reported to be involved in removal of a wide range of chlorinated phenols [161, 162]. PAHs and chiefly chlorinated aromatic compounds tend to accumulate because of their low solubility in water,

and are the main pollutants present in soil. Moreover, most of them (e.g. chlorophenols) are carcinogenic.

Although there is some evidence that PAHs can be oxidized by laccases to a considerable degree [138, 163], the low redox potential of this enzyme (450–800 mV) compared to those of peroxidases (>1 V) only allows the direct degradation of low-redox-potential molecules and not the oxidation of more recalcitrant aromatic compounds, including some PAHs that have too high potentials and large dimensions [151]. By means of an example, the degradation of 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) was tested in different conditions by Camoni and co-workers [164] with negative results. Some activity was observed for hydroxylated recalcitrant PAHs, for instance 2-hydroxydibenzofuran [165], hydroxyl-PCBs [166], and chlorinated hydroxybiphenyls [162, 167]. When the enzyme is able to modify a xenobiotic compound, such as PAHs and pentachlorophenol, the catalytic constants are very low and not useful for applications [138, 168].

Redox mediators could be used as alternative for the oxidation of compounds with high redox potentials, or to increase the rate of oxidation. At the same time, protein engineering techniques could be also used to optimize laccase catalysis for degradation of toxic xenobiotics, such as PAHs, but the starting enzyme should be the most stable and catalytically active against xenobiotcs. Amongst the various laccases, fungal ones seem to be the more adaptable to this aim, even if the expression of this enzyme requires an eukaryotic heterologous host (e.g. a yeast expression system). The advantage of fungal laccase is obviously the high reactivity of these enzymes, due to their elevated redox potential, and the great protein stability probably due to glycosylations and the extracellular nature of this oxidoreductase. One of the most studied fungal laccase, the enzyme from *T. versicolor*, is also the most active against xenobiotics [138]. Starting from this laccase, it would be of interest to design an enzyme that is either more efficient in catalysis or capable of oxidizing a wider number of toxic compounds. The *T. versicolor* laccase structure is a monomer, organized in three domains, with the trinuclear copper cluster (T2/T3) embedded between domains 1 and 3 [146], and a redox potential of about 780 mV [138]. This fungus expresses four laccase iso-enzymes that show different features and activity, but share 97 % of sequence homology: lccα (GenBank Accession No. AY693776), lccβ (GenBank Accession No. Y18012), lccγ (GenBank Accession No. D84235) and lccδ (GenBank Accession No. X84683) [169].

Between these isoforms, lccβ seems to be the more active toward some polycyclic aromatic hydrocarbons [169] and was chosen for the following protein engineering. In fact, it would be useful to have an enzyme able to metabolize a large number of PAHs to develop a biosensor for the detection of this kind of pollutants in the environment. This enzymatic isoform is particularly suitable for this purpose, because it has already been successfully expressed in yeast (e.g. *Pichia pastoris*), showing high stability in different conditions [169]. Fungal laccases of *T. versicolor* have been already used for biosensing [158, 159, 170], and also in Organic Phase Enzyme Electrode (OPEE) [171], because it is able to retain some activity also in solvent-water mixture [172].

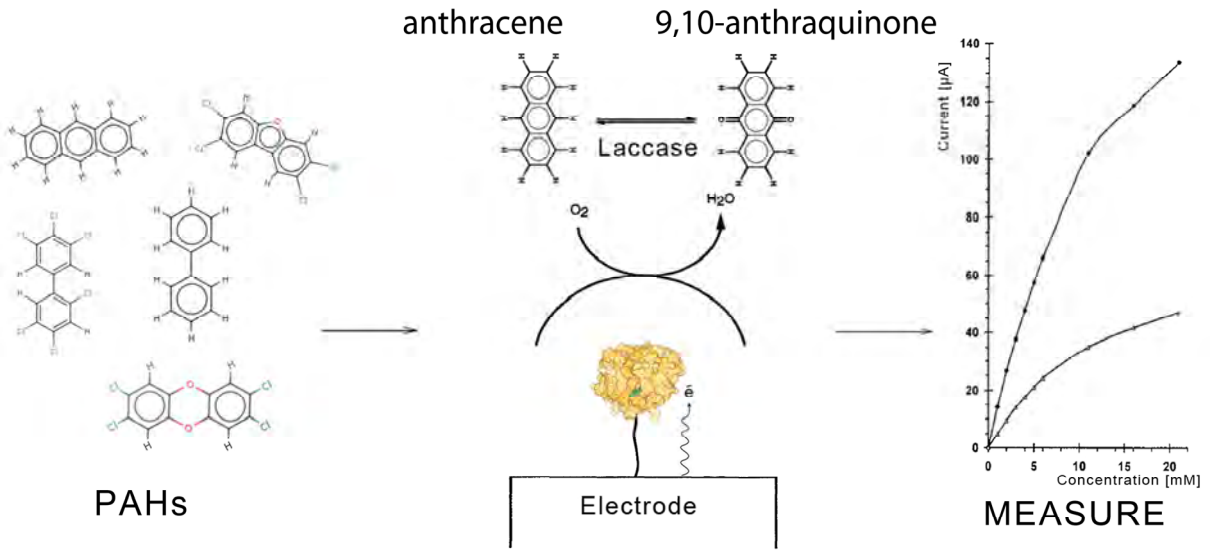

Figure 3.10: General scheme of a biosensor for PAHs detection based on a modified laccase enzyme.

In order to make lccβ more active against toxic and persistent xenobiotics, a combination of computational docking and molecular biology techniques was used to generate rationally engineered fungal laccases with increased ability to process large and persistent aromatic compounds. These mutated laccases were produced by heterologous expression in *P. pastoris* and characterized by means of biochemical assays. The main idea, represented in Figure 3.10, is to apply the obtained enzyme in a biosensor for environmental monitoring.

4 - Materials and Methods

Vibrio fischeri: bioluminescent bacteria and broad acute toxicity

V. fischeri DSMZ 7151 (NRRL B-1117) cultures were set-up into 500 mL Erlenmeyer flasks, using 200 mL of SW medium (see annex A) as working volume, and 2.5 % of inoculum with an optical density 620 nm (OD_{620}) of 0.8-1.0 RU, unless differently stated.
During the experimental period, the strain was stored at 4 °C on SW agar plates and maintained by monthly subcultures. Long-term storage was at -20 °C in 12.5 % glycerol.
The microorganism was grown at 20-30 °C and 130 rpm, in microaerobic conditions. The sterile glucose solution used in fed-batch was 100 g/L. Cell growth was monitored by measuring the OD_{620} (HP 8452A Diode Array Spectrophotometer). pH was recorded with a Crison 2001 pHmeter. The bioluminescence was visually estimated.

Pseudomonas fluorescens: siderophores and metals toxicity

Preliminary tests: influence of culture conditions on biomass growth and siderophore production

P. fluorescens DSMZ 50090 (ATCC 13525) was streaked on a DSM1 agar plate (see annex A) and grown overnight at 20 °C (preculture phase). The plate was, then, re-suspended with 10 mL of saline solution (0.9 % NaCl), and used as inoculum. During the experimental time-course the strain was stored at 4 °C on DSM1 agar plates and subcultured monthly. Long-term storage was carried out at -20 °C in 12.5 % glycerol. The cultures were prepared using succinic acid medium M78 medium (see annex A) [122], or substituting the carbon source with glucose (4 g/L). To evaluate the effect of different initial pH, the phosphate buffer (K_2HP0_4 - KH_2P0_4) was modified in the culture medium. Cultures were set-up at 15-30 °C and 130 rpm, in microaerobic conditions, and 1-10 % of inoculum with an optical density at 620 nm (OD_{620}) of 0.8-1.0 RU in 500 mL baffled Erlenmeyer flask, in 500 mL BOD bottle, or in 96 well plate (Corning Incorporated - 3799).
Cell growth was monitored by measuring OD_{620} (HP 8452A Diode Array Spectrophotometer, or Biotek PowerWave 340 Microplate Reader in case of 96well plates). pH was recorded with a Crison 2001 pHmeter. Pyoverdine was estimated by measuring the OD_{400} of the cultures supernatant obtained by centrifugation (Centrifuge 4217, AIC) at 3000 rpm, 20 °C, 10 minutes [122, 173]. The amount of pyoverdine was determined transforming the OD_{400} according to the Lambert-Beer law (ε_{400nm}= 20,000 L/mol*cm) [122]. Succinic acid concentrations were determined with a high performance liquid chromatography (HPLC) (Kontron Instrument) equipped with an ion exchange column (Hamilton HC-75 H, 305x7.8 mm), at 50 °C, using 5 mM H_2SO_4 as mobile phase [174]. Each sample was filtered (0.22 µm, cellulose acetate, Sartorius Stedim Biotech GmbH) and analyzed on HPLC using refractive index and UV/Vis (210 nm) detectors. The samples concentration was determined calibrating the system with succinic acid standards in the concentration range 5-0.625 g/L.

Evaluation of the interaction between *P. fluorescens* and Fe^{3+}, Cu^{2+} and Zn^{2+}

Minimum inhibitory concentrations (MICs) were determined on DMS1 agar plates, using a modified Kirby-Bauer method [83], and in liquid media, using 96well plates [175]. For the modified Kirby-Bauer method, the area of the inhibition halo allowed to calculate the values of MICs for $CuSO_4$, $ZnSO_4$, $FeCl_3$, $Fe_2[SO_4]_3$. For the determination of the MIC in 96 well plates DSM1 or M78 media were used, supplemented with increasing concentrations of each metal (up to 10 mM). The cultures were

incubated for 48 hours, and growth was monitored by measuring the OD_{600}, as stated above. To study the effect of metals on bacterial growth and pyoverdine production, cultures were set-up in 500 mL baffled Erlenmeyer flask or in 96well plates, and the culture media was supplemented with different Fe^{3+}, Cu^{2+} or Zn^{2+} concentrations (up to 6.25 mM). The concentrations for these tests are always referred to the metal ion.

Characterization of PolyVinil alcohol (PVA) gel immobilisation of microbial sensible elements

Polyvinyl alcohol (PVA) cryo-gels and cryo-films

A 10 % PVA solution was prepared by melting the polymer (Mowiol 28-99, Sigma Aldrich) in distilled water (dH_2O) at 90 °C for 90 minutes. PVA gels were formed by 'freezing-thawing' method [176]. A defined amount of solution was poured on a glass surface and mechanically casted by means of a Mayer rod [177] to obtain a nominal thickness of 100 and 400 µm. To determine diffusivity through the PVA matrix, PVA gels were casted on different supporting material by means of a Mayer rod: a paper disk or a nylon net was put in between the glass surface and the Mayer rod. Thereafter, the casted gel was put at -20 °C for 2-24 hours, and then thawed at 25 °C for 15-20 minutes. The freeze-thaw cycle was repeated two times, and the obtained cryo-gels were directly used wet, or after air-drying (films). The thickness of cryo-films was established by means of a coating thickness gauge (QUANIX 7500, Automation, GmbH).

PVA biodegradation test

The ability of *P. fluorescens* to metabolize PVA as a carbon source was tested in flask cultures. The cultures were set-up in 500 mL baffled Erlenmeyer flask (as above), using succinic acid medium M78 medium (see annex A) [122], or substituting the carbon source with PVA (4 g/L). Cultures growth was also compared with a negative control (without C source).

Growth calibration curve (OD_{620} against dry cell weight), and evaluation of biomass dispersion in PVA gel

Cultures were set-up in 500 mL baffled Erlenmeyer flask, as specified above, and grown overnight. The biomass was recollected by centrifugation (18000 rpm, 4 °C, 10 minutes), and the pellet was washed and resuspended with dH_2O. A biomass stock solution with an OD of 0.8 RU was prepared and serially diluted to prepare 5 biomass standards (OD_{620} 0.2-0.8 RU). The OD_{620} of these standards were recorded and 100 mL was filtered in triplicate on dried pre-weighted 0.45 µm cellulose acetate filter disk (Sartorius Stedim Biotech GmbH). These disks were dried to constant weight at 105 °C and gravimetrically evaluated. The calibration curve was prepared by plotting dry cell weight against OD_{620} (Figure 4.1).

A biomass stock solution (OD_{620} 0.8 RU) was prepared, as above. A 10 % (w/v) PVA solution was prepared as previously mentioned and mixed with the biomass stock solution and casted to form cryo-gels, as stated above. To evaluate the biomass dispersion inside the PVA matrix, different gels and films were prepared by mixing the biomass (15 mg) previously stained with Methylene blue with various amount of 10 % (w/v) PVA solution (1:1, 1:2, 1:5, 1:10, biomass to PVA ratio).

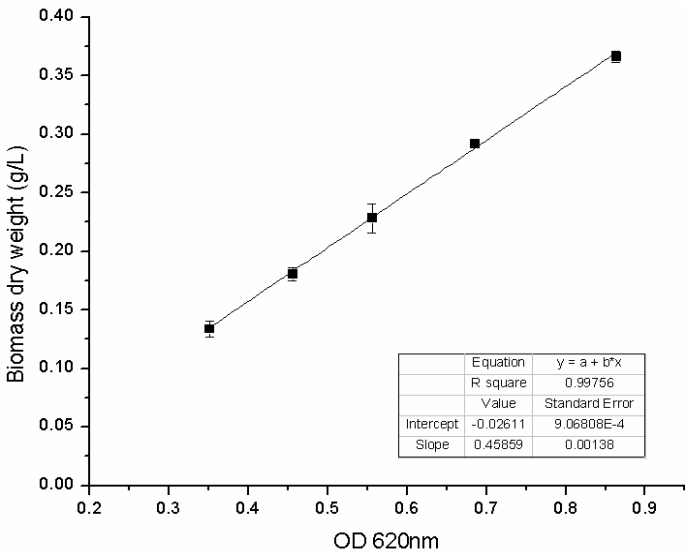

Figure 4.1: Calibration curve biomass dry weight versus OD_{620}.

Determination of PVA gel diffusivity

A diffusion chamber was used for the determination of the Methylene blue diffusivity through the PVA gel (Figure 4.2). This apparatus is composed by two compartments (A, 118.2 mL; B, 142.2 mL) separated by a septum: the gel was mounted on one side of the septum. The two compartments were subsequently filled with dH_2O, and stirred. In one side of the camber 50 µL of 10 g/L Methylene blue was added.

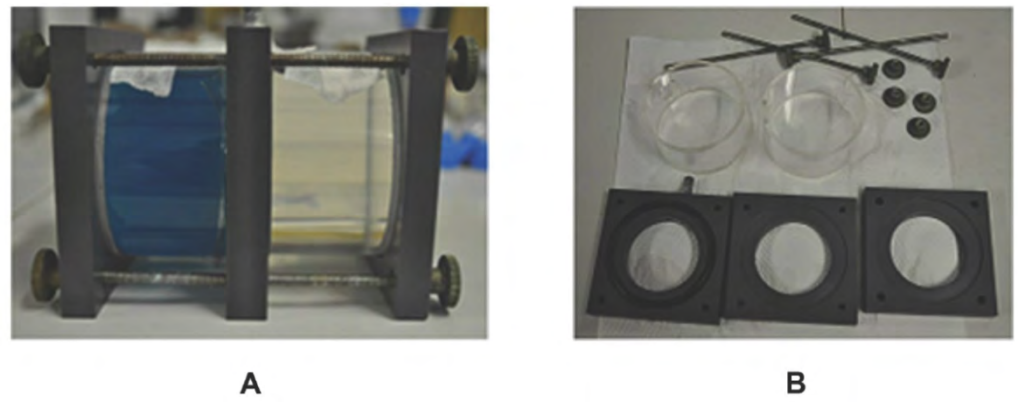

Figure 4.2: Images of the diffusion chamber.

To determine the diffusivity the following system of equations must be solved (assuming no volume variation in both compartments):

$$\begin{cases} \dot{n} = D\dfrac{(c-c')}{\Delta_x}A \\ V_1\dfrac{dc}{dt} = -\dot{n} \\ V_2\dfrac{dc'}{dt} = \dot{n} \end{cases}$$

$\dot{n}$ is the diffusive flow rate through the membrane [mol/s], D is the diffusivity coefficient [m^2/s], Δ_x the gel thickness, c and c' are the concentrations in the two compartments, A is the gel area, V_1 and V_2 are the volumes of the two compartments, dc/dt and dc'/dt are the variations of concentration during time in the two compartments. This system could be approximated. If D is the diffusivity and

dc/dx is the concentration gradient in the gel, *J*, the dye flux through the gel, is described by the mono-dimensional form of the Fick's law:

$$J = D\,\frac{dc}{dx}$$

If c_t' is the dye concentration in the low concentration compartment at time *t*, c_{t0}' is the dye concentration in the same chamber at time t_0, and *V* is the compartment volume, $\dot{n}$ is the average dye flow rate over time, represented by this relation:

$$\dot{n} = \frac{\left(c_t' - c_{t_0}'\right)}{(t - t_0)} * V$$

The flow (*J*) is then obtained dividing the flow rate over time ($\dot{n}$) by the gel area (*A*):

$$J = \frac{\dot{n}}{A}$$

Since the dye concentrations changes over time, then, the concentration gradient at time *t* will be approximated following this relation:

$$\frac{\Delta c(t)}{\Delta x} = \frac{\left(c(t)' - c(t)\right)}{\Delta x}$$

In this formula, *c'* is the dye concentration in the low concentration compartment at the time *t*, *c* is the dye concentration in the high concentration compartment at the same time *t*, while Δ_x is the gel thickness. The dye concentration (*c* and *c'*) were determined spectrophotometrically (OD_{670}), taking 3 mL of sample from both side of the camber. After the measurement, the samples were re-introduced into the corresponding chambers. The concentration of dye was calculated by reference to a calibration curve prepared using standards (0-5 mg/L).

Spectrophotometric determination of PVA in water samples [178]

PVA forms a green complex with iodine in the presence of boric acid. The colour develops immediately (at 25 °C.) and it is stable for at least 4 hours. The sample was transferred into a 50 mL volumetric flask and eventually diluted with dH_2O to approximately 25 mL. The solution was then treated with 15 mL of 3.8 % (w/v) boric acid solution. Suddenly, 3 mL of iodine solution (12.5 g/L resublimed iodine in 25 g/L potassium iodide) was added and the mixture was diluted to 50 mL with dH_2O. The OD_{690} of the mixture was measured against a reference solution prepared by diluting 15 mL of 3.8 % (w/v) boric acid and 3 mL of iodine solution to 50 mL with water. The concentration of PVA is calculated by reference to a calibration curve prepared using standards (0-20 mg/L), and relating mg/50 mL PVA and OD_{690}.

Determination of polymer release from PVA gel in water

The trials were set-up in 1 L stirred jar, at 30 °C, with 500 mL of dH_2O, by supporting the PVA gels on nylon net and fixing it at the air/water interface with sewer thread (Figure 4.3). Two gels (gelA) were used after rinsing (three times, 10 minutes, dH_2O), two (gelB) directly (no wash). Every 24 hours a sample (30 mL) was taken from the jar for PVA release determination and the volume was re-established in the jar by adding the corresponding removed amount of dH_2O. This implied a dilution that must be taken into count during calculations. The PVA release was also evaluated on wash solutions.

Figure 4.3: Image of the PVA release test.

T. versicolor laccase protein engineering and PAHs recognition

Computational docking and rational design

The X-ray structure of *Trametes versicolor* laccase complexed with 2,5-xylidine (1KYA) [146] was used for the wild type enzyme calculations. The centre of the binding site was taken from the coordinates of the 2,5-xylidine complexed to the enzyme in the X-ray structure PDB. Mutants model structures were predicted by I-TASSER server [179] using the wild type as a template structure. The PDB files of wild type and mutant model structures were prepared for docking using the dockprep tool in UCSF Chimera package [180].

For computational docking, a selection of substrate and pollutants was used as small ligands: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-Dimethoxyphenol (2,6-DMP), anthracene (ANT), fluoranthene (FLUO), bisphenol A (BPA), pentachlorophenol (PCP), 2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD), dibenzodioxin (DD), and dibenzofuran (DF). These small molecules were prepared using MarvinSketch [Marvin 5.9.0, 2012 (http://www.chemaxon.com/)] to draw the chemical structure, add all hydrogen and save it in 3D coordinates, whereas OpenBabel [181] was used to convert it into Mol2 file format.

Computational docking was performed by means of SwissDock server (www.swissdock.ch) based on the docking software EADock DSS [182], with a user-defined box (15Ax15Ax15A) centred on the centre of the catalytic binding site of the enzymes as investigated region. Briefly, an efficient tree-based Dihedral Space Sampling (DSS) algorithm generates 15000 binding modes that are subsequently minimized (100 steps of steepest descent algorithm and 250 steps of adopted basis Newton Raphson algorithm) [182]. Redundant binding modes and binding modes making little or detrimental interactions with the protein are removed. Simultaneously, the CHARMM [183] energies of the remaining binding modes are estimated on a grid. Then, binding modes with the most favourable energies are ranked, taking account of the solvent effect using the FACTS implicit solvation model [184], and clustered by root mean square deviation (RMSD) with a distance cut-off of 2 Å. Finally, the most favourable clusters are dumped into the result file with a value of estimated ΔG of binding.

Starting from computational docking results and information on the enzyme catalysis, from the available literature, some mutations were selected for heterologous expression in *P. pastoris*.
Molecular graphics and analyses of docking results were performed with the UCSF Chimera package [180], or with Pymol [185].

Mutagenesis

The laccase gene (sequence accession number, D13372, [146]) of a *Trametes versicolor* laccase was used as a wild type starting DNA sequence. The wild type DNA have been synthesized by Genscript service (Genscript USA Inc.) and cloned into the vector pPICZαA (Invitrogen - Lifetechnologies Corporation) (Figure 4.4).
The QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene-Agilent technologies) was used to mutate the wild type laccase gene sequence in the vector pPICZαA, according to the manufacturer's protocol. The mutants F162A/L164A, D206N, F332A, F162A/L164A/D206N, F162A/L164A/F332A and F162A/L164A/D206N/F332A were constructed by PCR using the following primers:

- F162A/L164A Fwd: GCTTGGTCCAGCTGCTCCTGCGGGAGCAGATGC
- F162A/L164A Rev: GCATCTGCTCCCGCAGGAGCAGCTGGACCAAGC
- D206N Fwd: GGTTTCACTTAGTTGTAATCCAAACTATACCTTCTCTATTGACGG
- D206N Rev: CCGTCAATAGAGAAGGTATAGTTTGGATTACAACTAAGTGAAACC
- F332A Fwd: GCTATTAACATGGCCTTTAACGCCAACGGTACTAACTTTTTC
- F332A Rev: GAAAAAGTTAGTACCGTTGGCGTTAAAGGCCATGTTAATAGC

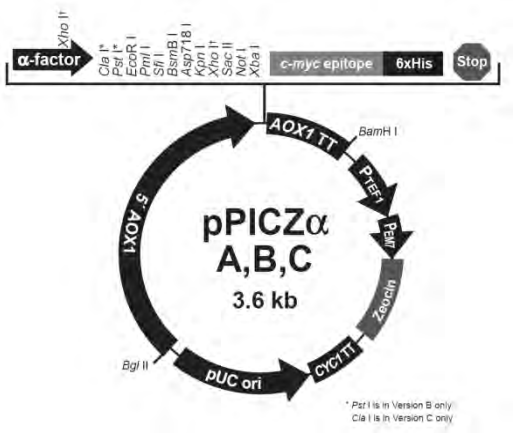

Figure 4.4: Image of the pPICZαA [186].

PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega Corporation) and used to transform DH5α *E. coli* cells. Transformed DH5α *E. coli* cells carrying wild type and mutated laccase gene were grown overnight in 5 mL LB medium (see annex A) supplemented with 100 µg/mL zeocin at 37 °C and 180 rpm. DNA minipreps were made with a GenElute Plasmid Miniprep Kit (Sigma-Aldrich) with an overnight bacterial culture. DNA quantifications were done with a NanoDrop 2000c (Thermo scientific). Each mutated sequence was checked by DNA sequencing by means of Microsynth AG services and BLAST alignment.

Yeast Transformation

Yeast competent cells were prepared as described by the manufacturer [186] (Invitrogen - Lifetechnologies Corporation). Briefly, *Pichia pastoris* X33 cells were grown overnight in 250 mL YPD (see annex A) at 30 °C and 200 rpm in a 1 L flask. The flask was inoculated with 0.1-0.5 mL of an

overnight culture or with 0.1 mL of -80 °C stock culture. The cells were grown to an OD_{600} of 1.2-1.5 and recollected by centrifugation at 1800 rpm for 5 minutes at 4 °C. The pellet was washed with 250 mL of ice-cold, sterile water, centrifuged and then resuspended with 125 mL of ice-cold, sterile water. After another centrifuge, the pellet was washed in 20 mL of ice-cold 1 M sorbitol, centrifuged and then resuspended in 1 mL of ice-cold, sterile 1 M sorbitol for a final volume of about 1.5 mL.
The pPICZαA vectors carrying the laccase genes (5-20 µg/µL) were cleaved with PmeI (NewEngland Biolabs Inc.), purified with Wizard SV Gel and PCR Clean-Up System (Promega Corporation) and resuspended in dH2O to avoid salt failure during electroporation. The cleaved DNA was used to transform P. pastoris competent cells (40-80 µL of competent cells with 0.5-2 µL of linearized DNA) by electroporation (25 µF, 200 ohm, 1.5-2 kV) with a GenePulser apparatus (Bio-Rad Laboratories Inc.). Immediately after electroporation 1 mL of ice-cold 1 M sorbitol was added to the cuvette and the content was transferred to a sterile 15 mL tube, incubated for 2 hours at 30 °C without shaking.
The electroporated cells were spread onto YPDS agar plates (see annex A) supplemented with 100 µg/mL zeocin and incubated at 30 °C for 72-96 h. Transformants (carrying wild type and mutated laccase gene) were analyzed to determine the phenotype (Mut^S or Mut^+) as outlined in the Pichia Expression kit manual, and screened for production of laccase. Briefly, colonies were patched on MD agar plate (see annex A) and on minimal methanol MM/Cu/ABTS agar plate (see annex A) containing 0.5 % (v/v) methanol to induce expression of laccase, 0.3 mM $CuSO_4*5H_2O$ and 0.2 mM ABTS (Sigma Aldrich). Colonies that produced the largest green halo on the MM/Cu/ABTS plates (indicative of the secretion of active laccase) were chosen. Production of laccase was verified by growing the transformants in 250 mL flasks and analyzing the supernatant from methanol-induced cultures by enzyme activity assays, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel activity assays utilizing ABTS (zymogram). The best laccase producers (wild type and mutants) were grown in 50 mL BMGY to an OD_{600} of 20, and then the biomass was recollected by centrifugation, washed twice with phosphate buffer saline pH 7.2 (PBS) and resuspended in 7.5 mL BMGY and 2.5 mL of glycerol. This cells stock solution was dispensed, frozen in a liquid nitrogen bath, and stored at -80 °C.

P. pastoris culture conditions

P. pastoris transformants that had a Mut^+ phenotype were grown 16-24 hours in 50 mL BMGY (see annex A) at 30 °C and 150 rpm to an OD_{600} of 1.0-1.5 (preculture phase); 0.2 mL from a -80 °C stocks were used as inoculums. Overnight cultures were harvested by centrifugation (3000 rpm, 10 min, 4 °C) and washed twice with phosphate buffer saline pH 7.2 (PBS). The pellets were resuspended in 500 mL BMM supplemented with 0.3 mM $CuSO_4$ (see annex A) to an OD_{600} of 1.0 (methanol induction phase). Transformants were cultivated at 20 °C and 150 rpm, and methanol (100 %) was added daily (final concentration 0.5 % (v/v)) to maintain induction. Samples were taken daily for spectrophotometric determination of cell growth, protein concentration (see below) and laccase activity. During laccase production in *P. pastoris*, cell growth was monitored spectrophotometrically by measuring the OD_{600} (Biophotometer, Eppendorf AG).

Optimization of culture conditions for protein expression

The effect of copper on laccase activity was evaluated in BMM media supplemented with various concentrations (0-0.5 mM) of $CuSO_4$. The effect of buffering the cultures was assessed by comparing the growth and enzyme expression with MM and BMM media. The effects on laccase expression of temperature (20-30 °C), and methanol feed (final concentration 0.5-2 % (v/v)) in BMM medium were also investigated.

SDS-PAGE, zymogram and westernblot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 10 % (w/v) polyacrylamide gels under reducing and denaturing conditions with a Mini-PROTEAN II system (Bio-Rad, Laboratories Inc.). 15 µL of sample (supernatant or purified laccase) was loaded on the gel together with 15 µL of sample buffer (100 mM Tris-HCl pH 6.8, 15 % glycerol, 2 % SDS, 0.015 % Bromophenol Blue, 5 % β-mercaptoethanol, 100 mM dithiothreitol), without thermal treatment. The proteins in the gel were stained with Coomassie Brilliant Blue R-250 or by silver staining. The molecular weight of the enzyme was estimated by using a molecular weight marker (Precision Plus Protein Standards, all blue, pre-stained, Bio-Rad Laboratories Inc.).

Zimogram analysis was performed by using a 10 % (w/v) polyacrylamide gels under reducing and denaturing conditions as for normal SDS-PAGE. The gel was run and then washed for 10-15 minutes in dH_2O and then stained. The ABTS staining was performed by incubating the gel in a solution containing 0.4 mM ABTS in 50 mM acetate buffer, pH 5.0, on a shaker for 15-30 min at room temperature.

For the westernblot, a regular SDS-PAGE gel was prepared and run. Then, the gel was transfer (1 h) in transfer buffer (25 mM Tris-HCl, 0.2 M glycine, 10 % methanol, 0.01 % SDS) at 4 °C in the Mini-Protean Electrophoresis Module Assembly (BioRad) and blocked in blocking solution (PBS + 0.1 % Tween20 + 5 % milk) 1 h or more (overnight). Then, the membrane was developed by incubating 1-3 h at RT or overnight at 4 °C with Anti-His Tag (1 g/mL in PBS + 0.1 % tween + 5 % BSA + 0.05% NaN_3), and subsequently 1 h in the secondary antibody (Anti-Mouse Peroxidase conjugated, diluted 1:10000 in washing buffer + 2 % milk). The membrane was rinsed in washing buffer (PBS + 0.1 % Tween20). Then, the membrane was incubated in washing buffer supplemented with 2 mL of Super Signal Pico Chemiluminescent Substrate Pierce (1 mL of Enhancer + 1 mL of Superox). LAS 4000 was used to read the chemiluminescence of the membrane.

Enzyme activity assays

Two substrates have been used in different conditions to test the enzymatic activity of laccase enzyme in 96 well plates (Corning Incorporated - 3799) at 22 °C: a phenolic one (2,6-DMP), and a non phenolic one (ABTS). The oxidation of ABTS led to an absorbance increase at 420 nm (ε_{420nm}= 36,000 L/mol*cm) [256], while the oxidation of 2,6-DMP led to an absorbance increase at 468 nm (ε_{468nm}= 49,600 L/mol*cm) [256]. One unit is defined as the amount of enzyme that oxidizes 1 µmol of substrate per minute. The effect of temperature on laccase stability were evaluated incubating laccase samples at 4, 22 °C and room temperature (>23 °C) for 96 h. The measurements were performed twice.

ABTS reaction mixture: 20-100 mM sodium acetate pH 3.0-6.0 or 20-100 mM phosphate buffer pH 6.0-8.0, ABTS 0.1 mM, enzyme sample 50 µL (culture supernatant or diluted purified enzyme), water up to 240 µL.

2,6-DMP reaction mixture: 20-100 mM sodium acetate pH 3.0-6.0 or 20-100 mM phosphate buffer pH 6.0-8.0, 2,6-DMP 1 mM, enzyme sample 50 µL (culture supernatant or diluted purified enzyme), water up to 240 µL.

Laccase purification

Cultures were harvested by centrifugation (3000 rpm, 10 minute, 4 °C) 12-96 hours after the methanol induction. The supernatants were concentrated with concentrator (VivaSpin 15R, cut-off 5 kDa, Sartorius Stedim Biotech GmbH), or by means of a Cogent (Millipore, cut-off 50 kDa) and then dialyzed overnight (cut-off 5 kDa) against 20 mM sodium phosphate buffer pH 7.5, 0.5-1 M NaCl (buffer A). Dialyzed supernatants were loaded on an affinity column (Hi-Trap Chelating HP, 5 mL, GE

Healthcare) previously equilibrated with 0.1 M $NiSO_4$ and then with buffer A. When the sample were loaded, the column was washed with 20 mM sodium phosphate buffer, pH 7.5, 200 mM NaCl (buffer B), and then eluted with 20 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl, 200 mM imidazole (buffer C).

The laccase activity of eluted fractions were assayed as stated before, and the one with the highest activity was concentrated and loaded on a size exclusion column (Superdex 200 10/300 GL, 25 mL, GE Healthcare), previously equilibrated with 20 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl. The laccase activity of eluted fractions was assayed as stated before, and the ones with the highest activity were pooled and concentrated. The protein concentration was determined by measuring the OD_{280}, and using calculated laccase parameter (MW= 54.5 kDa; ε_{280nm}= 60,975 L/mol*cm) [187]. The samples were eventually precipitated by acetone precipitation.

Decolourization assays

Purified enzymes (wild-type and laccase mutants) were tested for dyes decolourization on 96well plates (Corning Incorporated - 3799). Dyes stock solutions were prepared according to the table below (Table 4.1).

Table 4.1: Dyes stock solutions and related information.

Dye	MW [g/mol]	λ [nm]	stock sol.	Final concentration [µM]	pH
Sudan black B	456.54	600	5 mM in 10 % (v/v) ethanol	1000	5.0
Ponceau S (acid red 112)	760.57	500	0.01 % (w/v) in 0.05 % (v/v) acetic acid	26.3	2.0
Eosin Y (acid red 87)	691.85	500	0.008 % (w/v) in 1.5 % ethanol	23.1	4.0
Alcian blue 8G (Ingrain blue 1)	1298.86	580	0.375 % (w/v) in 1.125 % (v/v) acetic acid	577.4	2.0
Coomassie brilliant blue R250 (acid blue 83)	825.97	600	5 % (w/v)	12107	8.0
Safranin O (basic red 2)	350.84	500	0.005 % (w/v)	28.5	5.0
Xylene cyanol (acid blue 147)	538.61	600	0.1 mM	20	5.0
Toluidine blue	270.374	660	0.01 % (w/v) in 0.78 % ethanol	74	4.5
Methylene blue	319.85	675	0.005 % (w/v) in 75 mM sodium acetate pH 5.2	31.3	5.0
Poly R-478	n.d.	520	0.04 % (w/v)	n.d.	5.0
Bromophenol blue	669.96	600	0.125 mM	25	5.0
Hematoxylin (natural black 1)	302.28	500	0.041 % (w/v)	271.3	4.5
Methyl green	653.24	600	0.1 % (w/v)	306.2	4.0
Remazol brilliant blue R	626.54	600	0.5 % (w/v)	1596	4.0

The enzymatic decolourization of dyes was performed in presence or absence of 1 mM redox mediator (or 5,5'-Hydrazinebistetrazole (HBT), Glycine, Cysteine, Imidazole) in 20 mM sodium acetate buffer pH 3.0-6.0, containing 8 μg/mL of purified laccase. The reaction mixtures were incubated at room temperature and the decolourization activity of wild type and mutated laccases was determined spectrophotometrically as a relative decrease of absorbance. The wavelengths of tested dyes are indicated in Table 4.1. To determine the maximum wavelength of absorption of dyes, spectra between 800 and 200 nm were spectrophotometrically determined. Controls without enzyme and in the presence of different mediators were included. All reactions were performed at least in duplicate.

5 - Results and discussion

Vibrio fischeri: bioluminescent bacteria and broad acute toxicity

Environmental temperature changes along with seasons and this influences the growth and the behaviour of a microbial culture. In order to take advantage of the bioluminescent reaction to detect pollutants in the environment, a steady-state culture must be obtained, and the influence of physical-chemical parameters on the growth must be known. *V. fischeri* is a marine bacterium, well adapted to oceans and seas, where it could be applied for a real-time monitoring application. The Mediterranean Sea temperature oscillates between 15 and 21 °C during winter and summer respectively [188]. Initially, to evaluate growth conditions related to the possible application field, 20 and 30 °C were chosen as tested temperatures.

In different batch trials conducted at 20 °C, the stationary phase of growth was reached in less than 20 hours, with an OD_{620} around 3.5: is possible to see very reproducible growth and pH trends and a light emission that start around an OD_{620} value of 2.2-2.4 RU, and is stable for 25-30 hours. Instead, if the batch is performed at 30 °C, the exponential phase last almost the same time (less than 20 hours), reaching similar maximum OD_{620}(around 3.5) at the stationary phase, but a very dim (or no) light emission.

Concerning pH, it is reduced during the early growth phase (15-20 hours), then it increases along with biomass growth until the end of the trial, but with a slower rate during the stationary phase if the temperature is set at 30 °C. This kind of behaviour have been seen in all tested repetitions, possibly due to nitrogen uptake, or to consumption of organic acid up to that time excreted by bacteria, or both mechanisms. These considerations could not be easily verified in the complex medium used (SW).

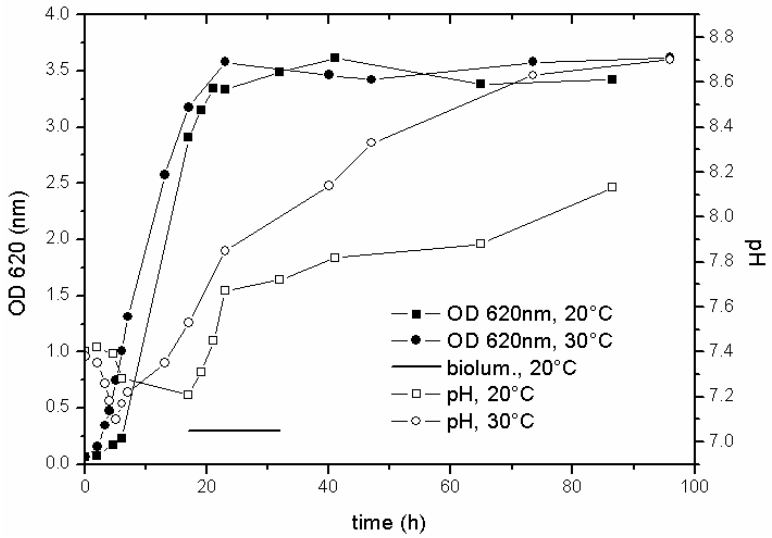

Figure 5.1: OD_{620} and pH of cultures performed at 20 ° and 30 °C

In Figure 5.1 is possible to see the growth curve of two tests, representative of a batch conducted at 20 °C and at 30 °C. At 20 °C the microorganism show a longer lag phase, but a similar growth in comparison with trials maintained at 30 °C. The pH trends are related to the growth of the cultures, with an acidification during the first part of the exponential phase of growth, and a following increase of pH due to metabolic activity of bacteria. Moreover, the bioluminescence of the cultures is different, with a stable light emission if the temperature is at 20 °C, and a very dim light if is 30 °C.

From these results is possible to say that the optimal growth temperature is 30 °C (reduced lag phase), but this value is not the best for the bioluminescent emission. The observed behaviour is probably related to enzymatic reaction, which is slower or inhibited at 30 °C, and requires high amount of energy that during the light emission are drifted from growth metabolism. At 20 °C, the growth is comparable, but the light is stable for 25-30 hours and, for this reason, during the following tests the temperature was kept at 20 °C.

As stated before (Chapter 3), the bioluminescence is quorum sensing dependent. This implies that the growth and the light emission could be affected by the amount of cells used in the inoculum of the cultures. Cultures with different amount of cells were set-up to verify this hypothesis. Different inoculum percentage influences the slope of the exponential growth phase, as expected, but also the pH trend [82] and the maximum growth.

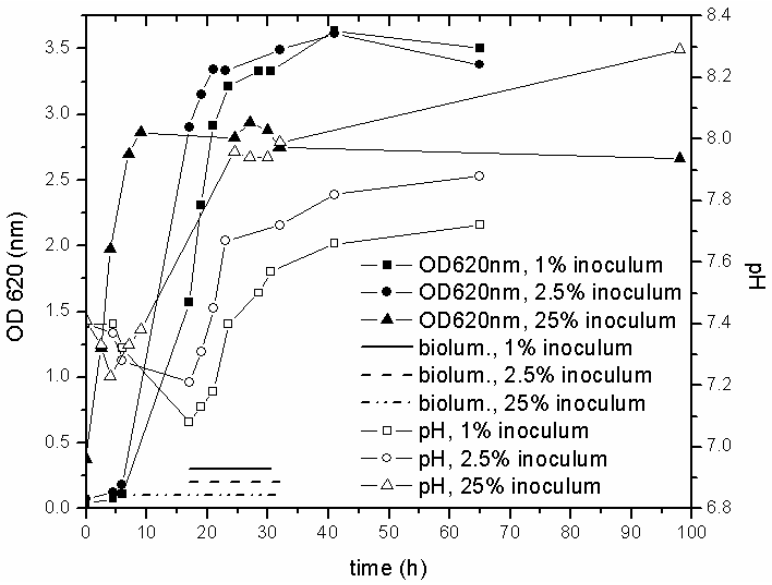

Figure 5.2: OD_{620} and pH of cultures started from different inoculum percentage.

In Figure 5.2 some trials performed with inocula ranging from 1 to 25 % (v/v) are reported. In this plot is possible to see that the higher the amount of biomass in the inoculum, the faster the exponential growth phase, and the earlier the pH start to increase. Although each culture was started from the same medium with identical pH (7.40), if 2.5 % (v/v) of inoculum is used, after 17 hours of culture, OD_{620} is almost double respect to a lower inoculum (1 % v/v), and pH is higher, while with an high inoculum (25 % v/v) the pH start to increase after only 4 hours, when the culture is in the middle of the exponential phase. It's also possible to notice that with a 25 % (v/v) of inoculum the maximum growth evaluated with the OD_{620} was lower in comparison with the other cultures: this is clearly visible after 24 hours of fermentation, when the pH of the culture started with a high inoculum is around 8.0. This effect could be due to an inhibiting factor, such as pH or a lack of nutrients.

The light emission of the cultures was similar, unless for the fermentation time they started to glow. The culture with a high inoculum (25 % v/v) started to glow after 4 hours, while the one inoculated with 1 % (v/v) of biomass started at 17 hours, when the remaining culture (2.5 % v/v inoculum) was already bioluminescent.

These differences between cultures could be due to the inoculum: the lower the inoculum percentage, the longer the adaptation during the lag phase. Regarding the bioluminescence, this hypothesis was confirmed in other tests. At the same time, to understand if pH inhibits the growth if

higher than a certain value, two cultures with different initial pH were set-up. The test is reported in Figure 5.3.

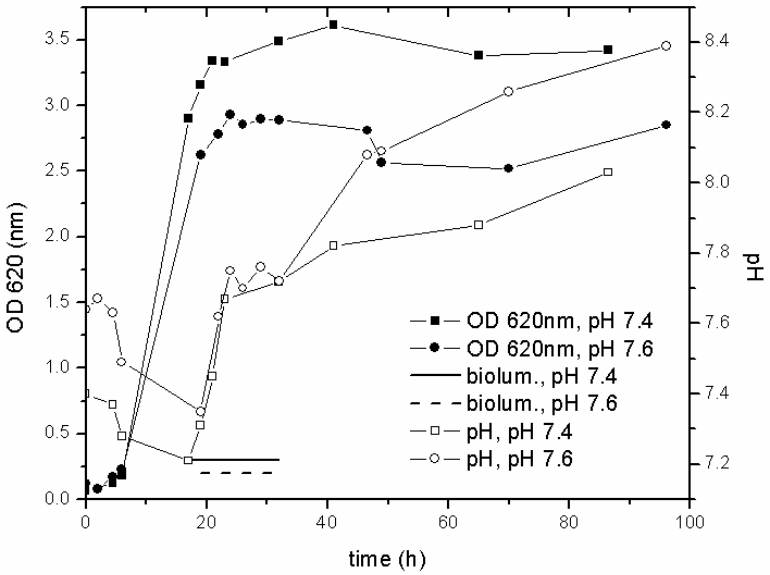

Figure 5.3: OD_{620} and pH of cultures set-up with different initial pH

The two cultures, started from the same inoculum (2.5 % v/v) showed a similar growth during the exponential growth phase, with a corresponding pH trend, and both cultures reached the stationary phase around 20 hours of fermentation, showing a comparable emission of light. The culture started from pH 7.4 get to a higher maximum growth (OD_{620} around 3.5), meanwhile the one started from pH 7.6 achieve an OD_{620} lower than 3.0. It is possible to see that both cultures reached the stationary phase when the pH is approaching 7.8. From this trial it seems that a very little difference of pH (0.2) is able to significantly influence the growth of the microorganism. At the same time, is also important to remember that nutrients usually are the main limiting factor for microbial growth during the late exponential phase: for this reason is not obvious that the pH is the only limiting factor.

Bioluminescence depends on temperature, pH, biomass, oxygen and nutrients, as reported [189, 190, 191]. In previously presented tests, the effect of temperature and biomass was evaluated. In all these cultures, the light emission was stable only for 25-30 h of fermentation that is a too short lapse of time for the application of this system in environmental real-time monitoring. To extend and maintain a stable emission of light, one promising methods could be the use of nutrient fed-batch, especially of the carbon source [189].

On the basis of previous tests, in the following trials the temperature was controlled at 20 °C, the inoculums was 2.5 % (v/v), while pH was uncontrolled and it ranges between 7.0 and 8.5, which is by far included into the recommended 6.0 to 9.0 range for bioluminescence [190]. The effect of glucose fed-batch was investigated: to verify the recovery of bioluminescence, a glucose feed solution was added when the culture reached a dim light emission (48 h), or no light emission (72 h). Biomass growth and pH trends of this test are reported in Figure 5.4. With an early feed (48 h), the emission of light was increased to previous brightness very rapidly (a few minutes), and it was maintained for at least a few hours, while the control culture (no feed) wasn't able to recover the bioluminescent emission, that slowly decreased to a not detectable level (Figure 5.5). The same effect was seen with a late glucose feed (72 h), but the recovered bioluminescence was lower in comparison with the one risen with an early glucose feed.

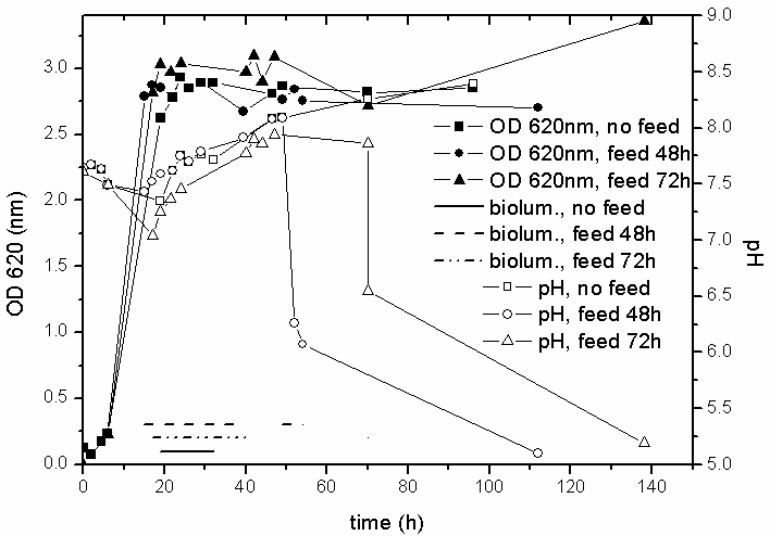

Figure 5.4: OD_{620} and pH of fed-batch culture

After the glucose feed, pH dropped to acid values, with a further decline of pH during the following hours of fermentation. Ruby and Nealson showed that, growing *V. fischeri* on media containing glucose as carbon source, pyruvate tends to accumulate with a pH drop [192]. It is realistic that also in this case acidification is related to the glucose consumption, used mainly as energy for the bioluminescent enzymatic reaction and not as energetic compound for bacterial growth, as shown by the little increase in the measured OD_{620} values. This hypothesis is reinforced by the fact that the bioluminescence, which dissipates a large part of cellular energy, was recovered almost suddenly in correspondence of the addition of nutrients.
Light emission was reduced a few hours after the feeding, maybe because the carbon source was depleted or was lower than a critical concentration. Oxygen, too, may have been limiting during the tests because its concentration in the flask was not controlled. Furthermore, pH came close to acid values, unfavourable to light emission [190]. Probably light emission was affected by all these parameters.

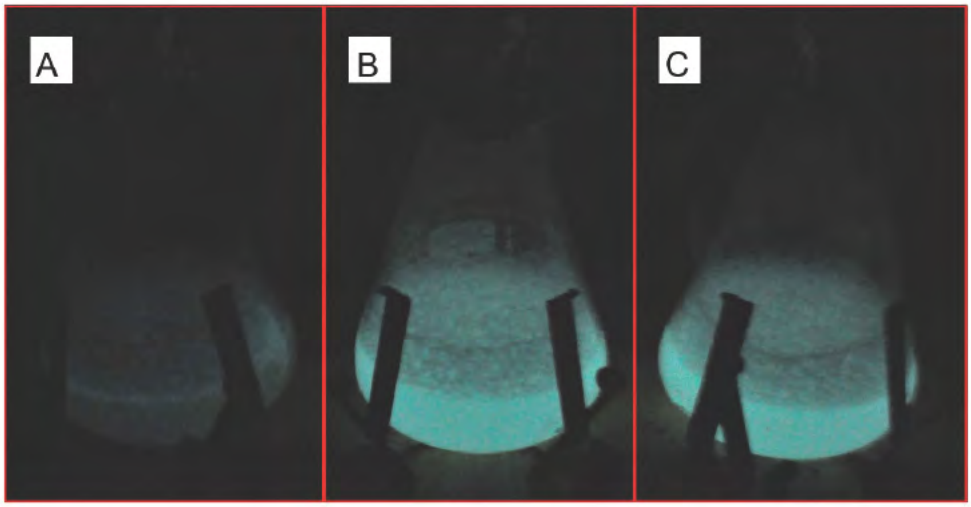

Figure 5.5: OD_{620} and pH of fed-batch culture

These last tests showed how is possible to extend the bioluminescent emission to make it useful to develop a biosensor for environmental real-time monitoring.

Conclusions

During these preliminary tests some bioluminescence controlling parameter (temperature, biomass amount and carbon source fed-batch) of *V. fischeri* were investigated and some considerations on its growth were made.

The bioluminescent emission were visually seen for 25-30 hours during fermentation at 20 °C, starting from an $OD_{620}$of 1.5 (1 % v/v inoculum, Figure 5.2), reached during the exponential growth phase: the culture became rapidly bright as soon as the quorum sensing mechanism activate the expression of the Lux operon, with a later slow decline during the stationary phase. Other trials, conducted at 30 °C, showed a comparable growth but an irregular emission of light, probably because the optimal temperature for bioluminescent emission is lower than 30 °C. Moving forward from these considerations, other temperature values should be investigated, taking into account a useful comparison with environmental data.

Regarding pH, a reproducible pH trend was seen, although, the higher the amount of biomass in the inoculum, the faster the growth during the exponential phase, and the earlier the pH start to raise. These tests also stand out a possible inhibitory effect of pH higher than 7.8-8.0. To evaluate this phenomenon, other tests conducted with pH control (by means of a fermenter) should be planned.

In order to obtain a stable bioluminescent light emission, a glucose fed-batch was investigated. This approach was effective, with a very rapid recovery of luminescence, but the adding of an unbuffered solution dropped the pH of the culture medium. For this reason, to assess the effect of the recovery of the luminescence, and also the nutrients consumption and influence, a buffered minimal medium can be used: nitrogen and carbon sources should be monitored only in a minimal medium, and a useful correlation with bioluminescence and pH should be made. At the same time, oxygen influence, that wasn't controlled during tests, must be investigated too, to complete the evaluation of the system for its application as a biological sensing element in a biosensor for environmental monitoring.

Pseudomonas fluorescens: siderophores and metals toxicity

Preliminary tests: influence of culture conditions on biomass growth and siderophore production

The second biological sensing element tested was based on the regulation of pyoverdine produced by *P. fluorescens*. Growth and behaviour of a microbial culture is always influenced by various physical-chemical parameters: among them, nutrients, temperature, and pH are the most relevant. The study of the effect of these physical-chemical parameters, related to the environmental ones, is one of the first step during the assessment of a new sensing element, especially if it is biological built on. By knowing the influence of these variables on the studied system, it is possible to evaluate its applicability for the development of a portable device.

Carbon source influence

The pyoverdine production seems to be regulated not only by the concentration of iron, but also by the presence of different carbon sources: starting from the pioneering work of Meyer and Abdallah [122], or Cox and Adams [193], until the more recent ones [194], succinic acid is the favourite substrate for pyoverdine production, whilst glucose is regarded as an inhibitor of this mechanism. One possible explanation was proposed by Meyer and Abdallah [122]: they have proved that only Fe^{3+} contamination regulates the siderophore production, and not the nature of a substrate, previously classified into 'chromogenic' and 'anti-chromogenic'. Afterwards, they have also demonstrated that different carbon sources require different amount of iron for the catabolic

assimilation of energy (e.g. 0.20 and 0.14 mg_{Fe3+}/g dry biomass for succinic and citric acid, respectively). This implies that the carbon source influence directly the growth, and indirectly the pyoverdine, that is functionally related to the growth behaviour of *P. fluorescens*. Furthermore, a high pyoverdine production was recently achieved by means of glucose as C source. Cultures with glucose and succinic acid were set-up to evaluate this effect and chose the optimal carbon source for the following tests.

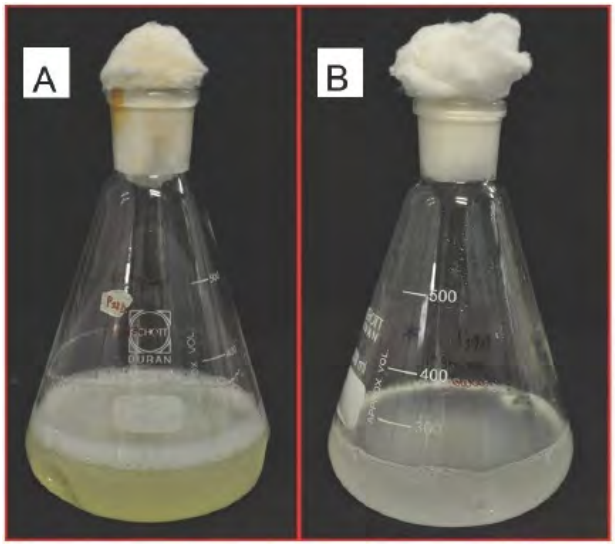

Figure 5.6: Flasks cultures of P. fluorescens grown with different C sources: succinic acid (A), and glucose (B).

In both types of cultures, biomass growth (OD_{620}) was similar until 65 hours of fermentation, then, for the following time-points, the culture grown with succinic acid showed a significant drop of the OD_{620} value, while the culture with glucose remained stable during the last part of the stationary phase (Figure 5.7). The same results were obtained in different trials: in each run conducted with succinic acid the OD_{620} fell down when the pH was higher than 8.4. Probably, basic pH value has an inhibitory effect on growth of *P. fluorescens*. At the same time, this inhibition could be due to a lack of nutrients, or to the secretion by the microorganism of toxic metabolites. The constant raise of pH value in these cultures could be imputable to the consumption of succinic acid, buffered at pH 7.0 with NaOH: is realistic that the depletion of the organic acid left an excess of OH^- in the culture media, with a consequent increase of pH. Other authors [120] reported that *P. fluorescens* cultures grown in succinic acid reach high pH values at the stationary phase (8.0-8.8). This effect was never seen with glucose; indeed the pH trend of cultures grown with glucose was different: an acidification during the exponential phase and a following increase in the stationary one were observed. The most probable hypothesis is that the pH behaviour is linked to glucose and ammonium consumption: it is known that the uptake of nitrogen or the excretion of organic acid by bacteria could increase the pH during fermentation, as already observed in *V. fischeri* cultures.

In these experimental tests, one of the most interesting results is related to the siderophore production, reported in Figures 5.6 and 5.7. The pyoverdine secretion (OD_{400}) increased from the beginning of the test, especially during the exponential growth phase, and until the stationary phase, when a plateau was reached. Succinic acid supported a higher production of pyoverdine (nearly doubled) in comparison with glucose. One possible explanation of this effect is that the pyoverdine molecule is composed by a succinate moiety [195, 206]: the presence of this compound in the culture media represents an advantage for the microorganism that avoids the biosynthesis of part of the siderophore molecule, saving energy for growth and cellular metabolism. This outcome highlights

the direct influence of carbon source on microbial growth and on siderophore production. The close association between these two variables was also highlighted by the trend of pyoverdine biosynthesis which was related to the growth curve.

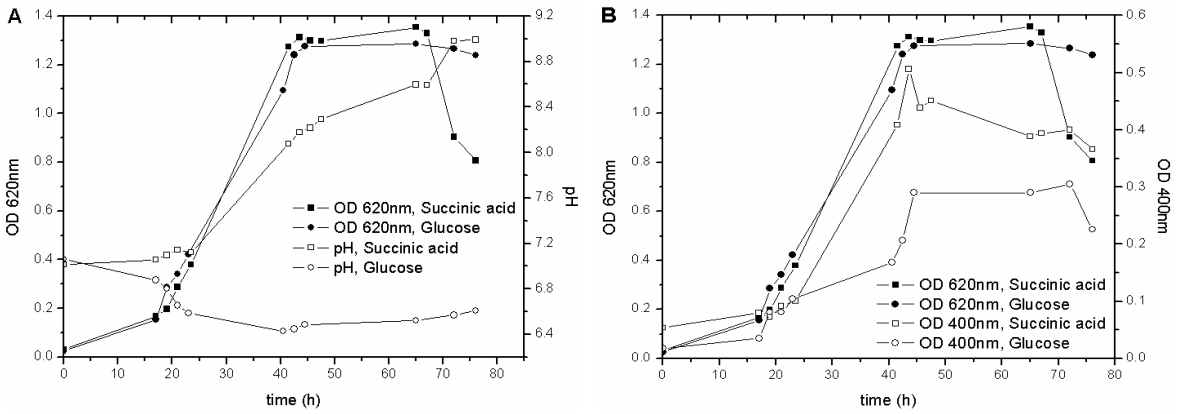

Figure 5.7: Influence of different C sources on biomass growth, pH behaviour (A) and pyoverdine production (B).

The obtained results showed that a higher production of pyoverdine is reached with succinic acid as carbon source, although glucose give better results in terms of culture stability, once the stationary phase is reached. The level of pyoverdine produced could be an advantage for a future application in a biosensor: the higher the production of this molecule, the wider the dynamic range of the system for the final application. For this reason the C source of the culture media for the following tests was 4 g/L succinic acid.

Influence of temperature

The temperature, that in the environment changes along with seasons, was the second physical-chemical parameter studied. In order to assess the influence of temperature (range 15-30 °C) on biomass growth and siderophore production, different tests were performed in BOD bottles or in 500 mL baffled Erlenmeyer flasks.

In Figure 5.8 the results of tests carried out in BOD bottles are reported: growth of *P. fluorescens* was markedly slower than in previous tests conducted in 500 mL baffled Erlenmeyer flasks, probably because of different mixing rates (100 and 130 rpm for BOD bottles and baffled Erlenmeyer flasks, respectively), or to different mixing methods (stirring versus orbital shaking). Otherwise, from these tests is possible to see that the growth was higher in the temperature range 15-25 °C. This was confirmed by the biomass dry weight obtained at the end of each tests, reported in Table 5.1.

Table 5.1: Final biomass dry weight of cultures maintained at different temperatures.

Temperature [°C]	**15**	**20**	**25**	**30**
Biomass dry weight [mg/L]	567	489	547	305

The pH rose during the whole test for each temperature values (data not shown), and highest values were obtained at 30 °C. This effect should be due to a higher stress of the microorganism grown at a temperature near to its upper limit of growth. In fact, from literature it is known that *P. fluorescens* is not pathogenic because its optimal growth temperature is lower than 37 °C, differently from the related pathogenic *P. aeruginosa* [196].

In Figure 5.8 B the pyoverdine production is reported, too. The pyoverdine content at 15 and 20 °C was three to six times higher than in cultures maintained at 25 and 30 °C. The effect of temperature on pyoverdine production was well studied in *P. aeruginosa*: this strain has a higher optimum growth temperature, near to 37 °C [196] and an optimal temperature for pyoverdine production at 30 °C [197]. The results obtained with *P. fluorescens* grown at different temperatures remark this behaviour also for this strain: the optimal growth temperature (25 °C, on OD_{620} and cell dry weight basis) was higher than the optimal temperature for pyoverdine production (15-20 °C).

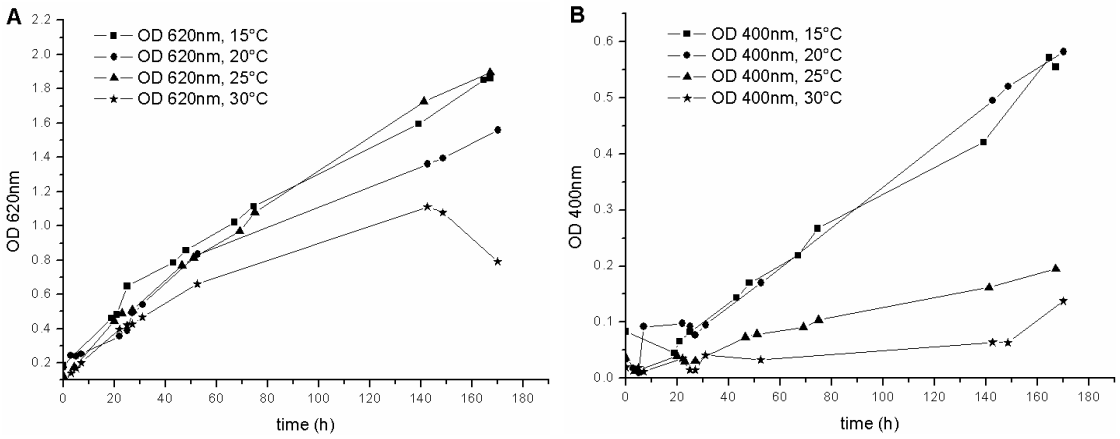

Figure 5.8: Biomass growth (A) and pyoverdine production (B) in cultures set-up in BOD bottles.

Similar results were achieved with cultures conducted in 500 mL baffled flasks. In Figure 5.9 it is possible to see the growth and pH behaviour of two tests, representative of a trial conducted at 20 °C and at 30 °C.

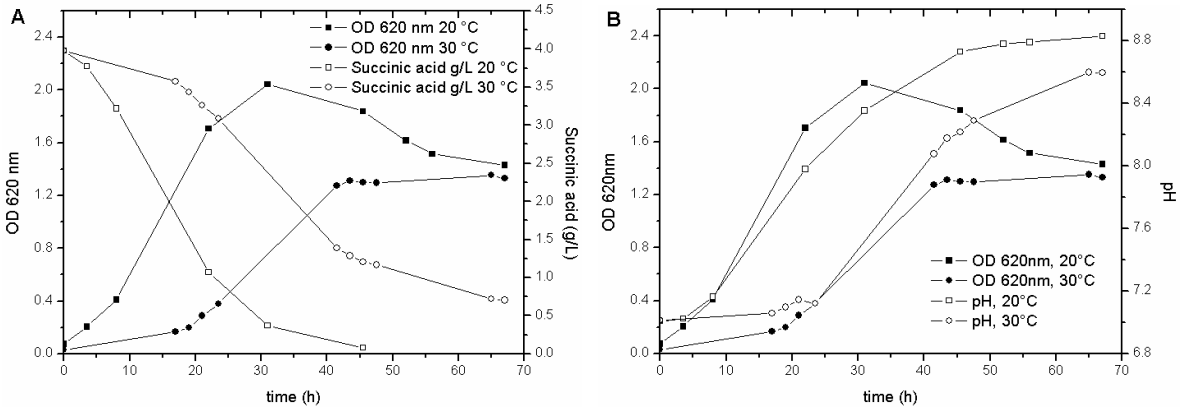

Figure 5.9: Biomass growth, C source concentration (A) and pH trends (B) of cultures performed at 20 and 30 °C.

The stationary phase was reached in less than 20 hours in culture carried out at 20 °C (OD_{620} 2.0 RU), meanwhile at 30 °C the duration of exponential phase was longer (40 hours) and the maximum OD_{620} was only 1.35 RU (Figure 5.9). The pH trends were related to the growth of the cultures and to the carbon source consumption, with an increase of pH value due to metabolic activity of bacteria: a faster consumption of carbon source was observed at 20 °C in comparison with 30 °C. In the former culture the C source is almost depleted at 45 hours of fermentation (pH 8.7), on the contrary, in culture carried out at 30 °C the stationary phase was reached around 40 hours (pH 8.2) and at the end of the test, when pH value was 8.6, succinic acid concentration was 0.7 g/L. These results proved that C source limitation (succinic acid lower than 0.5 g/L), rather than high values of pH, had an

inhibitory effect on the biomass growth. The most relevant result of this test was in connection with the siderophore production: in the culture performed at 20 °C the pyoverdine is almost ten times higher than in the culture maintained at 30 °C (Figure 5.10). This outcome confirms the results obtained with test in BOD bottles.
From these results, it is possible to say that the optimal growth temperature is 25 °C (reduced lag phase and higher maximum growth in low mixing conditions, Figure 5.8), but this value is not the optimal value for pyoverdine production. The best temperature for the production of the siderophore is 15-20 °C.

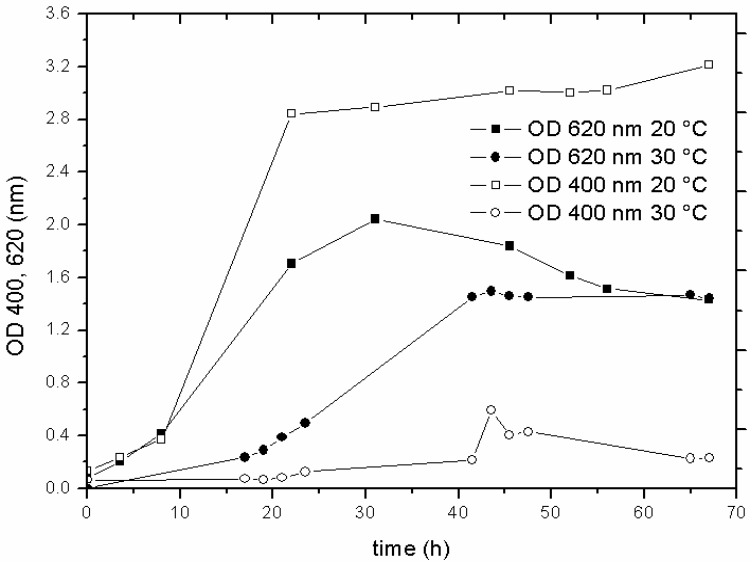

Figure 5.10: Growth and pyoverdine production of cultures performed at 20 ° and 30 °C

A different biomass growth and pyoverdine production was observed in cultures set up in BOD bottles or in baffled Erlenmeyer flask: in the latter, biomass growth and pyoverdine secretion were three to four times higher after 24 hours of fermentation than in BOD bottles. These two cultural devices are characterized by different mixing methods and different exchange area at the gas-liquid interface (A_{gl}). The working volumes were adjusted in BOD bottles to maintain the same gas-A_{gl} ratio of flasks culture, and probably *P. fluorescens* was more influenced by the mixing method. The mixing method is extremely related to the oxygen exchange between the headspace and the culture media: the more turbulent the mixing is, the higher the oxygen concentration dissolved in the medium, and the lower the limitations of this parameter on growth and siderophore production of the microorganism. For these reasons for the following tests only flasks were used.

Influence of the inoculum percentage

In every previous test the siderophore was produced starting from the early phase of microbial growth. This implies that the pyoverdine production could be affected by the amount of biomass used as inoculum. Cultures with different inoculum percentage (1-10 %) were set-up to verify this hypothesis (Figure 5.11).
The higher the inoculum % (v/v), the higher the maximum growth, as expected, but also the higher pyoverdine production. By way of example, the maximum growth and pyoverdine production was reached by the culture started with 10 % (v/v) of inoculum, and this was the amount used for the next tests.

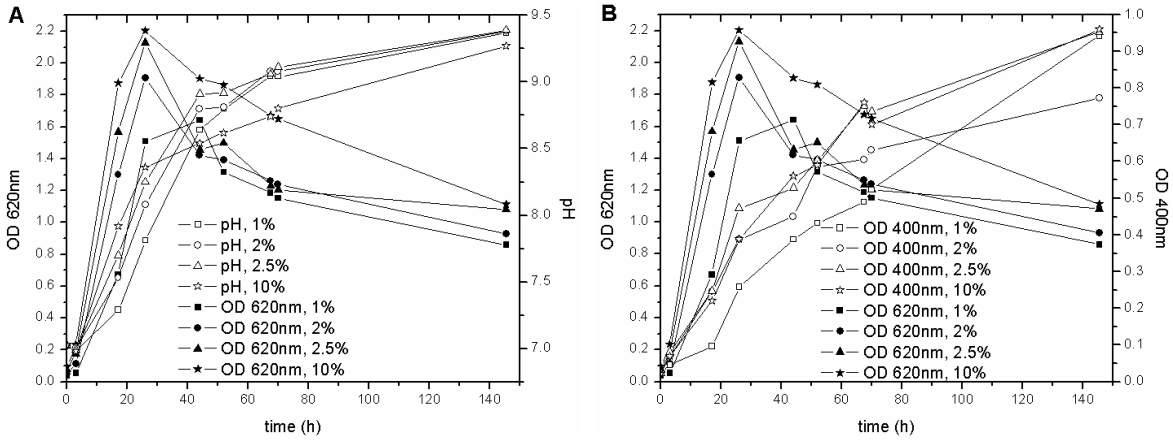

Figure 5.11: Growth, pH (A) and pyoverdine production (B) of cultures started with different inoculum % (v/v)

Influence of culture media buffered at different pH

Once optimal temperature and inoculum percentage for growth and siderophore production were determined, the influence of initial pH was evaluated by modifying the phosphate buffer in M78 medium.

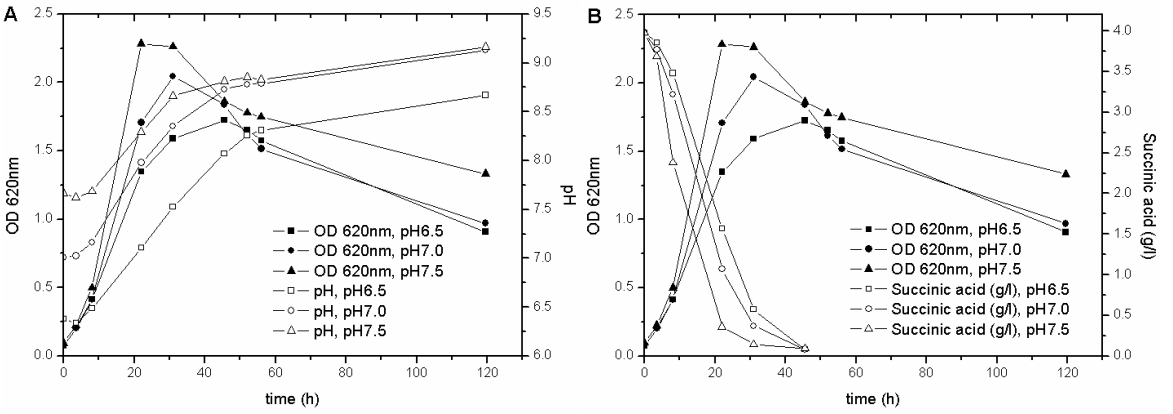

Figure 5.12: Biomass growth, pH (A) and pyoverdine production (B) of cultures started from different initial pH.

In Figure 5.12 the growth curves and pH behaviours of cultures started from media buffered at different pH are shown. Looking at the plot, the upper the initial pH value, the faster the growth of the microorganism during the exponential phase and the earlier the maximum growth are reached. The pH trends and the carbon source concentrations were related to the growth curve of the cultures: the stationary phase was reached after 20, 30 and 40 hours for pH 7.5, 7.0 and 6.5, respectively. Moreover, when the succinic acid was almost consumed (concentration lower than 0.5 g/L), after 20, 30 and 40 hours of fermentation, the pH recorded was 8.3, 8.3 and 8.0, respectively, and the OD_{620} started to diminish, as previously verified. Another time, this confirm that is more important to have a succinic acid concentration above a critical concentration (probably 0.5 g/L), than the pH below 8.4 to maintain the culture alive in stationary phase.

Looking at the plot in Figure 5.13, representing the growth and pyoverdine production, it is possible to notice that the siderophore content was very similar in the different cultures during the exponential growth phase, but once the stationary phase was attained, the lower the starting pH, the higher the pyoverdine secreted by the microorganism. Thanks to this test, it was possible to notice that initial pH values, similar to those usually found in aquatic environment, influences not only the

microbial growth, but also the siderophore system, when the culture is in stationary phase. Further experiments will be useful to fully investigate this influence.

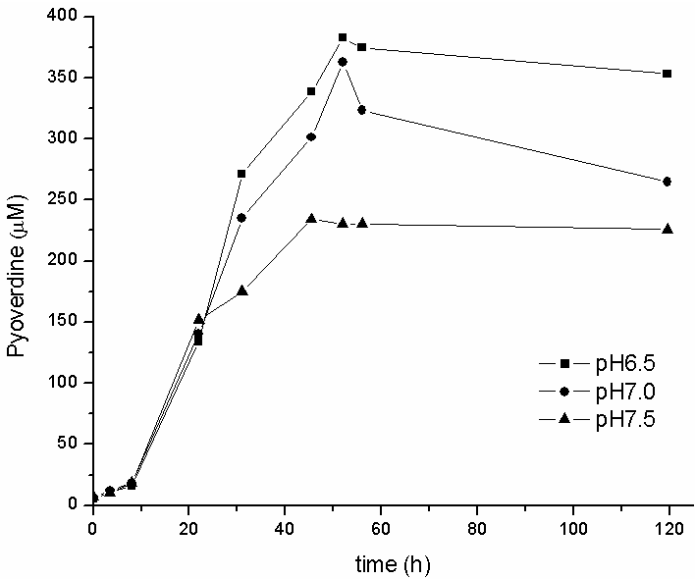

Figure 5.13: Pyoverdine concentration in cultures started with culture media buffered at different pH.

Conclusion

From these tests, it was clear that the pyoverdine production is influenced by carbon source, temperature, inoculum % (v/v), and initial pH. Growth of *P. fluorescens* in standard succinate medium (M78) was accompanied by secretion of pyoverdine from the beginning of the exponential growth phase, and ceased or was greatly reduced as the culture entered the stationary phase. Growth and pyoverdine production were higher with succinic acid as carbon source instead of glucose. A succinic acid concentration above 0.5 g/L was found to be mandatory to maintain the culture alive in stationary phase.

A higher pyoverdine production was observed if the temperature was controlled at 15-20 °C, although the optimal growth temperature was 25 °C. The inoculum % (v/v) influences the slope of the exponential growth phase, the pH trend, the maximum OD_{620} value, as expected, but also the pyoverdine production: the higher the inoculum % (v/v), the higher the values obtained. The maximum pyoverdine production was reached by the culture started with 10 % (v/v) of inoculum.

The influence of initial pH value was studied by means of culture media buffered at different pH. This variable influences not only the microbial growth, but also the siderophore synthesis only during the stationary phase: the lower the starting pH, the higher the pyoverdine secreted by the microorganism.

Evaluation of the interaction between P. fluorescens and Fe^{3+}, Cu^{2+} and Zn^{2+}

Once the influence of physical-chemical parameters was evaluated, assessing the agreement with the environmental variables, the interaction between *P. fluorescens* and metals was studied. This set of tests was relevant to characterize the pyoverdine regulation at different concentration of metals, and then, to evaluate its applicability as a sensing element for environmental monitoring.

Determination of the Minimum Inhibitory Concentration (MIC) of Fe^{3+}, Cu^{2+} and Zn^{2+} on solid media

High concentrations of metals in the environment, due to human activities or to natural occurrence, create selective pressure for microorganisms that have developed several resistance mechanisms,

such as siderophore secretion. As a consequence, to study the interactions between metals and *P. fluorescens*, the first step is to determine the MICs of Fe^{3+}, Cu^{2+} and Zn^{2+}. MIC is generally defined as the lowest metal concentration for which the growth is inhibited after overnight incubation [175]. To establish this inhibitory concentration a modified Kirby-Bauer method was used (Figure 5.14).

The Kirby-Bauer semi-quantitative method, also called antibiogram, is based on the measure of the inhibition halo of a microorganism spread on agar plates, due to diffusion of the tested compound from a filter disk put on top of the plates [175]. In an attempt to create a quantitative assay, a modified Kirby-Bauer method was applied [83]: the disks were soaked with solution at increasing concentration of a given metal (0-0.75 M) and a linear fitting was used to calculate the MIC (Figure 5.15).

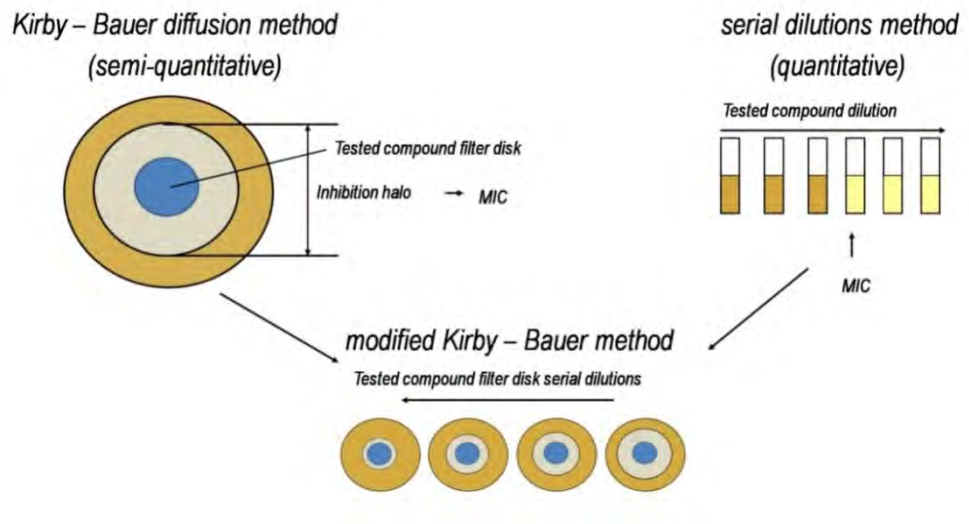

Figure 5.14: New modified Kirby-Bauer method for MIC determination [83].

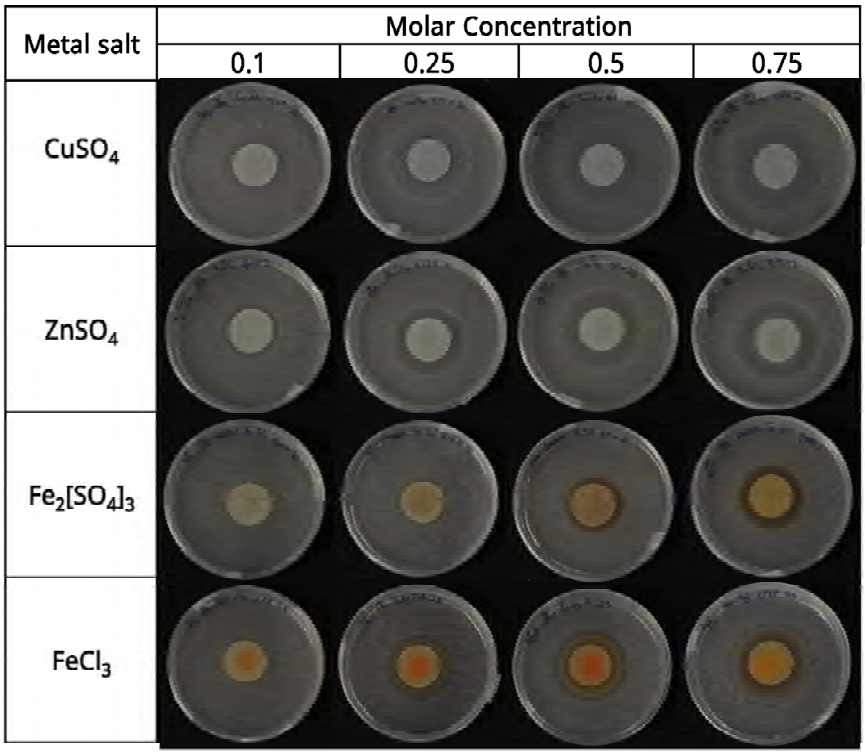

Figure 5.15: MICs agar plates.

In Figure 5.16 the relation between metal concentration and the net inhibition halo is reported: the higher the concentration of the metals on the disk, the larger the inhibition halo. The area of the inhibition halo allowed to calculate the values of MICs for $CuSO_4$, $ZnSO_4$, and $Fe_2[SO_4]_3$, that were 46.30, 54.40 and 74.11 mM respectively. These values correspond to 2.94, 3.56 and 3.65 g/L of metal ions. The plot presented shows that for $Fe_2[SO_4]_3$ a linear correlation between metal concentration and the net inhibition halo subsists. At the same time, for $CuSO_4$ and $ZnSO_4$ a non linear correlation between metal concentration and the net inhibition halo exists. This could be due to a large number of effects. First of all, the molecular weight and the hydrodynamic molecular radius influence the ions movement inside the matrix, which act like a molecular sieve. Moreover, the method is built around the diffusion of the tested compound: every mass transfer limitations could be interfering with this process. Furthermore, the solid media were prepared using agar to harden the culture media. This gelling agent is usually rich in SO_4^{2-} ions, which probably could interfere with the migration in the solid matrix of positively charged ions.

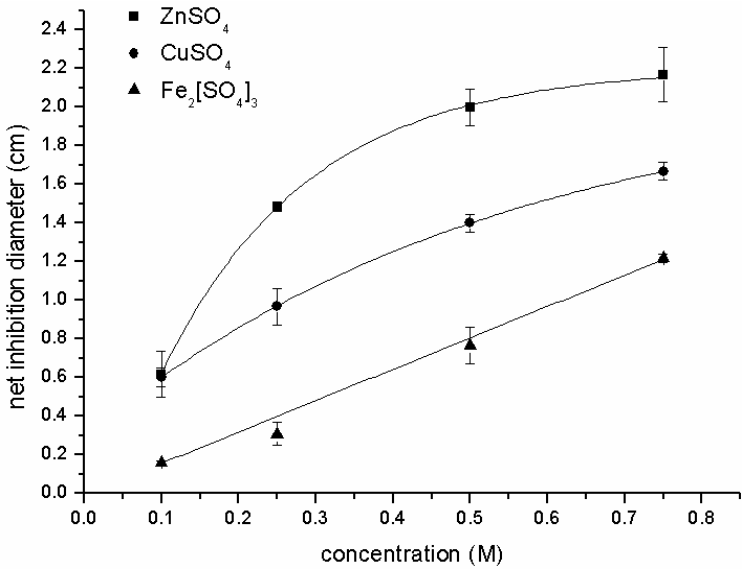

Figure 5.16: Cu^{2+}, Zn^{2+} and Fe^{3+} net inhibition diameter halo.

This new modified Kirby-Bauer diffusive method of MIC assessment revealed an order of strain's metal sensitivity ($Cu^{2+} > Zn^{2+}$) similar to the one reported by Poirier and co-worker [119] for copper and zinc (*P. fluorescens* BA3d12), but a fair correlation with previously published results obtained in 96 well plates [198]. The obtained values were ten times higher on a solid medium than in more homogeneous liquid cultures: probably mass transfer limitations and absorption influenced more the results on agar plates. Poirier and colleagues also reported that diffusion, complexation and availability of metals differ in solid culture media in comparison to liquid one: in the latter the free toxic metal ions evoke lower inhibitory concentrations than in solid medium where these ions are less available [119]. At the same time, this method should be useful to assess MICs simulating the bioavailability of metals in soil, where they tend to be absorbed to particles or complexed to organic compound, such as organic acid (e.g. oxalic acid, or humic acid) [3].

Regarding Fe^{3+}, a counter-ion effect has been observed for Cl^- and SO_4^{2-}. Two different Fe^{3+} salts were tested to evaluate this influence. A lower inhibition was observed for $Fe_2[SO_4]_3$ (74.11 mM, or 4.14 g/L of Fe^{3+}), in comparison with $FeCl_3$ (65.35 mM, or 3.65 g/L of Fe^{3+}), as represented by a slighter slope of the regression line (Figure 5.17). This test clearly showed that SO_4^{2-} is less toxic than Cl^- as

counter-ion. In fact, the iron used during the test was in the same oxidation state, and the only difference during the tests was the counter-ion.

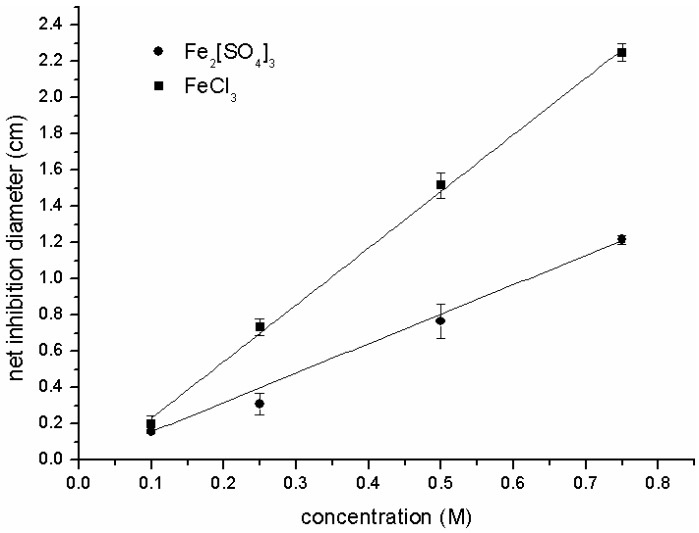

Figure 5.17: $Fe_2[SO_4]_3$ and $FeCl_3$ net inhibition diameter halo.

The values of MICs determined with this experiment are very high if compared with the maximum allowed concentrations of metals in water, indicated by the European (EU) or the World Health Organization (WHO) regulations ((98/83/EC), WHO Guidelines). This means that *P. fluorescens* is able to grow in the range of allowed concentrations of metals in water: then, the system is fully applicable for environmental monitoring, if only growth is considered. The next step toward the development of a system based on pyoverdine is the study of its production in metal supplemented cultures. Starting from this consideration the interaction of *P. fluorescens* with metals was investigated in liquid cultures.

Determination of the Minimum Inhibitory Concentration (MIC) of Fe^{3+}, Cu^{2+} and Zn^{2+} on liquid media

The growth and pyoverdine production were investigated in cultures set up in 500 mL baffled Erlenmeyer flasks, with metal supplemented culture media. The chosen range of concentrations used for these tests was also related to the MICs determined in 96 well plates for the same strain by Workentine et al. [198]. Regarding iron, in this previous work the MIC, corresponding to 6.25 mM, was evaluated using $FeSO_4$. Instead, in this test $FeCl_3$ was preferred because the pyoverdine is only able to bind ferric ions, while its affinity for ferrous ion is very low [114].

In Figure 5.18 growth and pH behaviour of cultures supplemented with $FeCl_3$ are reported. *P. fluorescens* was able to grow at each tested concentration, and then, no toxic effects were recorded in the range 0-6.25 mM, corresponding to 0-350 mg/L of Fe^{3+}, differently from what reported by Workentine [198]. Moreover, a faster growth and higher maximum OD_{620} values were seen by increasing the concentration of ferric ion in solution, in comparison with the control (0 mM $FeCl_3$). It is known that the presence of low concentration of metals could be an advantage during microbial fermentation: particularly, iron seems to be important for growth of *P. fluorescens* [110, 122]. This was also clearly demonstrated by Fallahzadeh and co-worker [197]: a very low concentration of Fe^{3+} and Zn^{2+} in the tap water used for the culture media improved growth and pyoverdine production of a related *Pseudomonas* (*Pseudomonas aeruginosa* 7NSK2).

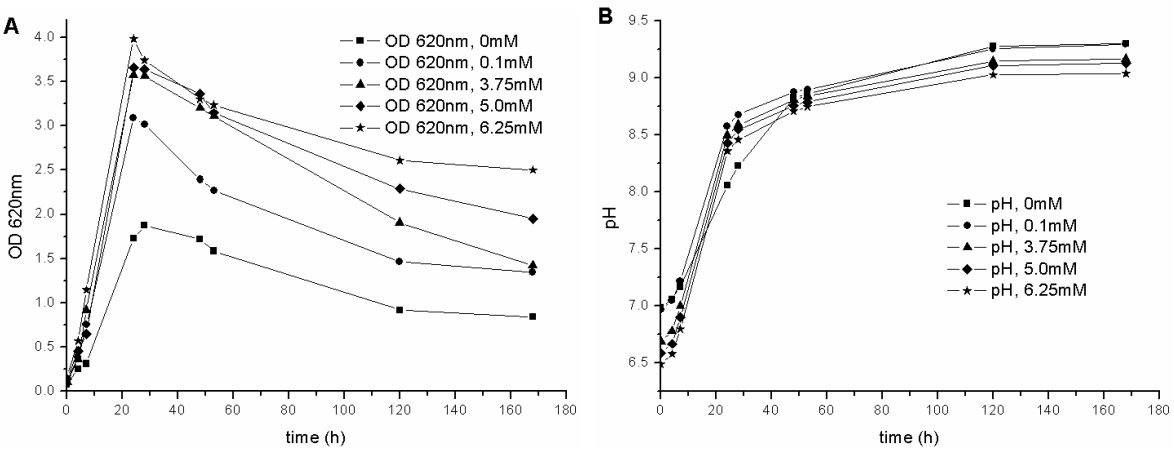

Figure 5.18: Biomass growth (A) and pH trends (B) in cultures supplemented with $FeCl_3$.

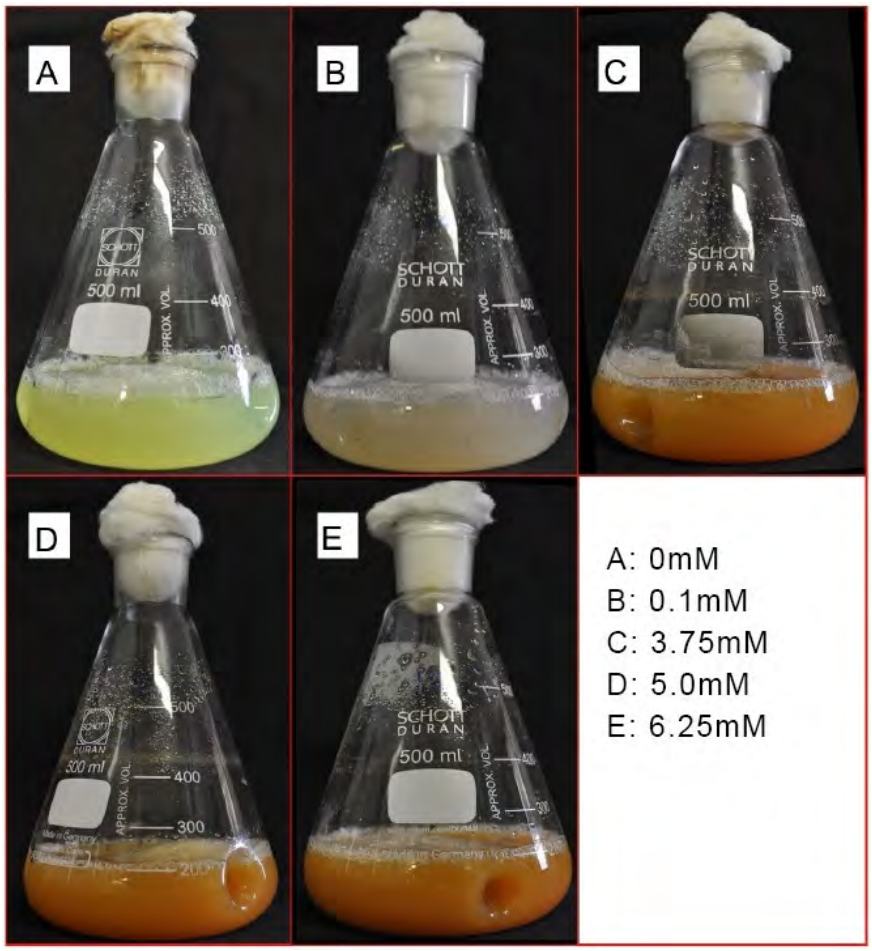

Figure 5.19: Flasks of cultures supplemented with $FeCl_3$.

The pH trends of all cultures were very similar (Figure 5.18), with a slightly different initial pH for cultures in the range 3.75-6.25 mM: the pH of metals stock solution was not adjusted to 7.0, with a resulting acidification of the culture media of these flasks. This difference didn't affect the behaviour of the microorganism, so that after 24 hours of fermentation the pH of all cultures was around 8.5.
Some solubility problems were encountered using $FeCl_3$ in flask tests (Figure 5.19). The solubility of $FeCl_3$ in water is 912 g/L [199], but this holds if this is the only compound present in solution: it is realistic that by adding concentrated salts solution to the culture media, already rich in salts, the solubility product was easily reached, forming insoluble precipitates in cultures with more than 3.75

mM (or 210 mg/L) of Fe^{3+}. Hence, it is reasonable that in these flasks the concentration of bioavailable metal was lower than the nominal concentration added. This hypothesis was supported by the similar growth shown by the cultures in the range 3.75-6.25 mM, and by the carbon source consumption. Growth and succinic acid depletion in these flasks was also greater in comparison with the control culture (0 mM $FeCl_3$).

In Figure 5.20 the pyoverdine content of cultures supplemented with Fe^{3+} is reported. Looking at the plot is clear that the siderophore was produced only in the control flask (0 mM Fe^{3+}). In the other cultures the pyoverdine amount in was very low and similar to the initial content present in the inoculum. These results confirm that the critical concentration of Fe^{3+} that inhibits the pyoverdine production (pyoverdine critical concentration or PCC) is lower than 0.1 mM (or 5.6 mg/L) [122].

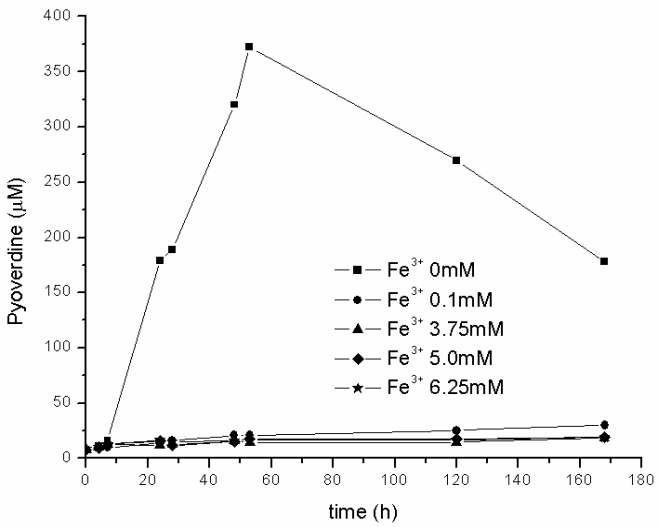

Figure 5.20: Pyoverdine content in cultures supplemented with $FeCl_3$.

A similar experiment was carried out with $CuSO_4$ (Figure 5.22). The chosen concentration range was related to the MIC determined by Workentine et al. [198], and corresponding to 3.13 mM Cu^{2+} ions.

In Figure 5.21 growth, carbon source consumption and pH behaviour of cultures supplemented with $CuSO_4$ are reported. *P. fluorescens* was able to grow only in the range 0-0.1 mM, corresponding to 0-6.0 mg/L of Cu^{2+}. pH trends and carbon source depletion were similar for the control culture (0 mM of Cu^{2+}) and the other cultures supplemented with less than 0.1 mM, or 6.0 mg/L of Cu^{2+}.

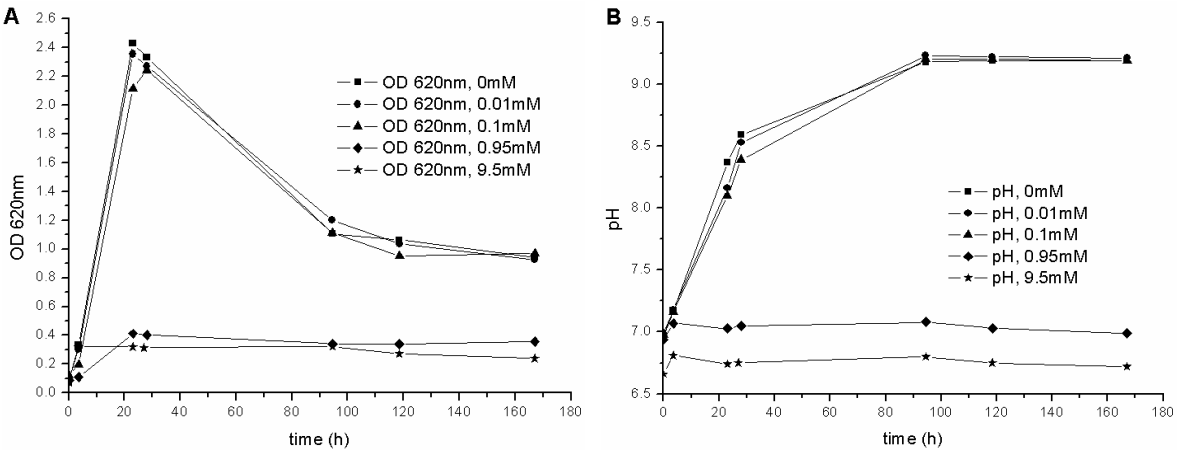

Figure 5.21: Biomass growth (A) and pH trends (B) of cultures supplemented with $CuSO_4$.

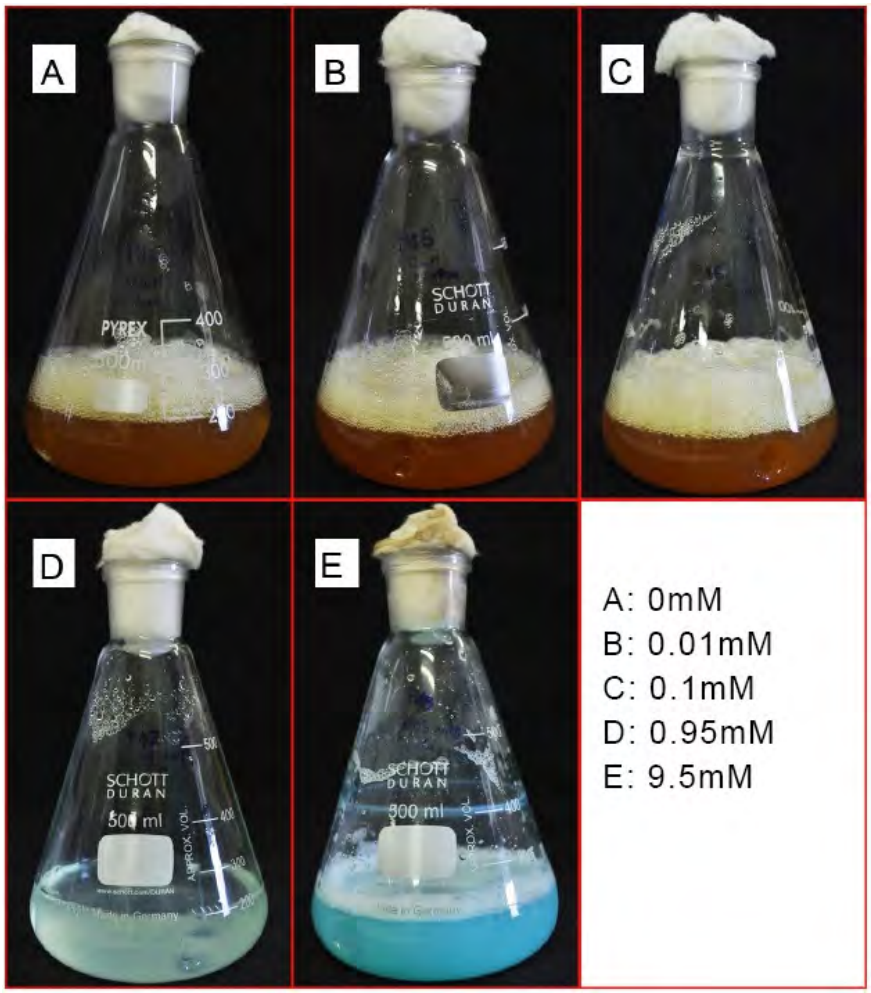

Figure 5.22: Flasks of cultures supplemented with $CuSO_4$.

Some toxic effects were recorded for $CuSO_4$ concentration higher than 0.1 mM (or 6.0 mg/L of Cu^{2+}). Biomass growth in cultures supplemented with 0.95 and 9.5 mM of $CuSO_4$ (or 60 and 600 mg/L of Cu^{2+}) was completely inhibited by the presence of metal in solution. This inhibition was clear also from the recorded pH values, as well as from the negligible carbon source consumption. This means that the MIC for $CuSO_4$ (concentration range 0.1-0.95 mM) is roughly three times lower than the one reported by Workentine and co-worker [198], but agrees with the one reported by Poirier [119].
Furthermore, for this test some problems related to $CuSO_4$ solubility in the medium were encountered. The solubility of $CuSO_4$ in water is 220 g/L [199], and, as stated before for $FeCl_3$, the concentration of bioavailable metal was lower than the nominal concentration added in those flasks supplemented with 0.95 and 9.5 mM of $CuSO_4$.
In Figure 5.23 the pyoverdine content of cultures supplemented with $CuSO_4$is reported: the siderophore was produced only in flasks in which the microorganism was able to grow (0-0.1 mM Cu^{2+}). In these flasks the pyoverdine production was clearly not influenced by the presence of $CuSO_4$ in the culture media: the siderophore content was similar to the control flask (0 mM Cu^{2+}), except for a slightly higher concentration in culture supplemented with 0.01 mM Cu^{2+} during the exponential growth phase. A higher pyoverdine content (+ 185 %) was revealed also by Braud and co-worker [110] at 12 hours of fermentation, in a culture of *Pseudomonas aeruginosa* PAO1 (ATCC 15692) supplemented with the same concentration of copper. These results showed that growth is inhibited for Cu^{2+} concentration higher than 0.1 mM, while no effect on pyoverdine regulation were observed for the lower concentrations tested.

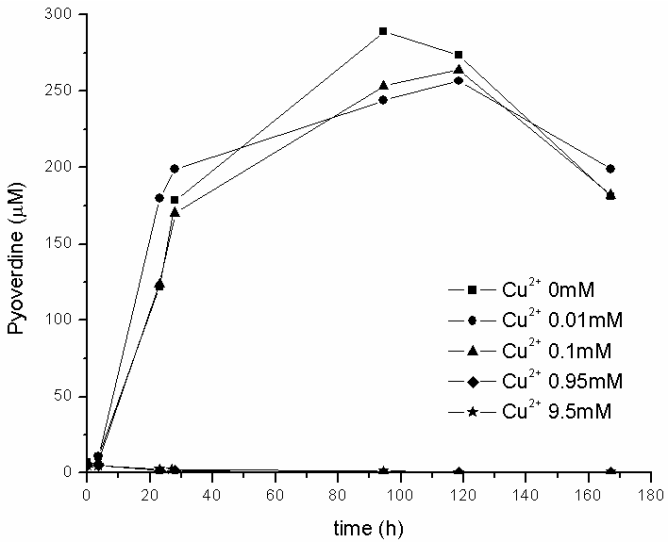

Figure 5.23: Pyoverdine content in cultures supplemented with $CuSO_4$.

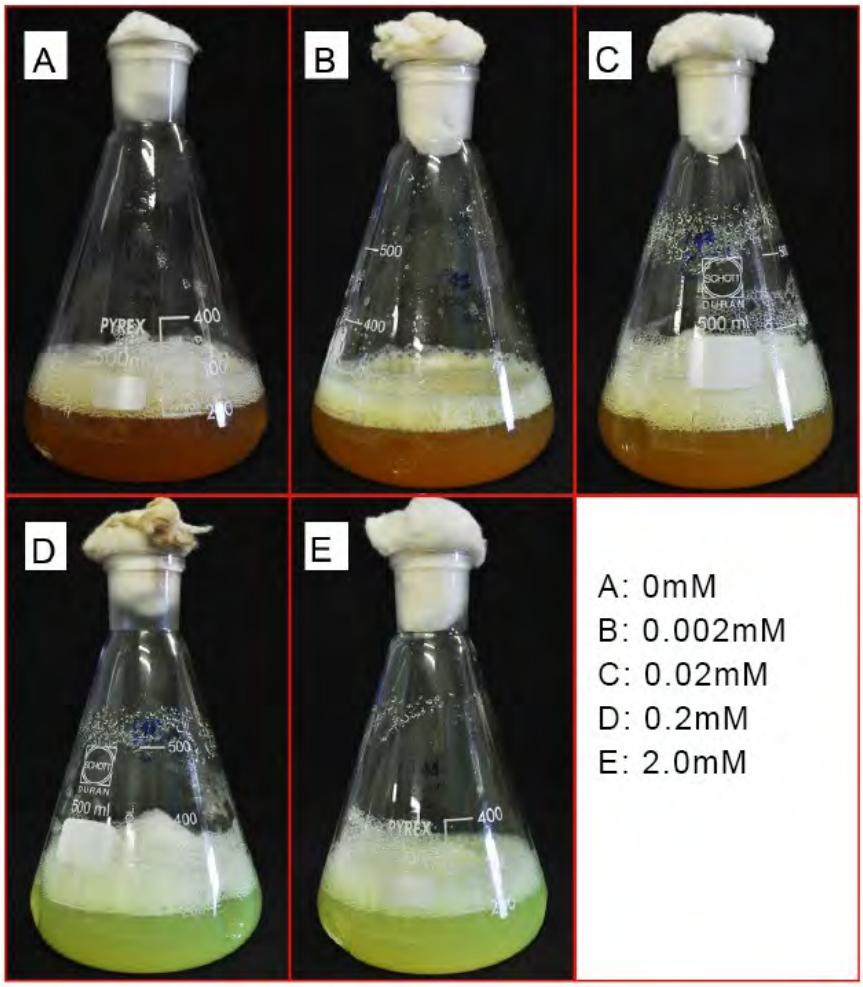

Figure 5.24: Flasks of cultures supplemented with $ZnSO_4$.

Growth and pyoverdine production were investigated in cultures supplemented with $ZnSO_4$ (Figure 5.24), in a concentration range related to the MIC determined in 96 well plates for the same strain by Workentine et allies [198], and corresponding to 1.56 mM Zn^{2+} ions. In Figure 5.25 the biomass growth (OD_{620}) and pH behaviour of cultures supplemented with $ZnSO_4$ are reported. *P. fluorescens* was able to grow at each tested concentration, and then no toxic effects were recorded in the range

0-2.0 mM, corresponding to 0-130 mg/L of Zn^{2+}. On the contrary, a slightly faster growth and higher maximum OD_{620} values were seen by increasing the concentration of zinc ion in solution, in comparison with the control (0 mM $ZnSO_4$). The cultures conducted with 13 and 130 mg/L (or 0.2 and 2 mM) of Zn^{2+} also maintained higher maximum OD_{620} during the stationary phase, and a greenish colour for a longer time, in comparison with control flasks that became brown coloured (Figure 5.24). According to these results, the MIC of $ZnSO_4$ is higher than the values reported by other authors [119, 198]. In the range of tested concentrations, no solubility problems were recorded.

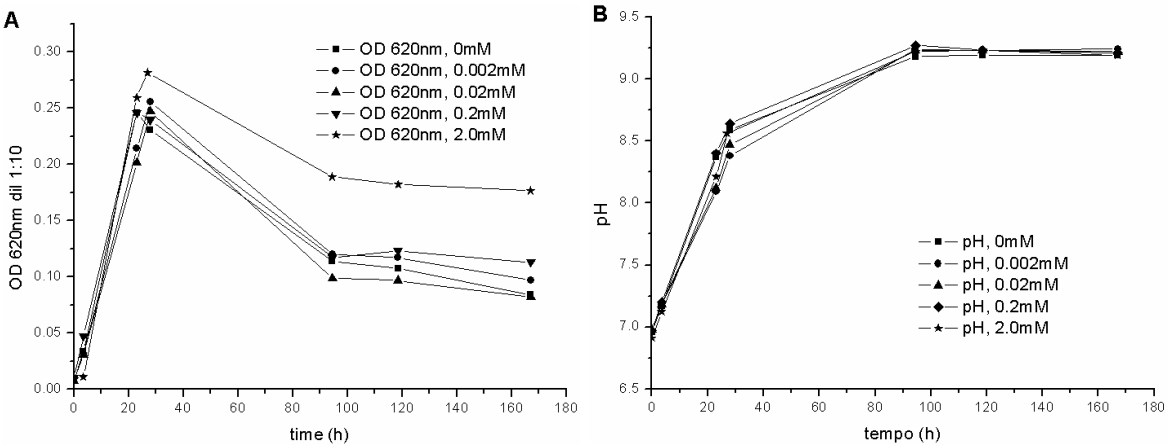

Figure 5.25: Biomass growth (A) and pH trends in cultures supplemented with $ZnSO_4$.

In Figure 5.26 the pyoverdine content of cultures supplemented with Zn^{2+} is reported: the siderophore was produced in every flask, and, then, the pyoverdine production was not influenced by the presence of $ZnSO_4$ in the culture media. The siderophore concentrations were similar in each flask, except for a greater concentration of cultures with 0.02 and 0.002 mM Zn^{2+} during the stationary growth phase. An increase in pyoverdine production was also reported by Braud and co-worker for cultures supplemented with 10µM Zn^{2+} [110]. These results disclose a possible stimulatory effect of zinc on pyoverdine production, but further investigations are required.

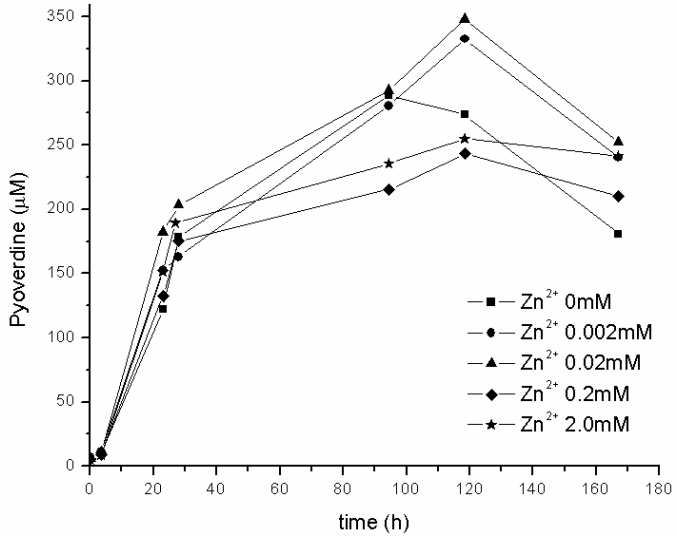

Figure 5.26: Pyoverdine content in cultures supplemented with $ZnSO_4$.

These experiments carried out in 500 mL baffled flask unveiled that MICs determined in liquid cultures are different from those obtained on agar plates. One possible reason of this discrepancy is

that MICs determined on solid media are probably influenced by mass transfer limitations. Moreover, higher (for Fe^{3+} and Zn^{2+}) or lower (for Cu^{2+}) concentrations seem to be inhibiting the growth of the microorganism in comparison with MICs determined by other authors in microtiter plates [119, 198]. For this reason, the MIC was evaluated also in 96 well plates, and the results obtained are reported in Table 5.2. The MICs were evaluated by the serial dilutions method [175] (Figure 5.14), using two different culture media: a complex (DSM1) and a minimal (M78) media.

Table 5.2: 96 microwell MICs results.

Culture media	MIC	$FeCl_3$	$Fe_2[SO_4]_3$	$CuCl_2$	$CuSO_4$	$ZnCl_2$	$ZnSO_4$
DSM1	mM	1.5	3	1.5	1.5	1.5	2.5
	mg/L	83.8	167.5	95.3	95.3	98.1	163.5
M78	mM	>10	>10	<0.5	<0.5	<0.5	<0.5
	mg/L	>558.5	>1117	<31.7	<31.7	<32.7	<32.7

These tests clearly shows that microbial growth is influenced by the presence of different metals, but also by the culture media: the MIC was higher for Cu^{2+} and Zn^{2+} if the complex medium (DSM1) was used, instead for Fe^{3+} the MIC was higher in the minimal medium (M78). According to Teitzel and Parsek [200] too, MICs depend on the culture medium used. Generally, highest values are obtained with a complex growth medium: probably, a high level of metal ions complexation by medium components occurred [119, 200]. This effect led to the reduction (for Cu^{2+} and Zn^{2+}) or increase (for Fe^{3+}) of the bioavailable fraction of metal in solution. At the same time, the iron concentration required for microbial growth, almost certainly not sufficient in the minimal medium, was re-established by supplementing the M78 medium. Instead, for Cu^{2+} and Zn^{2+} only a toxic effect was recorded. The obtained MIC values are similar to the one reported by other authors [119, 198]. Some solubility problems were recorded also in microwell plates for all salts ($FeCl_3$, $Fe_2[SO_4]_3$, $CuCl_2$, $CuSO_4$, $ZnCl_2$ and $ZnSO_4$). For this consideration, it is reasonable that the bioavailable concentration of metal in solution was lower than the nominal added concentration, and, moreover, this precipitate could have produced more false positive while measuring the OD_{600}.

In order to establish if the precipitates also occur without the presence of biomass, a test with the culture supernatant and different metal concentration was set up in 96 well plates, and the optical density was evaluated (OD_{600} and OD_{400}). When the solutions were mixed, a precipitate was suddenly formed at increasing concentration of metal, especially with chloride salts. In Figure 5.27 the OD_{400} is reported; similar trends were recorded at 600 nm.

The turbidity of the solution increased gradually, rising the added salts concentration. The solubility problems were much more important for iron and copper, and the ranking for the tested salts was: $FeCl_3$ > $Fe_2[SO_4]_3$ > $CuCl_2$ > $ZnCl_2$ > $CuSO_4$ > $ZnSO_4$. A reduced OD_{400} was seen in the range of metals concentrations 0-50 mg/L. Probably, pyoverdine in the supernatant (approximately 250 µM) was able to bind the metal ions: it is known that pyoverdine changes its optical properties if an ion is bound to its structure [110, 201]. In some instance, such as with Fe^{3+} or Cu^{2+}, the pyoverdine fluorescence is quenched by the binding of the metal, and its signal is proportionally lowered by the metal concentration [122, 110, 112,], whilst with other element, such as Al^{3+}, Cd^{2+} or Zn^{2+}, the binding to the siderophore increase the fluorescent emission, and shift the absorption maximum [110, 112]. It is reasonable that the effect seen in the range 0-50 mg/L could be related to this phenomenon.

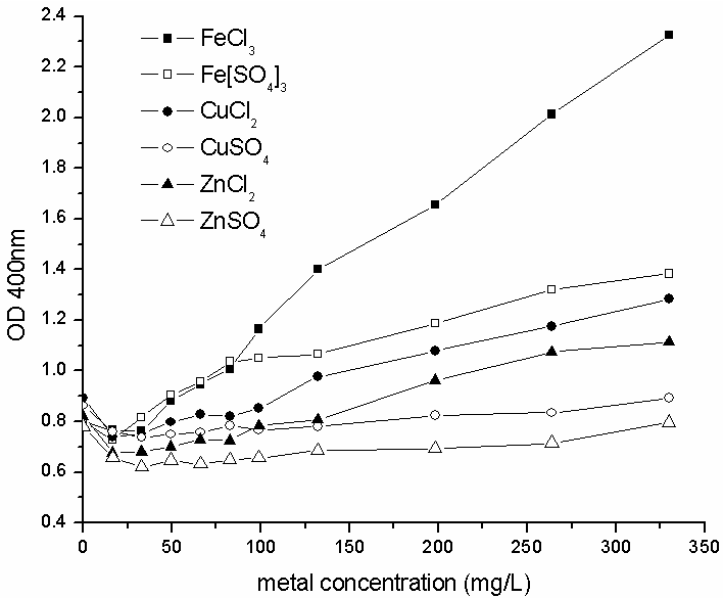

Figure 5.27: OD_{400} of supernatant mixed with different metal concentrations.

A new test was prepared in microwell plates in M78 medium to further verify the inhibitory concentration of Fe^{3+}, Cu^{2+}, Zn^{2+}, determined with previous test: a lower range of metals concentration and a highly diluted inoculum were applied (1% of inoculum) as recommended for MIC evaluations [175].

From this test it was possible to see that with a low percentage inoculum the microorganism didn't grow in the range 0-0.2 mg/L (or 0-3.6 µM) for Fe^{3+}, and 0-2 mg/L (or 0-31.5 µM) for Cu^{2+} (data not shown). At the same time, with low Zn^{2+} concentrations the microorganism was able to grow in the range 0-3 mg/L (or 0-46 µM). Furthermore, after 24 hours the pyoverdine content was directly proportional to Zn^{2+}concentration in the range 0.15-3 mg/L (or 2.3-46 µM) (Figure 5.28), but the control with no metal produced an amount of pyoverdine similar to wells supplemented with 0.75 mg/L (or 11.5 µM). Similar trends were recorded at 48 hours. This effect could be due to the binding of the siderophore to the metal at low concentrations, as reported above.

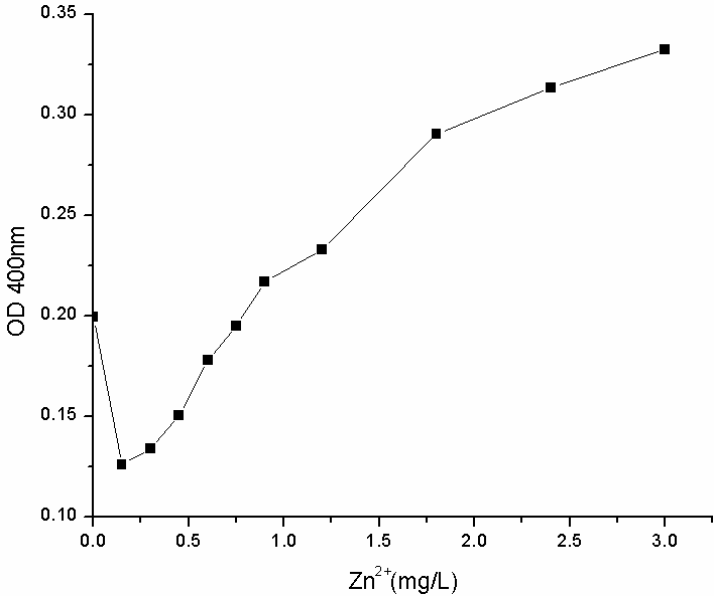

Figure 5.28: Pyoverdine (OD_{400}) at 24 hours in cultures treated with low Zn^{2+} concentrations.

This experiment revealed that for $Fe^{3+}$and Cu^{2+} the metal susceptibility of microorganism depends not only on the concentration of metal, but also on the inoculum percentage: the lower the biomass in the inoculum, the lower the MICs. To further investigate the influence of the initial biomass concentration on the sensitivity of this strain toward metals, a test with different inocula percentage was set up in 96 well plates. Moreover, the pyoverdine production chiefly depends on the amount of Fe^{3+} dissolved: if the concentration of this ion is above a critical value (non limiting iron or PCC), the pyoverdine system is repressed by the microorganism. This test was also useful to determine these PCC values. In Table 5.3 MIC and PCC (mg/L) for Fe^{3+}, Cu^{2+}, Zn^{2+}, related to different inoculum % are reported.

Table 5.3: MIC and PCC of 96 well plates cultures started with different inoculum %.

Metal	[mg/L]	Inoculum %			
		10%	5%	2.5%	1.25%
Fe^{3+}	MIC	/	0.12	0.04	0.02
	PCC	0.2	0.08	0.03	0.01
Cu^{2+}	MIC	/	0.8	0.3	0.2
	PCC	1.6	0.8	0.3	0.2
Zn^{2+}	MIC	/	/	/	/
	PCC	/	/	/	/

This experiment clearly highlights the importance of the inoculum % for the determination of MICs of $Fe^{3+}$and Cu^{2+}: the lower the biomass in the inoculum, the higher the susceptibility of the microorganism to the metal, and consequently the lower the MIC. PCCs values at 24 hours were very near to MICs for iron, and they were the same values for copper, unless for tests inoculated with 10% of inoculum. Regarding Zn^{2+}, the microorganism grew at every metal concentration and no MICs or PCCs were determined in the tested range (0-3 mg/L, or 0-46 µM). At the same time, it was possible to see that the correlation already seen in Figure 5.28 between Zn^{2+}concentrations and pyoverdine was present also in this test (Figure 5.29).

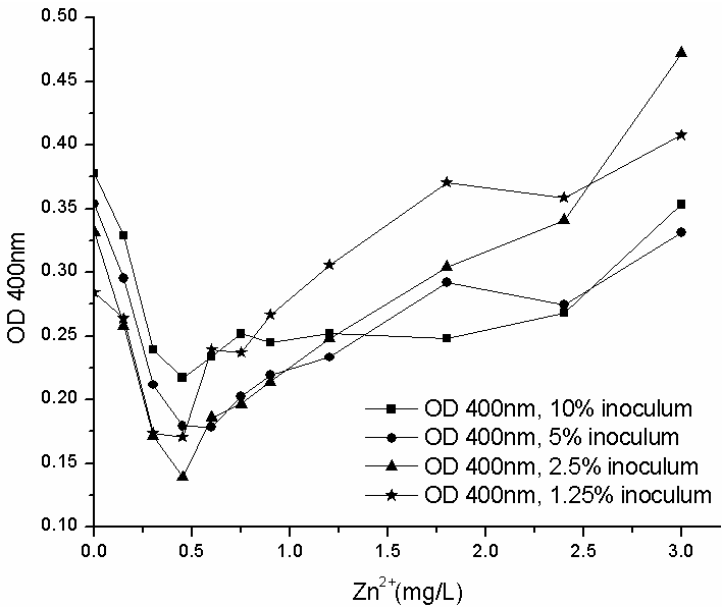

Figure 5.29: Pyoverdine (OD_{400}) at 24 hours in cultures treated with low Zn^{2+} concentrations.

The reasons of this effect are not clear. One hypothesis should be that from 0 to 0.5 mg/L Zn^{2+} the siderophore content secreted by the microorganism is enough to sustain growth without toxic effect of heavy metals. At 0.5 mg/L Zn^{2+} became toxic to the microorganism that increase its pyoverdine production as a protective mechanism. It is known that pyoverdine is effective in shielding the microbial cell of related *Pseudomonads* from Cu^{2+} and Zn^{2+} toxicity [109]. Unfortunately, in this work this effect was seen only for zinc, and not for copper. Further investigations will be required to fully understand the interactions between *P. fluorescens* and these metals.

From these results is evident that MIC values depend on several parameters, making difficult to compare data from different authors and experimental conditions [119]. For instance, regarding Cu^{2+}, Teitzel and Parsek [200] found a MIC of 127 mg/L for *Pseudomonas aeruginosa*, Chen et al. [202] 190 mg/L for *Pseudomonas putida*, whereas Tom-Petersen et al. [203] 3 mg/L for *P. fluorescens*. These data highlight the heterogeneity of MICs that are strongly related to the tested strain and conditions. This complexity of iron uptake is further confused by the simultaneous presence of different siderophores: pyochelin synthesis in *P. aeruginosa*, is repressed by 10 µM Fe^{3+}, Co^{2+}, Mo^{+6}, Ni^{2+} or Cu^{2+} [106], while pyoverdine synthesis is induced by 10 µM Al^{3+}, Cu^{2+}, Ga^{3+}, Mn^{2+} [110] or Ni^{2+} and 100 µM Co^{2+} or Zn^{2+} [106]. Teitzel et al. [204] verify with 2D-electrophoresis that exposure to Cu^{2+} up-regulates genes involved in the synthesis of pyoverdine and down-regulates those involved in the synthesis of pyochelin [109]. A similar analysis should be used also to investigate this effect in *P. fluorescens* ATCC 13525.

Further complications should be taken into account. Appanna and Pierre in 1996 showed that *P. fluorescens* was able to grow in a minimal medium supplemented with different high concentration of metals (Al^{3+}, Fe^{3+}, Zn^{2+}, Ca^{2+} mM Ga^{3+}), by elaborating a lipid-rich residue where most of the metals were immobilized [120]. Meanwhile, the same strain has been shown to initiate disparate detoxification mechanisms when each of these five metals was separately present: calcium was precipitated into $CaCO_3$, iron was embedded in a gelatinous residue, gallium and aluminium were complexed to a soluble organic conjugate, and finally zinc induced the synthesis of peptide-rich molecules where the metal was predominantly sequestered [205].

By comparing the concentration attained from these tests with those indicated in the EU Drinking water directive (98/83/EC) and in the WHO Guidelines for Drinking water quality, as reported in Table 5.4, is possible to see that MICs of Fe^{3+}, Cu^{2+} and Zn^{2+} are always above the indicated values. At the same time, the concentration of metal ions required to inhibit the pyoverdine production (PCC) is very near to the recommended thresholds for iron and copper. Unfortunately, this value was not determined for zinc.

Table 5.4: Comparison between MIC, PCC and EU and WHO drinking water regulations (mg/L).

Metal	MIC	EU	WHO	PCC
Fe^{3+}	> 83.8	0.2	0.3	0.2
Cu^{2+}	6.3-31.7	2.0	2.0	1.6
Zn^{2+}	3.0-32.7	/	3.0	/

Conclusions

The interactions between *P. fluorescens* and metals were initially studied through the determination of MICs of Cu^{2+}, Zn^{2+} and Fe^{3+} with a new modified Kirby-Bauer method on agar plates. The obtained results poorly correlates with MICs reported by other authors. Moreover, the sulphate ion showed a lower toxicity in comparison with the chloride one. Then, experiments carried out in liquid cultures proved that MIC values obtained in 96 well plates are more similar to literature ones: probably on agar plates the results were influenced by mass transfer limitations. This test revealed also that *P. fluorescens* metal susceptibility depends on the concentration of metal, but also on the inoculum percentage: the lower the inoculum percentage, the higher the susceptibility of the microorganism to the metals.

The MIC and PCC obtained values were compared to those indicated in the WHO Guidelines for drinking water quality and in European directive 98/83/EC: MICs of Fe^{3+}, Cu^{2+} and Zn^{2+} are always above the threshold specified, whilst PCCs are very near to the recommended thresholds for iron and copper. The PCC was not determined for zinc in the tested range of concentration (0-3 mg/L, or 0-46 μM) and conditions.

This kind of results highlighted that this sensible element should be further investigated (e.g. with other metals and differently combined), but optimistic outcomes make pyoverdine regulation useful for the development of a biosensor able to monitor metals in the environment.

Characterization of PolyVinil alcohol (PVA) gel immobilisation of microbial sensible elements

The immobilization of the biological sensing element is a key step for the development of a biosensor. Different methods have been used, depending also on the nature of the biological sensing element. Enzymes and microorganism have been mainly immobilized by physical adsorption, covalent linking, entrapment or encapsulation in polymer matrix. Since the beginning, these methods have been applied also for biosensors development.

Tests with a dialysis membrane were set up to evaluate an unusual system for biomass immobilization. By means of a tubular dialysis membrane (cut-off 12 kDa) it should be possible to create two separated compartment: one with the biomass, producing pyoverdine in the culture medium, and one with only pyoverdine (1,160 Da [206]) dissolved in the medium. Hence, the cut-off of the membrane was chosen to separate biomass from the secreted siderophore. Unfortunately, this test showed some sealing problems after 48 hours, despite it was repeated several times, and for these reasons the OD_{620} increased also outside of the membrane (data not shown). This clearly demonstrates the effectiveness of this system to separate biomass and siderophore for 48 hours, but sealing limitations affect its real applicability. Due to these doubtful results, this system was not fully evaluated. Instead, PVA gel immobilisation of microbial sensible elements was characterized.

Preliminary test with PVA

A widely known method of biomass or enzyme immobilization is by physical entrapment. Natural polymers, such as agar, alginate, chitosan or k-carrageenan, or synthetic ones, like polyacrylamide, polyurethane, acrylate/vinyl acetate, polyethylene glycol polymer, or silica biocers have been widely employed for the entrapment of microorganisms [176, 177, 207]. Each of these methods has some advantages, but also drawbacks. By way of example, the mechanical strength of hydrogels (e.g. agar, polyacrylamide) is rather weak, poisonous chemicals are used during polymerization (e.g. TEMED, glutaraldeyde), or the monomer itself is toxic (e.g. acrylamide). In other system there are no toxicity problems, but physical-chemical disadvantages: for instance, the requirement of Ca^{2+} and Al^{3+} ions for stabilization of alginate gels can form precipitates with other salts present in cultural media [176].

Polyvinyl alcohol (PVA) is a polymer of great interest because of its characteristics, and for its related application (e.g. pharmaceutical, biomedical, and separation) [208]. Furthermore this polymer is nontoxic to organisms and can be cheaply produced at industrial scale [176, 209], and the immobilization by means of this polymer have been frequently investigated also for its optical and mechanical properties [210].

PVA gels can be prepared by chemical or physical cross-linking [209]. Chemical cross-linking is based on cross-linkers such as boric acid or UV light [207], whereas the most common method to produce PVA gels by physical cross-linking is the so-called 'freezing-thawing' process [208]. The PVA gels are poor in strength, but they are usually hardened by soaking the polymer in saturated boric acid solutions (around pH 4.0) for a long time, and this exposure could cause serious cell injuries [207]. Alternatively, hardening of PVA gel by iterative freezing-thawing was developed by Nambu [211]. The formation of a gel network by physical cross-links can be mainly explained by the formation of hydrogen bonds between hydroxyl groups of PVA skeletons and water molecules, and the creation of crystallites [176, 209]. This method does not require the presence of toxic compounds, and result in a rubber-like gel, with better mechanical strength than PVA gels cross-linked by chemical or irradiative techniques, and a high degree of swelling in water [176, 209].

The performance of the immobilized cells is the protocol used for their immobilization [212]. The properties of the gel may depend on the molecular weight of the polymer, the concentration of the aqueous PVA solution, the temperature, time length, and the number of freezing/thawing cycles [213, 214]. Some preliminary tests were performed to evaluate the use of this polymer for the entrapment of microbial cells. At first, the polymer amount in water solutions was studied, by casting gels with PVA 7 to 14 % (w/v). At the same time, the temperature, the time and the number of freezing/thawing cycles were established. A 10 % (w/v) of PVA was chosen as a polymer concentration, associating this solution with three freezing/thawing cycles, correspondingly to -20 and 25 °C. Subsequently, plastic and glass surfaces were assessed for casting with a Mayer rod [177], or coating by spin coating [210]. The glass surface was better if compared with plastic one, probably because of the hydrophilic nature of the polymer, whereas casting by means of a Mayer rod seemed to give a better result in comparison with spin coating in terms of homogeneity and thickness.

Afterwards, to evaluate the biomass dispersion inside the PVA matrix, gels and films were prepared by mixing the biomass (15 mg) previously stained with Methylene blue with various amount of 10 % (w/v) PVA solution (1:1, 1:2, 1:5, 1:10, biomass to PVA ratio). The gels made with an equal biomass to PVA ratio (1:1) didn't solidify and was not further investigated. The other gels were observed with an optical microscope, but their thickness and opacity were too high to allow a qualitative evaluation of biomass dispersion. At the same time, some gels without biomass were soaked in Methylene blue and washed twice with dH_2O revealing that PVA gels naturally adsorb the dye: the resulting coloured background interferes with the light of the microscope. Better results were achieved with films obtained by drying the gels previously analyzed: the microscope showed that the biomass was homogeneously dispersed inside the PVA matrix for films with 1:5 and 1:10 biomass to PVA ratio; the films made with a ratio of 1:2 showed only a partial and patchy distribution of the biomass.

The air-dried gels (films) thickness was evaluated, to establish the loss of water after drying in comparison with the original gels. This test is also a good index of release of water by a PVA gel left directly in air during environmental applications. The dried films are more transparent than the gel itself, and once put in dH_2O they became extremely flexible, soft but resistant: they probably recover part of the water they lost during the drying process. Because of technical issues (too small variation in weight and volume to be measured with normal instruments) it was not possible to evaluate the swell and de-swell behaviour of these films. In Table 5.5 the thickness of films obtained in different conditions is reported.

Table 5.5: PVA films thickness

	A	B	C	D	E
Biomass to PVA ratio	/	/	1:2	1:5	1:10
Gel thickness [µm]	100	400	400	400	400
Film thickness [µm]	6.66	26.79	21.84	35.85	35.99
Standard deviation	0.54	1.60	2.39	2.01	2.16

The films casted without biomass showed a uniform thickness that was only the 6.6 % of the original gels thickness, with both Mayer rods used. This effect is due to the evaporation of water during the air-drying process, but also to other mechanism related to the hardening process. As supposed by Ariga and co-workers [176], volumetric changes and PVA variations in the polymer concentration in PVA gel observed during iterations of freezing-thawing may result in enhancement of the gel strength. The films casted with biomass (15 mg mixed with different amount of PVA) showed a uniform thickness, unless the one with a biomass to PVA ratio of 1:2: this film was thinner than the other films, with a non uniform distribution of the thickness, probably due to the non homogeneous dispersion of the biomass inside the gel during casting.

PVA biodegradation test: use of PVA as C source in *P. fluorescens* cultures

The immobilization of a microorganism in a supporting matrix requires that the microorganism itself is not able to degrade the compounds used to trap the cells. The ability of *P. fluorescens* to metabolize PVA as the sole carbon source was tested in flask cultures. In Figure 5.30 the PVA biodegradation test are presented. The growth of the microorganisms was recorded only by the positive control (4 g/L succinic acid), while the cultures set-up with 4 g/L PVA or without carbon sources showed only a little growth, probably due to some nutrients included in the inoculum. The same results were seen for the pH of the cultures, showing values comparable to previous tests for the positive control and a slight increase for the other two cultures. This support the outcomes obtained by monitoring the growth (OD_{620}).

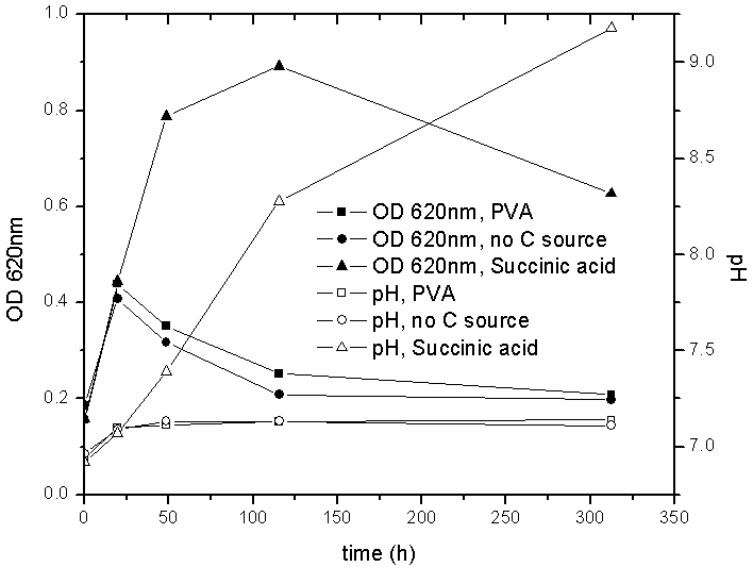

Figure 5.30: Biomass growth and pH trends of PVA biodegradation tests

From these results is possible to say that *Pseudomonas fluorescens* DSMZ 50090 in the tested conditions (for more than 13 days in M78 media) is not able to use the PVA present as the only carbon source. This is in contradiction with other outcomes related to the biodegradation of PVA by *Pseudomonads* [215].

Determination of PVA gel diffusivity

Once the inability of the microorganism to degrade the entrapment matrix was established, other tests were performed to characterize this kind of immobilization. The first test was the determination of the Methylene blue diffusivity (permeability coefficient) through PVA gel. Nanoporosity, in fact, is essential for preserving microbial viability and coating reactivity [177]. This kind of test is important to establish if the nanoporosity of the matrix is big enough to allow nutrients and pyoverdine to permeate inside and outside the gel, and then if this immobilization material is useful for the development of an optical biosensor, or if some mass transfer limitations make it unreliable. The measures were facilitated by spectrophotometric determination of the dye during the time course of the test. At the same time the chosen compound (Methylene blue) shows an hydrodynamic molecular radius comparable with that of other compound, such as glucose (0.36nm) [216].
From the different tests performed a value of $1.64*10^{-10}$ ± 0.78 $*10^{-10}$ m^2/s was determined. This value is similar to values obtained for other dyes in liquid media or in PVA gels [209]. For this reason it is possible to state that the mass transfer limitations should be insignificant for molecule with a similar hydrodynamic radius and/or a molecular weight comparable to that of Methylene blue (319.86 g/mol). The same kind of test should be repeated by substituting the dye with pyoverdine, to assess if a similar result is consistent with these outcomes. Entrapped enzymes and microorganisms still retain their activities and react with substrates species that diffuse through the pores of the sol-gel matrix [212]. At the same time, the diffusion through PVA gels could be also linearly influenced by the polymer molecular weight [217], or by trapped biomass. Further investigation will be required to validate this polymer as an immobilization matrix (e.g. biomass leakage evaluation, trapped cells viability, pyoverdine production in cultures supplemented or not with metals).

Determination of polymer release from PVA gel in water

To evaluate the behaviour of the PVA gel into water environment, a test for the determination of polymer release from PVA gel in water was performed. This is a rough assessment of the strength of the gel in aquatic system. For this test the spectrophotometric determination of PVA by Finley [178] was used, as reported in Chapter 4.
Two gels (gelA) were used after a washing step. The mean release of these gels was about 82.3 mg during the whole washing step, corresponding to 1.44 mg/cm^2. This means that a little amount of PVA was released probably from the gel surface, because it was not polymerized, or not fully cross-linked to other polymer chains.
In Figure 5.31 the PVA release over a period of 570 hours is reported as $mg/h*cm^2$. The plotted values indicate that the polymer release is almost completed in 90 hours, with a following small variation in the PVA concentration determined in water. This result highlight the previous hypothesis of PVA surface release: the gel seems to be stable in water, with a little release in the first 90 hours. The washing pre-treatment seems to be not really important, unless for a less amount of polymer leakage at the first time-point (5.7 and 24.2 mg for gel A and B respectively).
This test showed that the total release (almost completed in 90 hours) was 8.6 and 9.9 % leakage of PVA from gel A and B, respectively, in comparison with the initial mass of the gel. This means that the PVA release could not be taken into account and this material could be fully applied for biomass immobilization.

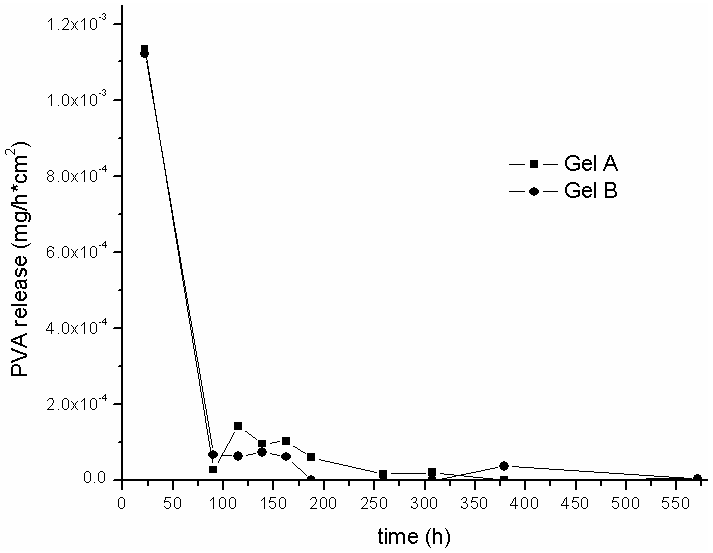

Figure 5.31: PVA release rate.

Conclusions

At first, a 10 % (w/v) of PVA was chosen as polymer concentration, associating this solution with three freezing/thawing cycles, correspondingly to -20 and 25 °C, and casting the gels with a Mayer rod, because of the resulting homogeneity and thickness.

Subsequently, the biomass dispersion was evaluated in air-dried gels (films): 15 mg of biomass were homogeneously dispersed inside the PVA entrapment matrix by mixing the biomass with 5 or 10 volumes of 10 % (w/v) PVA solution. The films thickness was evaluated, to establish the loss of water after drying in comparison with the original gels, as index of the release of water by a PVA gel left directly in air. The films showed a uniform thickness that was only the 6.6 % of the original gels thickness, with both Mayer rod used, due to the evaporation of water and to hardening process [176]. The ability of *P. fluorescens* to degrade the entrapment matrix used to immobilize the cells was tested in flask cultures, using PVA as the sole carbon source. No growth was recorded in this test.

The next test was the determination of the Methylene blue diffusivity (permeability coefficient) through PVA gel. From the different tests performed a value of $1.64*10^{-10}$ ± 0.78 $*10^{-10}$ m^2/s was determined. The diffusivity of Methylene blue showed that the PVA porous matrix is compatible with nutrients and small molecules diffusion, because this dye has an hydrodynamic molecular radius comparable with that of other compound, such as glucose (0.36 nm) [216]. A similar test should be repeated by substituting the dye with pyoverdine, to validate these outcomes.

To evaluate the behaviour of the PVA gel into water environment, a test for the determination of polymer release from PVA gel in water was performed. This test showed that the total release (almost completed in 90 hours) was 8.6 and 9.9 % leakage of PVA from gel A and B, respectively, in comparison with the initial mass of the gel. This means that the PVA release should not be taken into account and this material should be fully applied for biomass immobilization.

All these tests showed that the PVA could be a good entrapment matrix for the development of a biosensor for environmental monitoring, although further investigation will be required (e.g. biomass leakage evaluation, trapped cells viability, pyoverdine production in cultures supplemented or not with metals).

T. versicolor laccase protein engineering and PAHs recognition

Computational docking and rational design

In this thesis, sequence and 3D model structure analyses were used as guidelines for the site-directed mutagenesis experiments on lccβ laccase isoenzyme of *T. versicolor*, the last biorecognition element investigated. A lot of detailed structural data are available to allow such engineering, and a rational design approach was applied to change the substrate specificity of this enzyme.

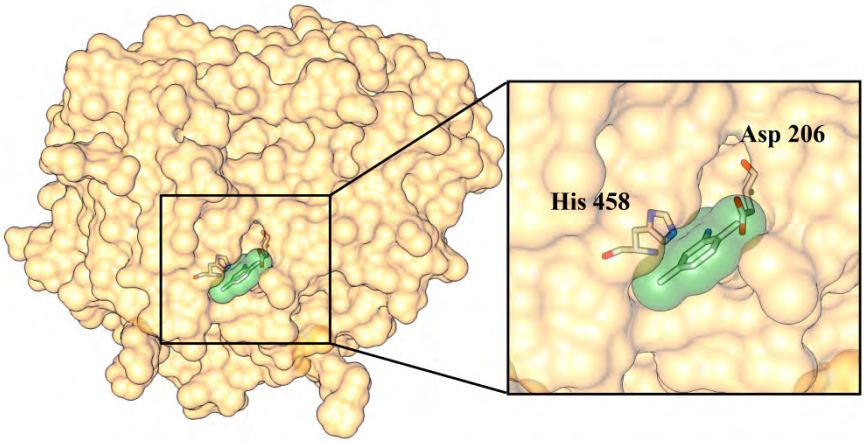

Figure 5.32: Structure of a T. versicolor laccase complexed with 2,5-xylidine (1KYA) [146].

Amongst many published X-ray structures of laccases [218, 219], there is also a structure of a *Trametes versicolor* laccase complexed with 2,5-xylidine (1KYA) [146]. The analysis of this structure revealed that the aromatic substrate (an aniline), in green the binding pocket, is in close proximity to the catalytic His458, with its NH_2 hydrogen-bonded to the Asp206 (Figure 5.32).

Substrate specificity usually results from the combination of the geometry and chemical nature of the substrate binding pocket that provides a suitable environment for the enzymatic reaction. The substrate binds the laccase enzyme in a small negatively charged cavity near the T1 copper, and seems that the negative charges could stabilize the radical cation products that are formed during the catalytic cycle [146]. The His458 has been suggested to be a primary electron acceptor, also because of its easy access to the molecule's surface. Another interesting residue in the complex is Asp206 that is hydrogen-bonded to the amino group of xylidine: all the fungal laccases have a conserved Asp/Glu in this position (Figure 5.36). This aminoacid is the only hydrophilic residue in the cavity and might play a role in substrate oxidation by accepting a proton from phenolic substrates [219]. The substrate likely first loses its proton, and then an electron is taken away by the T1 copper via the surface-exposed histidine. Alternatively, the electron is first extracted, leading to formation of a protonated phenoxy radical, which then loses its proton. This residue is, then, important for the catalysis of phenolic compounds. It is supposed that also the nature and position of substituents on the phenolic ring influence the efficiency of oxidation by laccase. In particular, electrophilic groups, especially in the ortho position, negatively affect the substrate's affinity for the enzyme [168].

This X-ray structure (1KYA) was used as a starting PDB file for the wild type calculations: computational docking simulations (SwissDock server [182]) with a selection of substrate (ABTS and 2,6-DMP) and pollutants (anthracene, fluoranthene, bisphenol A, PCP, TCDD, dibenzodioxin, dibenzofuran) were performed on the wild type enzyme to evaluate how these molecules interact with the catalytic site of the laccase. In fact, no laccase structures complexed with a pollutant are available. By using protein-ligand docking tools, [220] were able to screen potential laccase substrates and to discriminate well-known substrates as well as non-binder ligands, predicting also

new targets amongst a pool of pollutants. For this reason computational docking was performed, then, binding modes with the most favourable energies were ranked, and clustered by root mean square deviation (RMSD) with a corresponding value of estimated ΔG of binding. The obtained results showed that for some xenobiotics the binding site is properly recognized: for example, anthracene is able to enter in the binding pocket of the enzyme, positioning very close to the His458, as it is possible to see in Figure 5.33. This PAH is known to be metabolized by *T. versicolor* laccase, both in presence and absence of a mediator, forming a 9,10-anthraquinone [138].

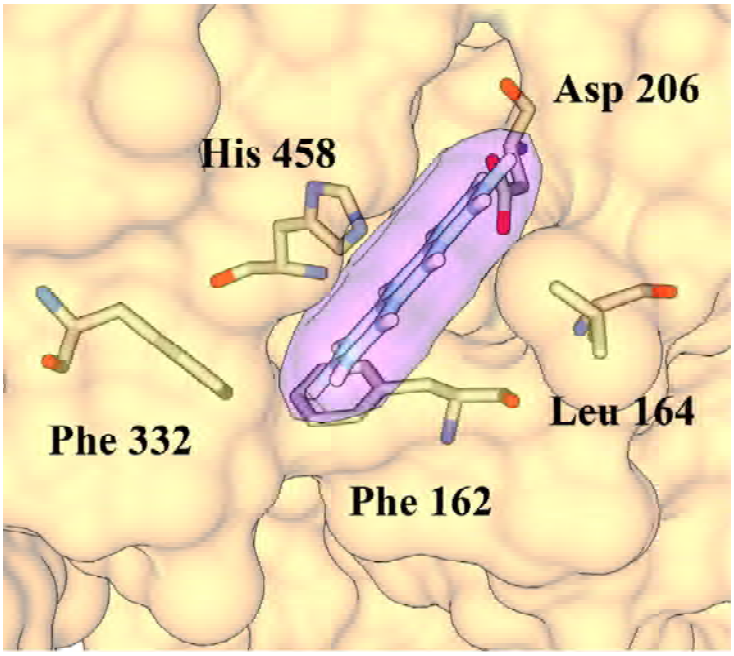

Figure 5.33: Prediction of enzyme-substrate interaction between WT laccase (1KYA) and anthracene.

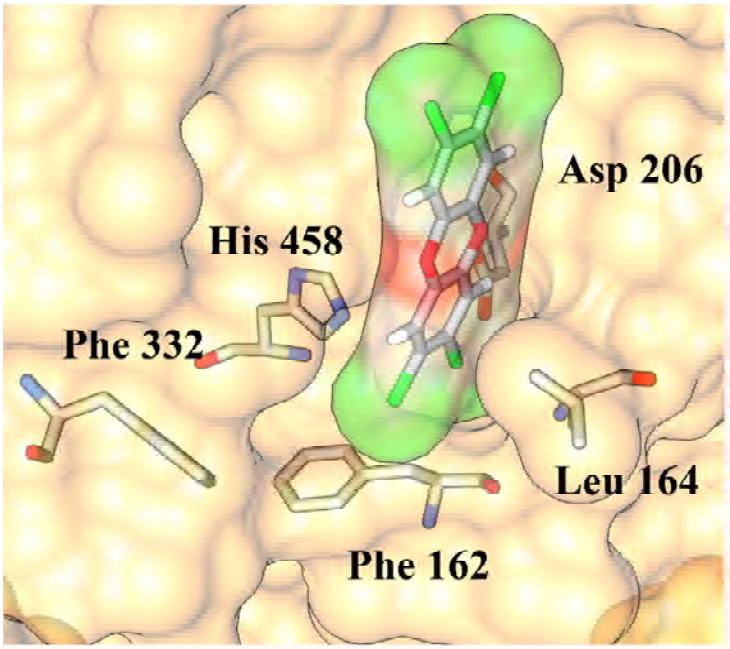

Figure 5.34: Prediction of enzyme-substrate interaction between WT laccase (1KYA) and TCDD.

Unfortunately, not every tested PAH was able to enter into the binding pocket, remaining far from the catalytic His458. As an example, the interaction between WT *T. versicolor* laccase and TCDD is

reported in Figure 5.34. The TCDD seems to remain mostly outside of the catalytic site, probably because is too big for the allowable space of the binding pocket, and maybe because of the repulsion between Asp206 and chloride atoms. Usually, steric hindrance and high redox potential negatively affect laccase oxidation rates [168].

Starting from computational docking results and available information on the enzyme catalysis, some mutations were selected for heterologous expression in *P. pastoris*. Mainly, two different kinds of mutations were realized: the first one, aiming to an enlargement of the binding pocket, and the other to change the electrostatic interactions. In the former group the bulky Phe162, Leu164 and Phe332 were mutated to the smaller but still hydrophobic Ala. This strategy was effectively applied to change the metabolization capability of a P450 enzyme: Sakaki et al. [221] obtained a rat CYP1A1 able to degrade TCDD, only by substituting a phenylalanine with a smaller alanine (F240A). At the same time, Leu164 was changed to Ala because, during computational docking simulations, the long side chain of leucin tends to bend toward the inside of the binding pocket, reducing the substrate accessibility. A similar effect was recorded for laccases from the Ascomycetes *Podospora anserina* [222], *Neurospora crassa* [223], *Melanocarpus albomyces* [224] and *Myceliophthora thermophila* [225, 226, 227]. In these cases, the C-terminal tail of the protein forms a plug obstructing the T2/T3 channel, and reducing oxidation ability of the enzyme.

In the second group the negatively charged Asp206 was changed in Asn, as summarized in Figure 5.35. F265A and F337A mutations were avoided because of previously made tests that showed the detrimental effect of these substitutions [169, 228].

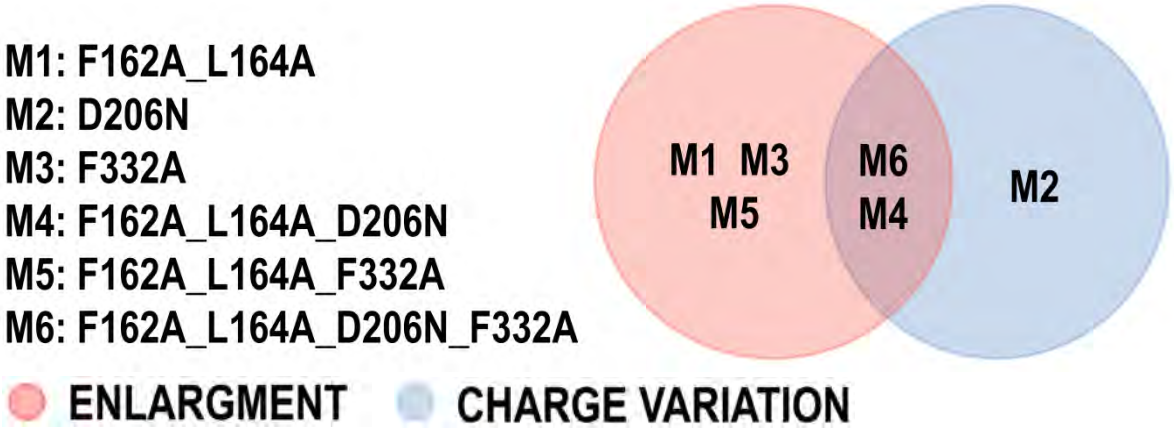

Figure 5.35: Prediction of enzyme-substrate interaction between WT laccase (1KYA) and TCDD.

The sequence alignment of the binding sites of fungal, bacterial and plants laccases (Figure 5.36) showed that the aspartate residue is well conserved in fungal laccases from basidiomycetes, while an asparagine is found in plants laccases. The Asp, as already said, is important to stabilize the cation intermediate formed during the oxidation of phenolic substrates, and it was changed with a positive one to evaluate the effect of this substitution. This residue in bacteria is substituted with an alanine.

Figure 5.36: Sequence alignment of WT laccase (1KYA) and fungal, bacteria and plants consensus.

Mutants' model structures were predicted using 1KYA as a template structure, and the interaction between the resulting models and substrates or pollutants were assessed by computational docking simulations, as stated above. The docking result of the interaction between M6 mutant and TCDD is representatively reported in Figure 5.37.

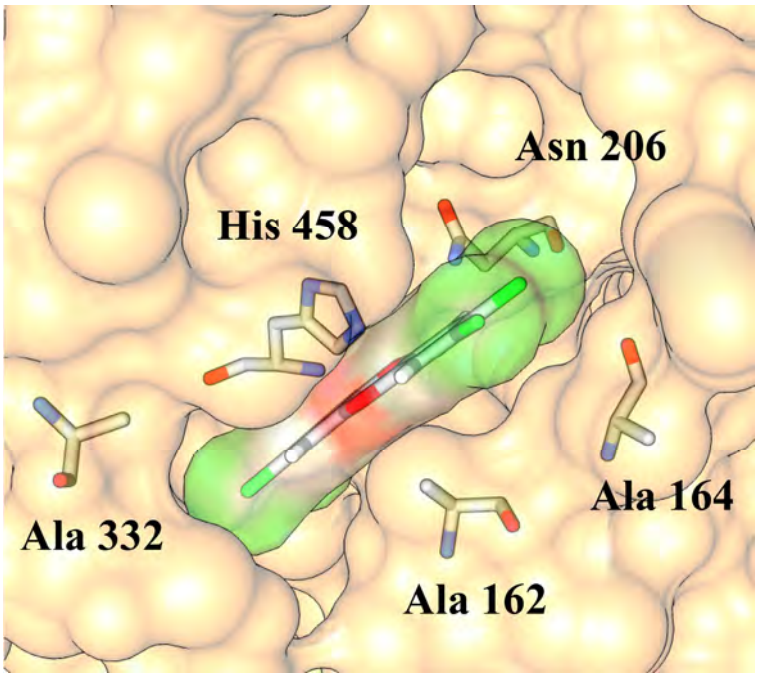

Figure 5.37: Prediction of enzyme-substrate interaction between WT laccase (1KYA) and TCDD.

The computational results of the interaction of this mutant with the TCDD show that the larger binding pocket allows the pollutant to enter in it, and to stay closer to the catalytic His458. The calculated free binding energy (ΔG) estimated by the algorithm is reported in Table 5.6, and the most favourable value (in bold) is reported for each substrate.

Table 5.6: Estimated binding free energy (ΔG) of laccase-ligand docking simulations.

LIGAND	ABTS	2,6-DMP	TCDD	ANT	PCP	DD	FLUO	DF	BPA
LACCASE									
WT	-8.06	-5.85	-6.22	-6.17	-5.93	-6.15	-6.36	-6.22	-6.58
M1	**-8.86**	-5.87	-6.40	-6.02	-6.45	-6.13	-6.05	-6.01	-6.48
M2	-7.31	-5.47	-6.77	-6.01	-6.49	-6.10	-6.32	-6.08	-6.32
M3	-7.39	-5.78	-6.61	**-6.36**	-6.34	-6.26	-6.52	-6.34	-6.77
M4	-7.69	-5.77	-6.87	-6.28	-6.65	-6.07	-6.20	-6.02	-6.36
M5	-8.34	-5.84	**-7.13**	-6.02	**-6.73**	-6.08	-6.23	-6.10	-6.78
M6	-8.32	**-6.36**	-6.88	-6.32	-6.70	**-6.32**	**-6.61**	**-6.52**	**-7.11**

ANT (anthracene), DD (dibenzodioxin), FLUO (fluoranthene), DF (dibenzofuran), BPA (bisphenol A).

The tabulated values illustrate that the specificity of *T. versicolor* laccase is affected by mutations. For a classical substrate (ABTS) the best values are obtained only by mutations F162A and L164A of M1 mutant. For bigger and carcinogenic PAHs, more substitutions positively influence the binding free

energy. By means of example, TCDD and PCP are more affine to M5 binding pocket, while BPA and fluoranthene are more affine to M6 binding pocket, if compared with other outcomes.
These results showed that the rational design of the laccase binding site have been virtually validated by means of docking simulations. Unfortunately, the success rate of the docking algorithms decreases as the ligand flexibility increases (e.g. increasing the number of possible conformations) [182], and many pollutants, such as dioxins, furans ad the most toxic PCB are coplanar and poorly flexible. The bioinformatics that build up the docking software declared that during tests the binding modes proposed by the algorithm are within 2 Å root mean square deviations (RMSD) to the experimental binding modes of crystal structures [182]. Furthermore, docking simulations are based on models and algorithms, and obviously must be validated with experimental data. For these reasons, and to demonstrate the possible industrial production of the enzyme, the selected enzymes have been produced and purified in a heterologous expression system.
As usual with any expression system, optimal expression conditions are dependent on the characteristics of the protein being expressed [186] and must be investigated to obtain acceptable amount of pure protein. Some tests were set up to find the most suitable expression conditions. An introduction to protein expression in *P. pastoris* is reported in annex B.

Optimization of *P. pastoris* culture conditions for protein expression

Protein expression was initially tested in *P. pastoris* GS115, using the pPICZαA vectors carrying the laccase genes. This strain is His^- and is usually used with vectors that complement this deficiency by carrying the functional gene (*his4*) necessary for the correct metabolism and growth of the strain on minimal media [186, 229]. Although usually 50 mg/L of a certain aminoacid is enough to sustain the growth of yeast cells [230], by using the pPICZαA vectors it wasn't possible to grow the GS115 transformants in MM, BMM, or BMGH supplemented with 0.04, 0.2 and 0.4 g/L histidine. *P. pastoris* GS115 transformants were able to grow only on YPDS or BMGY plates. For this reason the protein expression was tested in *P. pastoris* X33.
Preliminary experiments with agar plates confirmed that *P. pastoris* is a suitable heterologous host for expressing *T. versicolor* Lccβ and that the enzyme is secreted in an active form. *P. pastoris* X33 transformants formed a green coloured halo on MM/Cu/ABTS agar plates, differently from non transformed yeast, or yeast transformed with the empty pPICZαA vector: this clearly indicate that secreted laccase was responsible for the oxidation of ABTS in the medium.
Once the transformants were selected, the optimization of culture conditions for protein expression was necessary to achieve high yields of active enzyme. *P. pastoris* that had been stably transformed with the wild type *T. versicolor* Lccβ under control of the heterologous alcohol oxidase 1 (AOX1) promoter was cultured in small flask cultures (250 mL). The transformant with the highest laccase-secreting ability (WT) was selected for these preliminary tests. Controls with wild type X33 strain were also included in experiments using liquid cultures.
As reported by other studies [169, 231], an adequate amount of copper is required for laccase expression. The first step was the evaluation of different concentration of $CuSO_4$: during the induction phase, cultures with 0, 0.3 and 0.5 mM of $CuSO_4$ were set up in BMM medium and cultured at 30°C. At the end of the test (72 hours of induction) the enzymatic activity of the supernatants were evaluated: the higher activity was shown by the culture supplemented with 0.3mM of $CuSO_4$ with a specific activity of 6.49 UI/L, in comparison with 3.49 UI/L and 5.37 UI/L of cultures containing 0 and 0.5 mM of copper, respectively. No laccase activity was detected in *Pichia* cultures with empty pPICZαA vector.
This result confirms the Cu^{2+} requirement during laccase production. It is reasonable that copper ions in the growth medium did not influence the degree of protein synthesis, but the enzyme activity as

suggested by O'Callaghan and co-workers [232]. However, the biomass concentration of cultures decreased slightly as the copper concentration increased (data not shown), as previously reported by Hong and co-workers [233], especially if $CuSO_4$ was already added during the preculture phase, a 30-35 % decrease of biomass was obtained. For this reason, the copper was omitted in the preculture medium, differently from other reported tests [156, 233, 234].

Evaluation of a buffered (BMM) and an unbuffered (MM) culture medium

The control of pH during fermentation can be a relevant strategy to ensure high level of expression, mainly if the protein is secreted into the broth. Many effects have been related to the control of this variable: chiefly the influence of pH on protein activity and stability, or the detrimental effect of protease.

For the expression in *Pichia*, two culture media are recommended by the Invitrogen Guidelines [189]: an unbuffered medium (MM), and a buffered one (BMM). The latter is indicated if pH is important for protein activity, and a wide range of pH values could be tested in case of low level of expression. The MM medium is helpful to check if the protein is susceptible to neutral pH proteases: the pH in an unbuffered medium rapidly drops to 3.0 or below, inactivating neutral pH proteases [235], but without effects on biomass growth. To study the effect of pH on laccase expression, we incubated the WT transformant at 30 °C in a BMM (pH 6.0) and in MM media containing 0.3 mM $CuSO_4$, and the activity of culture supernatants was daily assayed. In Figure 5.38 the activity of supernatant samples during the induction phase is reported.

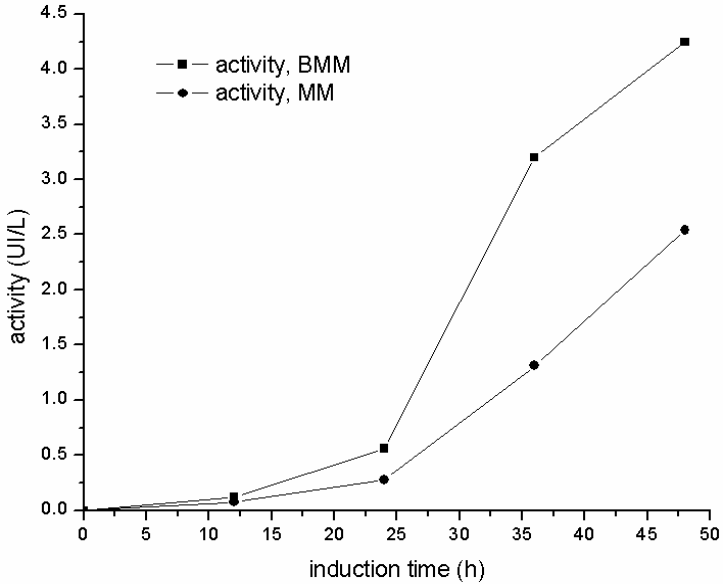

Figure 5.38: Laccase activity of supernatants of cultures conducted in BMM and MM media.

No laccase activity was detected in the culture medium before induction with methanol, whereas 12 hours after the induction with 0.5 % (v/v) methanol, laccase activity could be detected in the medium. A higher activity was determined in flasks cultured in BMM medium in comparison with the ones in which MM was used: the activity was almost double after 48 hours. The pH at this time-point was near to 6.0 in the BMM medium, while it was in the acidic range in the MM medium. This result agrees with other outcomes: Guo and collaborators reported that the optimal pH for laccase production was pH 6.5, while very little laccase activity was produced at pHs 4.0, 4.5 and 5.0 [236]. This effect should be due to the presence of proteolytic activity, higher at pH 5.0 than at pH 6.5 [234]. Kobayashi et al. also declared higher proteolytic activity at pH 4.3 than pH 5.9 [237]: probably

most of the proteases released by *P. pastoris* have acidic optimum value. Another way of obtaining a higher laccase production seems to be the presence of 0.6% (w/v) alanine: this phenomenon was highlighted for the first time by O' Callaghan [232], and confirmed by other papers [156, 233, 238]. The positive effect of alanine seems to be one more time the control of pH: in these tests with alanine pH remained constant for more than 7 days [232]. Furthermore, sorbitol and alanine were regarded as non-repressing carbon sources [239].
At the end of the test the whole cultures (MM and BMM) were centrifuged and the enzyme was partially purified by a single Hi-trap step. The activity of the fractions of the two purifications revealed higher values in the purified BMM laccase (26.65 UI/L, 0.23 UI/mg), in comparison with values obtained from the MM culture (16.5 UI/L, 0.17 UI/mg).
From these results it was clear that a higher yield of active enzyme is obtained by means of a buffered medium (BMM), compared to the unbuffered one (MM). For this reason the following tests were performed in BMM medium.

Determination of the optimal downstream time

Once the culture medium was chosen, the optimal time point for downstream was investigated with the WT transformant. The culture supernatants were assayed daily for 96 hours, and results are reported in Figure 5.39.

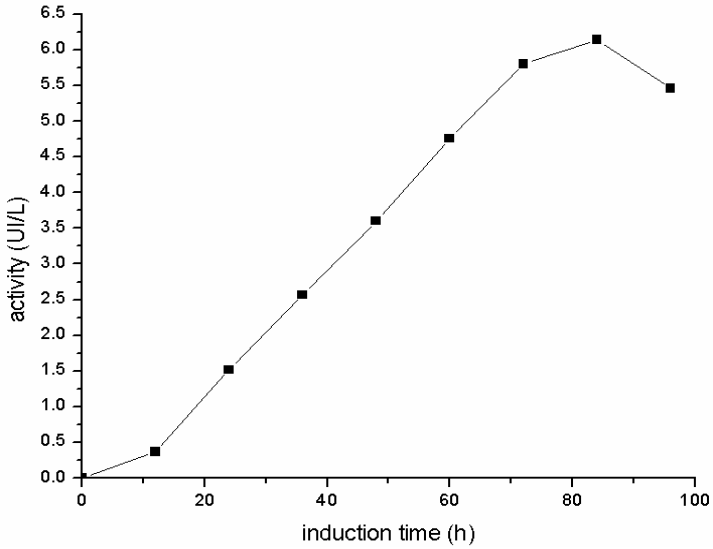

Figure 5.39: Laccase activity of supernatants of cultures conducted in BMM.

The plot reported shows that the higher activity in these flask tests was around 72-84 hours after induction (between the third and fourth day). Other flask tests led to similar results: Guo and collaborators [231] observed a maximum enzyme yield at the fifth day, over a seven-day fermentation. Higher yields were obtained by other authors in flask test after 13 days [156, 233, 234]: in these trials 0.6 % (w/v) alanine was added to support growth and increasing the level of expression. Kobayashi et al. [237] reported that the nitrogen starvation seems related to an augment of protease activity and a reduced amount of protein (human serum albumin, rHSA), restored by increasing the initial concentration of ammonia and phosphoric acid. It is reasonable that the nitrogen concentration could promote the expression of the protein, not only by supporting growth and reducing the release of protease in the culture media, but also by sustaining the protein synthesis. Both hypotheses are supported by the increased protein expression achieved by including casamino acids or alanine to the culture media [240].

In these initial expression experiments it was not possible to see a band on a Coomassie-stained gel. Usually, it is essential that by the time of expression studies, one or more sensitive assays for the detection of the protein of interest is ready to use. Good assays based on enzyme activity or on epitope recognition facilitate strain development and protein expression [230]. Furthermore, secreted proteins increase in the medium much more slowly than intracellular ones, and require at least two days to reach acceptable levels [230].
For these reasons, laccase fermentation time-points were loaded on two SDS-PAGE gels: one was coloured with the laccase substrate ABTS (zymogram), whereas the other was used to perform a westernblot analysis. The results are shown in Figure 5.40.

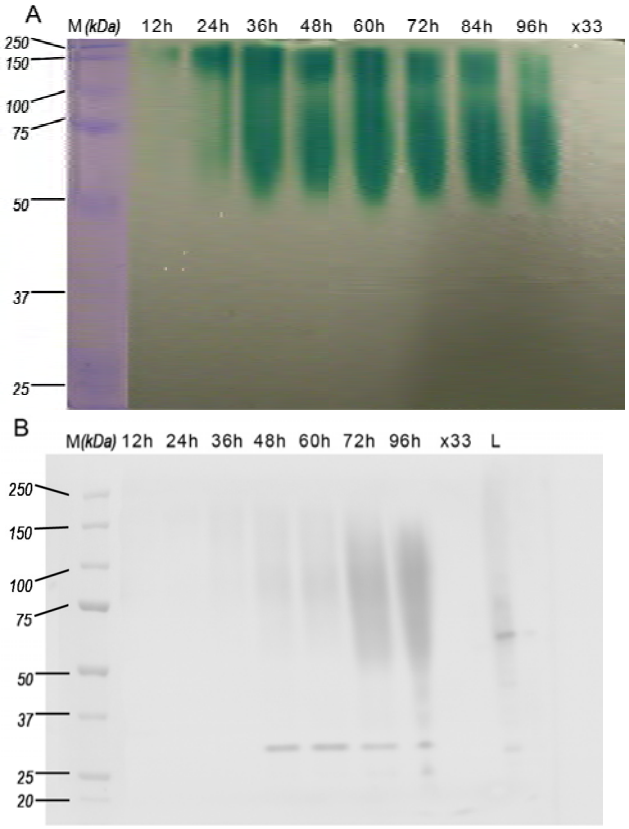

Figure 5.40: Zymogram (A) and westernblot (B) of time-points (X33: non transformed control; L: cell lysate).

The zymogram (Fig 5.40 A) shows that a more intense and bigger greenish halo was formed during the time course of the test. The halos, ranging between 55 and 250 kDa, tend to be more stretched after 48 hours of fermentation, and centred in the lower part of the smear, corresponding to lower molecular weight. The same kind of pattern is reported in the westernblot (Fig 5.40 B). Furthermore, after 48 hours a band corresponding to about 30 kDa appeared and seems to be present in each following time-point, but not visible in the zymogram, most likely because it corresponds to a not active enzyme. This band could be degraded protein that still contain the His-tag, and is recognized by the specific antibody during membrane hybridization, but is not able to metabolize the substrate ABTS because relevant parts of the enzyme (e.g. the binding site) are missing. Moreover, this band was not detectable in the non transformed wild type control (X33) grown in BMM, or in the sonicated cell pellet (L), sustaining the degradation hypothesis. Instead, a band with an approximate weight of 63 kDa was detected in the sonicated cell pellet. This band probably corresponds to the non glycosylated protein that is still inside the cell and that is still linked to the signal peptide (the

αMF). The band is not really intense, if compared to the smear, and this means that the secretion of the protein is quite good, not affecting the enzyme yield.
The smears on the gels were created by the variable amount of glycosylation: is known that the number of added sugar units is quite random, and generate a pool of protein with different molecular weights, usually hyper-glycosylated [241].
This analysis shows that the protein is produced, variously glycosylated, properly secreted and accumulated in the culture medium. No activity was observed for X33 control cells. Unfortunately, proteolytic degradation was seen after 48 hours, affecting yield and compromise protein quality. This effect could be reduced by lowering the temperature during fermentation [231], and this was further investigated.

Evaluation of the temperature on the laccase expression

The same transformant (WT) was also used to study the effect of temperature on laccase expression. The production of protein in yeast, and particularly of active enzyme, seems to be favoured by lower cultivation temperatures [231, 234]. It has been previously reported that a lower cultivation temperature improves the production of heterologous protein in *E. coli* systems, which has been attributed to aggregation problems at higher temperatures [242]]: lowering the cultivation temperature is habitually used for raising the amount of properly folded protein. Also Cassland and Jönsson [243] reported the improvement of *T. versicolor* laccase expression in *S. cerevisiae* by lowering the temperature.
Cultures were first grown at 30 °C in BMGY medium (preculture phase) and then the same amount of biomass from the preculture was transferred to BMM medium containing 0.3 mM $CuSO_4$ and cultured at 20, and 28 °C. The activity of samples was daily evaluated and the obtained values are reported in Figure 5.41.

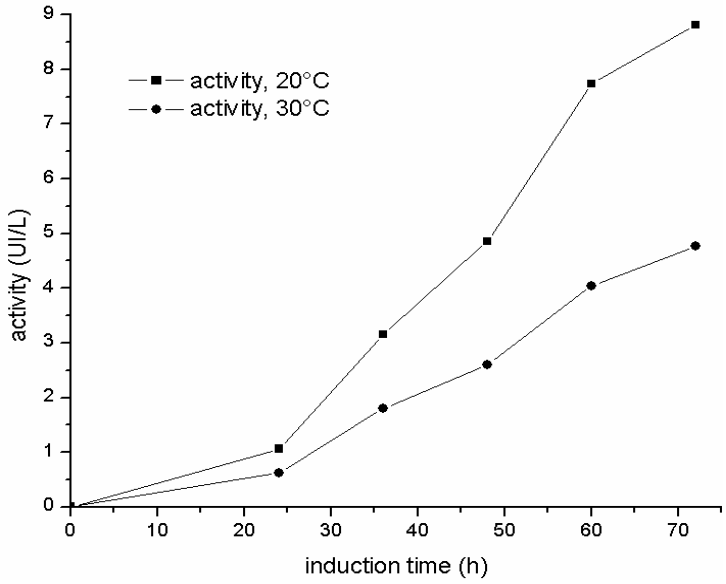

Figure 5.41: Laccase activity of supernatants of cultures conducted at 20 and 28 °C.

During the whole tests the determined activity was almost double for the flask maintained at 20 °C in comparison with the one kept at 28 °C, but a slower growth was recorded for the former flask (data not shown). The protein content was very similar in both conditions, but the cultures kept at 20 °C provided about two fold activity than those kept at 28 °C. Therefore, it can be concluded that cultivation at 28 °C was more beneficial for the growth of the yeast cells than cultivation at 20 °C, but

not for obtaining high laccase activity: the laccase activity was improved by decreased cultivation temperature, particularly the specific activity. Similar results were previously achieved by Hong and co-workers [234]. The lower temperature could be favourable because of a greater folding stability [234], a reduced protein degradation [229], a lesser protease activity, or a combination of these effects. Hong et al. observed on SDS-PAGE lower molecular weight protein bands in the culture medium at 28 °C than at 20 °C, probably due to protein degradation of higher molecular weight protein [234]. This confirms the previously mentioned hypothesis of protein degradation. A zymogram analysis was also performed loading on a gel the time-points of these cultures maintained at 20 and 28 °C. The result is reported in Figure 5.42.

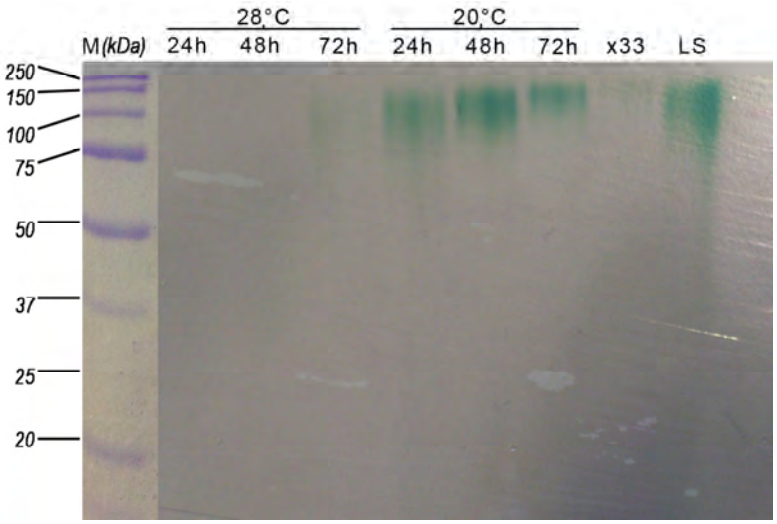

Figure 5.42: Zymogram of time-points related to cultures kept at 20 and 28 °C (X33: non transformed WT; LS: commercial T. versicolor laccase from Sigma Aldrich).

The zymogram shows that the activity halos were higher for samples taken from cultures maintained at 20 °C in comparison with the one at 28 °C. No activity was detected in the control X33 wild type, while was visible for the positive control (commercial *T. versicolor* laccase from Sigma Aldrich).
This test clearly showed that higher activity is obtained if the culture is maintained at 20 °C in comparison with the other at 28 °C. For the next tests the temperature was kept at 20 °C.

Evaluation of different induction strategies (see annex B)

The heterologous expression in *P. pastoris* through the AOX1 promoter requires that glycerol used during the preculture phase is replaced with methanol 0.5 % (v/v). The Invitrogen Guidelines recommend to induce the protein expression by adding methanol to 1 % (v/v) every day for Mut^S strains [244, 245] and up to 3 % (v/v) for Mut^+ [244], without any negative effect [189]. Different papers showed that methanol could negatively affect the production of active heterologous laccase [234, 236]: the results showed that 0.5 % (v/v) methanol was much better for producing active laccase than 1.0 % (v/v).
In Figure 5.43 the activity of cultures fed daily with different methanol concentration is reported. Variation of methanol concentration between 0.5 and 2.0 % (v/v) caused minor differences in cell growth than in laccase activity. The yeast was not inhibited by the used concentration and at the end of the test (96 hours), the laccase activity were 13.76, 11.82 and 10.72 for 0.5, 1.0 and 2.0 % (v/v), respectively. At the end of the test the whole cultures were centrifuged and the enzyme was partially purified by a single Hi-trap step. The activity (UI/L) and specific activity (UI/mg) of the eluted fractions revealed the following values for the three methanol feeding strategies: 0.5 % (v/v) (68.75 UI/L, 0.24

UI/mg), 1.0 % (v/v) (62.2 UI/L, 0.28 UI/mg) 2.0 % (v/v) (44.0 UI/L, 0.22 UI/mg). The higher purified laccase activity value was achieved by the culture fed with 0.5 % (v/v) methanol, in comparison with the other values obtained.

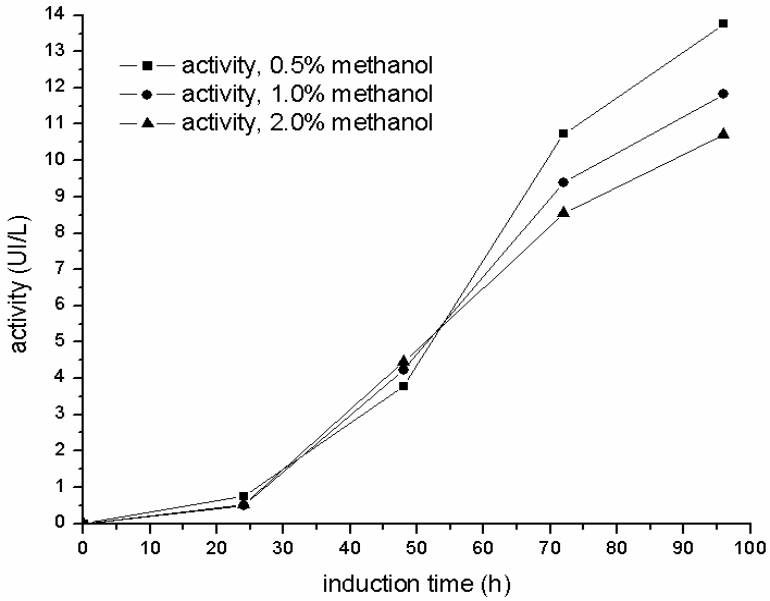

Figure 5.43: Laccase activity in supernatants of cultures fed with 0.5-2.0 % (v/v) of methanol.

Hong and colleagues [234] stated that the methanol accumulate gradually after 1 day when the concentration was 1.0 % (v/v) or higher. High methanol concentrations seem to be toxic to *P. pastoris* because of the generation of formaldehyde and hydrogen peroxide inside the cells [244, 246]. Hence, an accurate regulation of methanol concentration in *P. pastoris* cultures is mandatory not only to regulate the gene expression, but also to prevent the raise of methanol to levels that are toxic to the cells: a fed-batch strategy is frequently used to obtain high cell densities while keeping a low level of methanol. Several other complex feeding schemes were developed by using the dissolved oxygen spike method, with several drawbacks [247]. Alternatively, it is possible to analytically monitor the methanol concentration using HPLC or GC, but with higher costs and tricky on-line implementation, or to use methanol sensor systems [234].

The methanol concentration is hardly controlled in flask cultures, but for the following tests the methanol concentration was kept at 0.5 % (v/v) by daily feed of pure sterile methanol.

This test was the last one presented among preliminary tests set up to find the proper culture conditions for heterologous laccase expression. The optimal copper concentration required for expression was found to be 0.3 mM $CuSO_4$. At the same time, the buffered BMM culture medium led to higher activity and the maximum value was obtained after 84-96 hours of methanol induction.

The culture temperature revealed a marked effect on the production of active heterologous laccase. The mechanisms behind this temperature effect on the laccase expression and activity may be ascribed to release of more proteases from dead cells, poor stability, and folding problems at higher temperature (28 °C), in comparison with expression tests conducted at 20 °C. Finally, at 20 °C a daily methanol feed of 0.5 % (v/v) was found to be the best induction strategy for laccase expression in flask cultures.

Purification optimization

A purification protocol was evaluated and improved during these preliminary tests for the optimization of the heterologous expression of the WT laccase. The 'expression cassette' integrated

into *Pichia* cells contain a His-tag, useful for easy recovery of pure protein from the fermentation supernatant. The optimization of the purification was made by changing different conditions: buffers at different pH and containing various amounts of salts were tested in metal-chelate affinity chromatography (Hi-trap) on a FPLC system. Better results were obtained with 20 mM sodium phosphate buffer at pH 7.5 in comparison with lower pH (until pH 6.0). Some binding problems were limited by increasing the salt concentration. By means of example, the purification of WT cultured at 20 and 28 °C is reported. In Figure 5.44 the chromatograms of the Hi-trap step are shown.

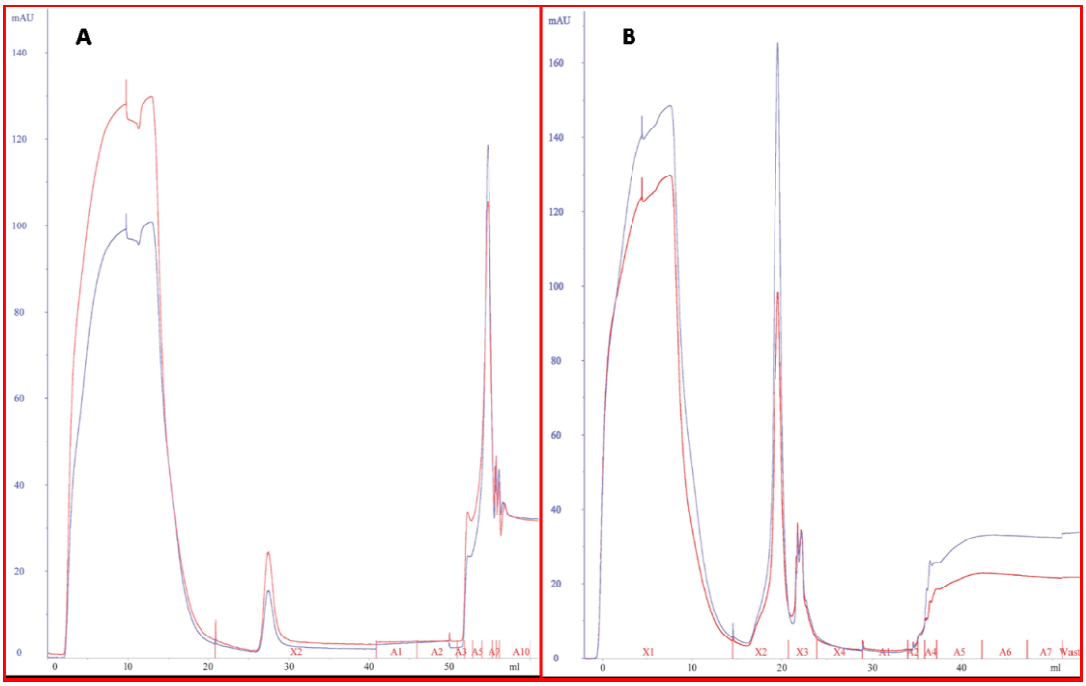

Figure 5.44: Hi-trap chromatograms related to cultures at 20 (A) and 28 (B) °C (OD_{280} in blue, OD_{260} in red).

At the end of the test the whole cultures were centrifuged and the enzyme was purified by a single Hi-trap step and a following size-exclusion. During the purification, the supernatant was loaded on the Hi-trap column, once its salt concentration and pH were adjusted with buffer A (20 mM sodium phosphate pH 7.5, 500 mM NaCl). Afterwards, the column was washed with buffer B (20 mM sodium phosphate pH 7.5, 150 mM NaCl). When this step was performed during the purification of the culture maintained at 28 °C (Figure 5.44 B)a higher activity was observed in the flow-through during the washing step (buffer B): the laccase was eluted, highlighting the poor binding to the column, probably due to poor quality of the protein obtained in these culture conditions. This effect was not seen with the supernatant of culture at 20 °C (Figure 5.44 A). These results were confirmed by activity tests on the eluted fractions and on the zymogram in Figure 5.45.

The activity of the eluted fractions revealed the following values: at 20 °C, 51.76 UI/L (0.24 UI/mg) for the eluted laccase, and 0.16 UI/L (0.001 UI/mg) in the flow-through of loading and washing steps, whereas at 28 °C, 33.72 UI/L (0.36 UI/mg) for the eluted laccase, and 16.32 UI/L (0.18 UI/mg) in the flow-through of loading and washing steps. The higher purified laccase activity was achieved by the culture maintained at 20 °C in comparison with the other at 28 °C, but the latter was affected by purification problems.

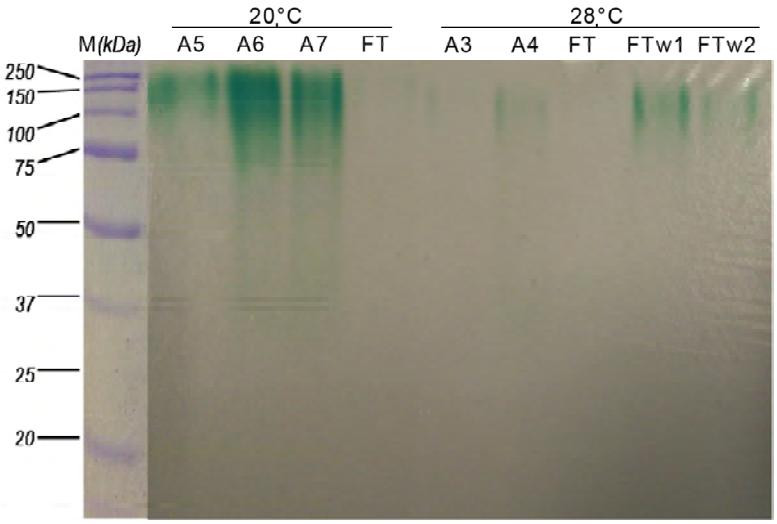

Figure 5.45: Zymogram of Hi-trap eluted fractions (An°; FT: flow-through; FTw: flow-through wash) related to cultures kept at 20 and 28 °C.

The zymogram shows that a more intense and bigger greenish halo was formed in Hi-trap fractions related to the culture maintained at 20 °C in comparison with the other ones correlated to the culture kept at 28 °C. A higher laccase activity was recorded in the flow-through during the washing step of culture at 28 °C (FTw1 and FTw2), and was probably related to a minor stability of the protein, or to its partial degradation. In fact, if the His-tag is lost only aspecific binding occur between the protein and the stationary phase of the column. Subsequently, the fraction corresponding to the highest activity (A6, Hi-trap 20 °C) was loaded on a size-exclusion column, to assess the molecular weight of the protein. Three main peaks were present on the chromatogram, but the activity of the fractions revealed that the active laccase was present only in the middle peak, centred at 12.5 mL (elution volume) and corresponding to a molecular weight ranging between 70 and 100 kDa. The other two peaks could be aggregated protein (first peak), and/or larger and thinner proteins present in the sample.

Mutants expression and purification

Once the optimal conditions for flask cultures and laccase purification were established, the laccase mutants were expressed for subsequent biochemical characterizations. The transformants were selected on MM/Cu/ABTS agar plates: the green halos were less intense and dark increasing the number of mutated residues (Figure 5.46).

Figure 5.46: Transformants selection on MM/Cu/ABTS agar plates.

It was reported that yeast transformed with the cDNA of isoform 4 of *T. versicolor* consistently produced green zones more rapidly (within 24 h) than those transformed with the cDNA of isoform 1,

suggesting that the former was secreted more efficiently than the latter, and/or was more efficient in oxidizing ABTS [248]. This means that a rough estimation of differences in the activity of mutated laccases can be made from the halos on MM/Cu/ABTS agar plates and from the time they appeared.

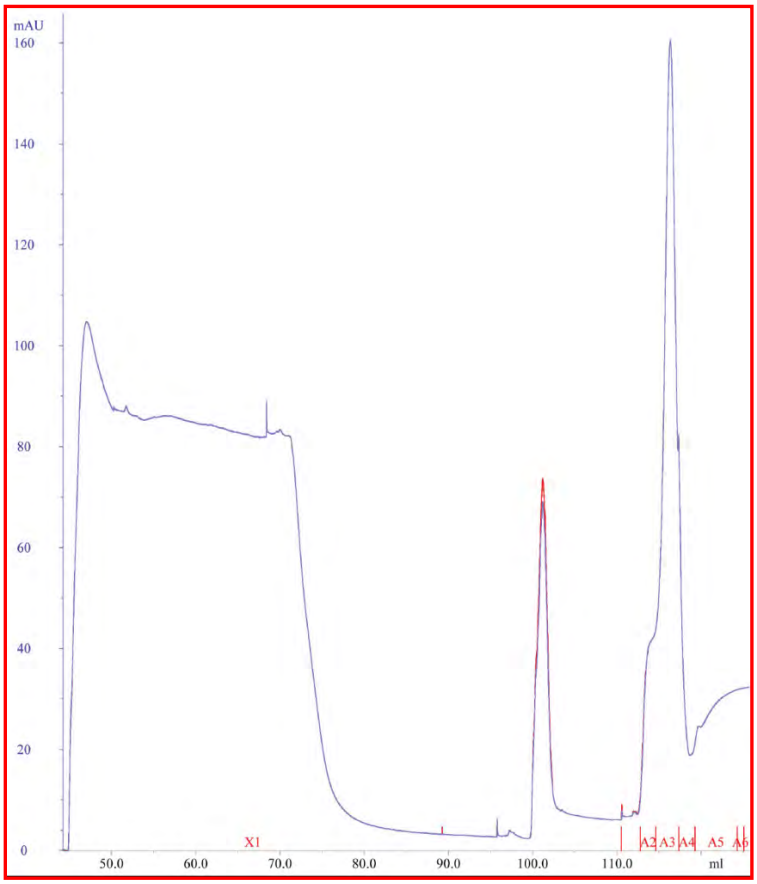

Figure 5.47: Hi-trap chromatograms of M1 mutant purification.

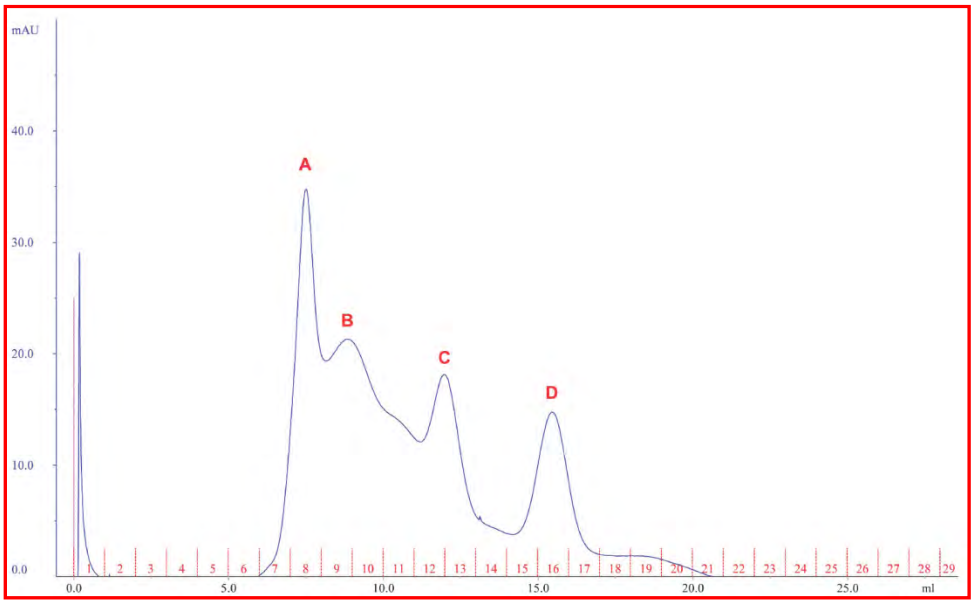

Figure 5.48: Size-exclusion chromatograms of M1 mutant purification.

Mut$^+$ transformants were cultured showing that growth curves of yeast expressing wild type and mutated laccases did not differ significantly (data not shown), then, the enzymes were recollected

from supernatants. During the following purifications the buffer B (washing step) was changed and the NaCl concentration was adjusted to 200 mM, instead of 150 mM. This reduced the leakage of the His-tagged protein from the column during the washing step. The salt concentration was also increased in the loading buffer (buffer A) to 1 M NaCl, to avoid leakage of the protein during the loading step, too. These tricks improved the level and quality of pure laccase, as is possible to see in Figure 5.47, representative of this better purification. In this purification the activity before and after the Hi-trap column were 5.7 and 166.4 UI/L, respectively, with a corresponding activity in the flow-through of 0.07 UI/L. The Hi-trap fraction with the highest laccase activity (A3) was concentrated and charged on a size exclusion column. In Figure 5.48 the chromatogram is reported.

Four peaks are visible in the chromatogram (A, B, C, D). The laccase activity was determined for each eluted fractions, and the purified laccase was present in the peak centred at 12.5 mL (C), corresponding to a molecular weight of about 100 kDa, and with an activity of 336 UI/L, and a specific activity of 1.7 UI/mg. A similar purification was achieved with mutated laccase isoforms.

The *P. pastoris* system of protein expression is usually able to produce 1-5 g/L of pure protein if properly optimized [189]. This yeast secrete very low levels of native proteins in the culture media [249], and this means that secreted heterologous protein comprises the vast majority of the total protein in the medium [250]. The concentration of protein secreted in the culture medium in this study was only 0.1-0.2 g/L, corresponding to a medium level of expression if compared to other reports [251, 252]. This level of expression and the overall purified laccase quality were probably affected by flask culture conditions. One of the most important parameter, not investigated in flask culture, is aeration, that is critical during the methanol induction [189]. The maximum agitation of the shaker was used, in combination with baffled flasks, but probably it was not enough to maintain a proper dissolved oxygen concentration of the culture medium that should be over 20 % [189].

Other issues almost certainly affected the expression. First of all, the laccase degradation by protease activity, or the incorrect cleavage of the secretory signal peptide (α-mating factor) could have been detrimental for protein yield. Even if a large number of proteins, and also laccase enzyme (see annex B), was successfully expressed and secreted using the αMF signal, unsuccessful processing of this signal peptide may occur, reducing the protein yield. A lower specific activity (25 %) was obtained with the heterologous signal peptide (αMF) in comparison with the native signal peptide of the fungus [253]. Usually the αMF signal includes two Glu–Ala repeats at the junction between the signal peptide and the NH_2-terminus of the mature protein of interest. This sequence is needed for the cleavage of the signal peptide from the rest of the protein because is recognized by the KEX2 protease, responsible of this modification. The proteolytic cleavage is enhanced if the sequence Glu-Lys-Arg-X (where X is Glu of Glu-Ala repeats) is placed before. Aminoacid other than Glu can vary the efficiency of this system (e.g. proline inhibits KEX2 cleavage) [254]. Furthermore, the Glu-Ala repeats are then cut by the STE13 protease, but the cleavage is not always efficient, and Glu-Ala repeats are left on the N-terminus of the expressed protein [189]. The additional NH_2-terminal residues may have also affected the activity of the recombinant enzyme: Guo and collaborators supposed also some interferences of the propeptide coded by the αMF with the folding of laccase [231]. Moreover, the molecular weight of the protein could have been erroneously determined by SDS-PAGE or size-exclusion mainly because of glycosylations. The molecular mass of the recombinant laccase was 85 kDa by SDS-PAGE and 100 kDa for the native enzyme by size-exclusion chromatography. The same values were found also by Brown and co-workers: although the endogenous KEX2 protease of *P. pastoris* efficiently removed the αMF signal peptide, the STE13 sometimes failed to cleave the Glu-Ala repeats encoded by the pPIC9 vector, leading to retention of a tetrapeptide at the N-terminus of the secreted enzyme [253]. The use of the αMF signal peptide added additional aminoacids, resulting in a 1.3 kDa increase of the molecular weight of recombinant laccase also in the paper of Hong [233].

Variable results have been reported in other studies comparing the *S. cerevisiae* αMF signal peptide to a native fungal signal peptide for directing secretion of recombinant laccase [253].
The laccase production described in the current study could probably be enhanced by further optimization of medium and culture conditions, especially by means of controlled conditions in a bioreactor instead of using shake-flasks. However, the main objective of this work was not to maximize protein production but to set up a heterologous expression system for the following protein engineering and rational design of the enzymatic binding site.

Mutants preliminary characterization: evaluation of the specific activity toward phenolic and non phenolic compounds

The recombinants and wild type laccases were purified and some characterizations were performed. First of all, the activity of laccase wild type and mutants was tested at different pH (3.0-8.0) with a phenolic (2,6-DMP) and a non phenolic compound (ABTS). The activity is a measure of the reactivity of the enzyme against a specific compound: the higher the activity, the higher the ability of the enzyme to metabolize the tested compound.
Oxidation of ABTS and 2,6-DMP proceeds presumably through different mechanisms as the oxidation of ABTS does not involve a proton transfer from the substrate, in contrast to the phenolic substrates. Recently, the oxidative reaction of 2,6-DMP by *Melanocarpus albomyces* laccase has been disclosed by Kallio et al. [219]. They produced X-ray structures of this laccase complexed with 2,6-DMP, at different time after soaking the crystals in a substrate solution, observing two main reaction product: a monomeric 2,6-dimethoxy-p-benzoquinone, and a C–O dimer of 2,6-DMP. They also suggested that phenolic substrates release a proton and an electron when they are oxidized to radical products by the enzymatic action of a laccase. These radicals can then further react non-enzymatically with other phenolic radicals or with smaller oxygen-based radicals: 2,6-dimethoxy-benzoquinone was probably formed following the latter reaction, typical of aerobic conditions [255], whilst C–O 2,6-DMP dimers were formed form through two oxidative couplings. In contrast to phenolic substrates, that form quinones or polymers, the ABTS oxidation proceeds in one step resulting in a blue-green coloured radical cation ($ABTS^{\bullet+}$) [256].
The activity profiles toward ABTS and 2,6-DMP are reported in Figure 5.49. Since recombinant proteins have been only partially purified, the specific activities values (UI/mg) were estimated using the total protein concentration (mg/mL) of the analysed enzyme solution.
Regarding ABTS activity, is possible to see in Figure 5.49 (A) that only a couple of mutants (M1 and M5) showed higher specific values in comparison with the WT laccase. By dividing the mutants in two groups, those with a larger binding site, and those with a mutated Asp206, distinctive trends are seen: the former group (M1, M3 and M5) has an activity profile more similar to that of the WT if compared with the second group (M2, M4 and M6), unless some differences at acidic pH. The WT, represented by filled star, has an optimal pH between 5.0 and 6.0, with a slight decrease of activity decreasing pH value. On the contrary, M1 (filled square), M5 (half filled square) and M3 (open square) have an optimal pH around 3.0 and the activity increases along with the drop of pH between 3.0 and 6.0. Similar profiles have been found also by other authors [156, 233]. The specific activity toward ABTS dramatically drops for all these iso-enzymes at pH higher than 6.0. The second group, M2 (filled circle) and M4 (open circle), has a different profile, that shows an higher activity at pH 3.0, but a faster decline of activity, that become very low at pH 6.0. The M6 mutant (half filled circle), instead, has a maximum at pH 5.0, but its activity is very low if compared to other enzymes (less than 25 % activity if compared to WT).

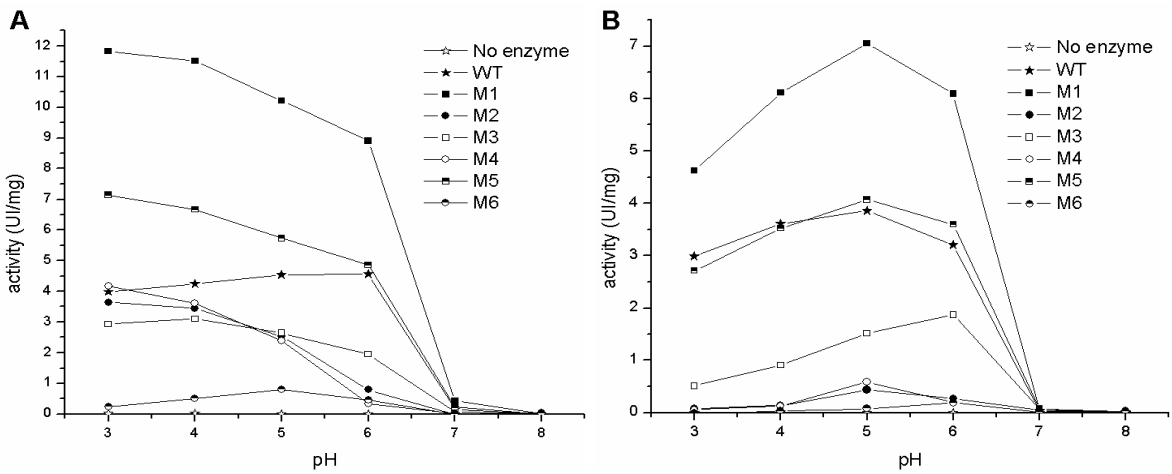

Figure 5.49: Enzymatic activity of WT and mutated laccases against ABTS (A) and 2,6-DMP (B).

In Figure 5.49 (B), the specific activity toward 2,6-DMP is reported. The pH activity profiles on 2,6-DMP are very similar for WT and mutants. A bell-shaped profile was observed, with a maximum at pH 5.0. This maximum is higher than the one reported by Jolivalt et al. [245], or by Madzak et al. [257] for the same enzyme obtained in yeast *Yarrowia lipolytica*. A higher (almost double) specific activity was recorded for M1 (filled square), compared to other enzymatic isoforms, whereas WT (filled star) and M5 (half filled square) showed very similar values. A lower activity was observed for the other mutants, especially for those with a changed Asp206: M2 (filled circle) and M4 (open circle) showed 8 times lower activity against 2,6-DMP in comparison with the WT, but no optimal pH shift was recorded for these two mutants as previously reported by Madzak et al. [257]. Moreover, it seems that the bell-shape profile is narrower than the one observed for the WT. Some differences were also found for M3 (open square) and M6 (half filled circle) that have a maximum at pH 6.0. At the same time, M6 showed a very low activity if compared to other enzymes, as reported above for ABTS. It is feasible that this mutant, the one that collect all the tested mutations, has the lower folding stability. In fact, it is known that the folding stability decreases by increasing the number of mutated residues.

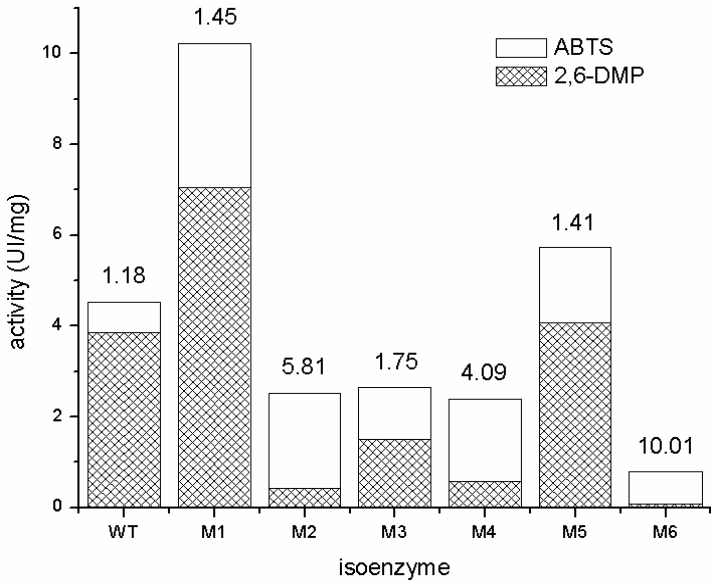

Figure 5.50: Enzymatic activity of WT and mutated laccases against ABTS and 2,6-DMP at pH 5.0. The ABTS/2,6-DMP ratio is reported on top of the column bars.

A comparison between the two substrates indicates that laccases have a higher reactivity against ABTS: the WT shows similar specific activity values, but in the reaction mixture a ten-fold higher 2,6-DMP concentration was used. This result agrees with those of Jolivalt et al. [245], that determined ten-fold higher K_M values for 2,6-DMP than for ABTS. According to these results, also Galli et al. [228] showed that the F332A mutant has lower activity for 2,6-DMP, with a 10-fold increase in K_M in comparison with the WT.

Moreover, all mutants, but especially those with a larger binding pocket, showed a further increase of activity toward ABTS in the same reaction conditions. By way of example, M1 has a specific activity of 10.2 UI/mg against ABTS, and of 7.0 UI/mg against 2,6-DMP at pH 5.0 (Figure 5.50). At the same time, by calculating the activity ratio between these two substrate (ABTS/2,6-DMP), the greater change in substrate specificity was registered by D206N mutants (M2, M4, M6).

Based on these results, it seems like that the mutation of Asp206 influences not only the oxidation of phenolic compounds, as previously reported [219], but also the ABTS radical activation. Xu [258] supposed that the bell-shaped activity-pH profile typically shown by fungal laccases with phenolic substrates is a consequence of two opposite effects. The ascending part of the curve at acidic pH is caused by the redox potential difference between the reducing substrate and the T1 copper, favoured, for a phenolic substrate, by higher pH. The descending part is generated by the binding of a hydroxide anion to the copper in the T2/T3 centre of laccase, which inhibits the activity at higher pH, combined with a drop in the oxygen reduction potential as pH rises. It is reasonable that a similar phenomenon affected the oxidation of ABTS: probably, the inhibition at pH higher than 6.0 observed for each iso-enzyme was due to a decreased oxygen reduction potential and to the binding of a hydroxide anion to T2 copper.

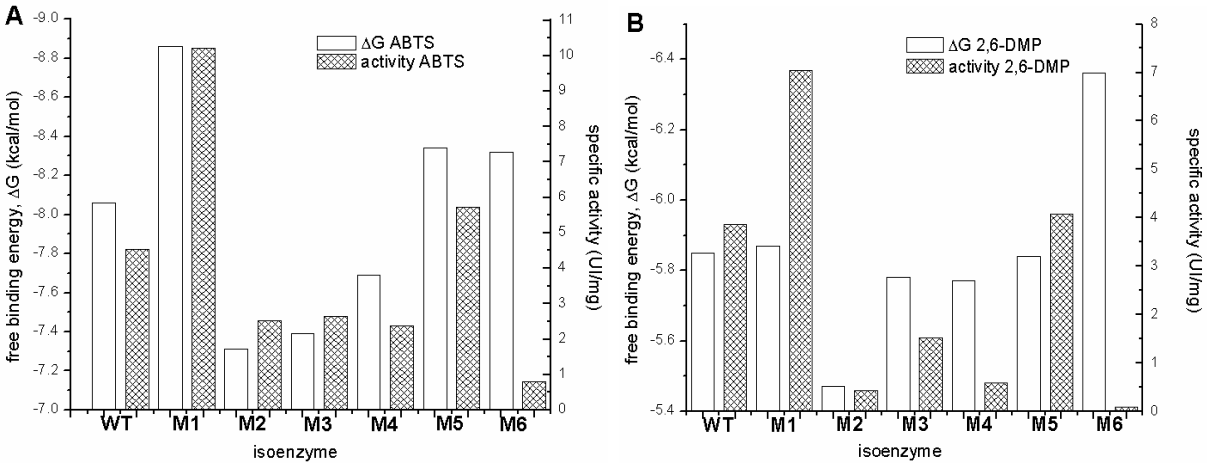

Figure 5.51: Comparison between enzymatic activity of WT and mutated laccases against ABTS (A) and 2,6-DMP (B) at pH 5.0, and binding free energy estimated during computational docking.

The dimensions of 2,6-DMP does not exceed the limiting dimensions of the binding pocket, and it was not expected that a larger catalytic site (Phe to Ala) would substantially affect the enzyme activity for this substrate. At the same time, ABTS, that is quite larger than 2,6-DMP, is bent and only partially buried inside the pocket in the X-ray structure of CotA laccase [259], and some differences could be expected by changing the enzymatic active site. Furthermore, by comparing these results with the computational docking simulations performed and indicated in Table 5.6, some interesting considerations could be made. The specific activity agrees with the estimated binding free energy calculated during computational docking, although the algorithm was much more reliable in evaluating the interactions of the non phenolic compound ABTS with laccase, than those calculated

for 2,6-DMP (Figure 5.51). At the same time, the software mainly evaluates the affinity between the two chemical structures, and was not able to assess the real catalytic activity. Particularly, the simulation failed to model the behaviour of M6 mutant, whose folding stability was probably affected by mutations. These results validate the synergic approach of rational design assisted by computational docking simulations.

A stability test was performed by evaluating the residual activity of laccase samples (WT and mutants) kept at different temperatures (room temperature (RT), 4 and 20 °C) for 96 hours (Figure 5.52).

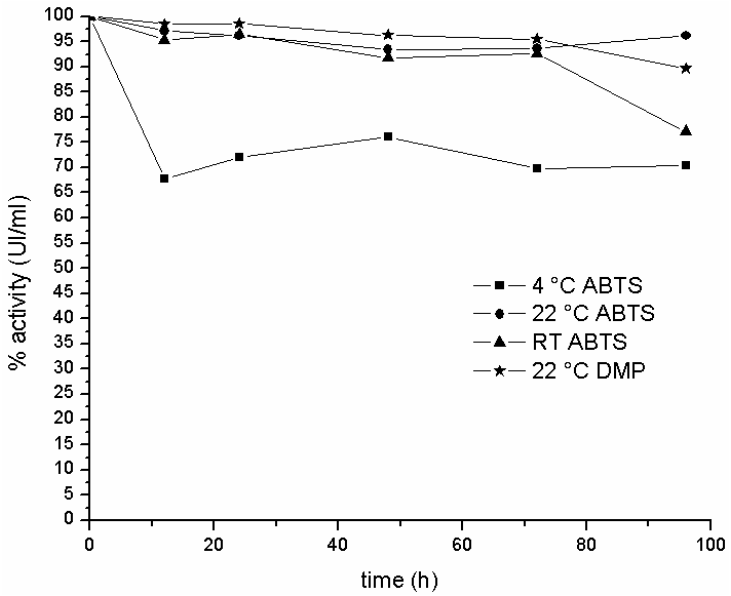

Figure 5.52: Laccase residual activity after incubation of enzyme at different temperatures.

The plot in Figure 5.52 , representative of one enzyme isoform (M1), show that the enzyme is very stable, and each mutant, unless M6, retain almost 80 % of activity after 96 hours. Probably, M6 has not a high folding stability. Galli et al. [228] reported that the half-life of F162A, F332A and double mutant (F162/F332) mutants are decreased to 37, 21 and 3 minutes, respectively, if compared to the wild type laccase.

All mutants showed a reduced stability after incubation at 4 °C: actually, after the first incubation the enzymatic activity didn't drop further. It could be that at lower temperatures the protein forms more aggregates than at 20 °C, or at room temperature. The protein stability of laccases is related to temperature, O_2 concentration and enzyme concentration: these parameters can obviously affect the enzymatic activity, but also protein aggregation [260]. Frozen sample have been also evaluated, but a dramatic drop of enzymatic activity was recorded. The same effect was previously reported by Shleev et al. [260] for highly concentrated frozen sample of *Trametes hirsute*: after thawing, the activity was 50% of the same sample left at room temperature for three hours. One possible explanation is that at temperatures below -18 °C, reversible autoreduction of the laccase T1 copper site occurs, reducing the catalytic activity of the enzyme [260]. These results confirm that the enzyme is more stable at room temperature (at least for 96 hours), than frozen, or kept at 4 °C, maybe for aggregation problems.

Evaluation of the reactivity against PAH by means of decolourization assay

One of the simplest and safer methods to evaluate the enzymatic activity toward aromatic pollutants involves mostly testing laccase breakdown of common aromatic dyes. This test is based on the

assumption that decolourization of dyes and biodegradation of aromatic pollutants is highly correlated [138, 261, 262]. Disappearance of colour is a simple and rapid way to measure laccase reactivity versus compounds with chemical structure resembling that of aromatic organic pollutants.
Decolourization of polymeric dyes has been proven to be a good indicator of the biodegradation of xenobiotics by peroxidases [261, 262, 263]. A clear relationship between a specific dye and pollutants oxidation by laccase has also been established. Alcalde et al [138] validate the use of Poly R-478 (R-478) by showing very similar time course through the comparison of decolourization data and HPLC data analysis of anthracene and benzo[α]pyrene biodegradation operated by laccase enzyme [138]. The decolourization of other dyes [264, 265] has been also proved to be related to PAH biodegradation: by means of example, Remazol Brilliant Blue R (RBBR) degradation has been related to PCB degradation [266]. Decolourization tests on more than one structurally different dye, and different pH values, may give more information about the catalytic activity of laccase against xenobiotics than activity toward phenolic and non phenolic compounds [267].
In the following tests, different dyes were used to evaluate the ability of wild-type and laccase mutants to decolourize industrial dyes. Initially, a preliminary tests was performed with commercial laccase (*T. versicolor* laccase from Sigma Aldrich), to evaluate the decolourization ability of the laccase against the selected dyes: dyes were tested alone, or with different mediators (HBT, glycine, cysteine and imidazole), and in reaction mixtures buffered at different pH. Some characteristics of these dyes [268] and some results from a first round screening are listed in Table 5.7, while their structure is shown in Figure 5.53. All these dyes have been selected because of their structure, and because they cause serious environmental pollution [265]. They are classified on the basis of the type of chromophore (e.g. Azo, Anthraquinone, Triphenylmethane), or of the textile dying process (e.g. reactive, acid or natural) [269].

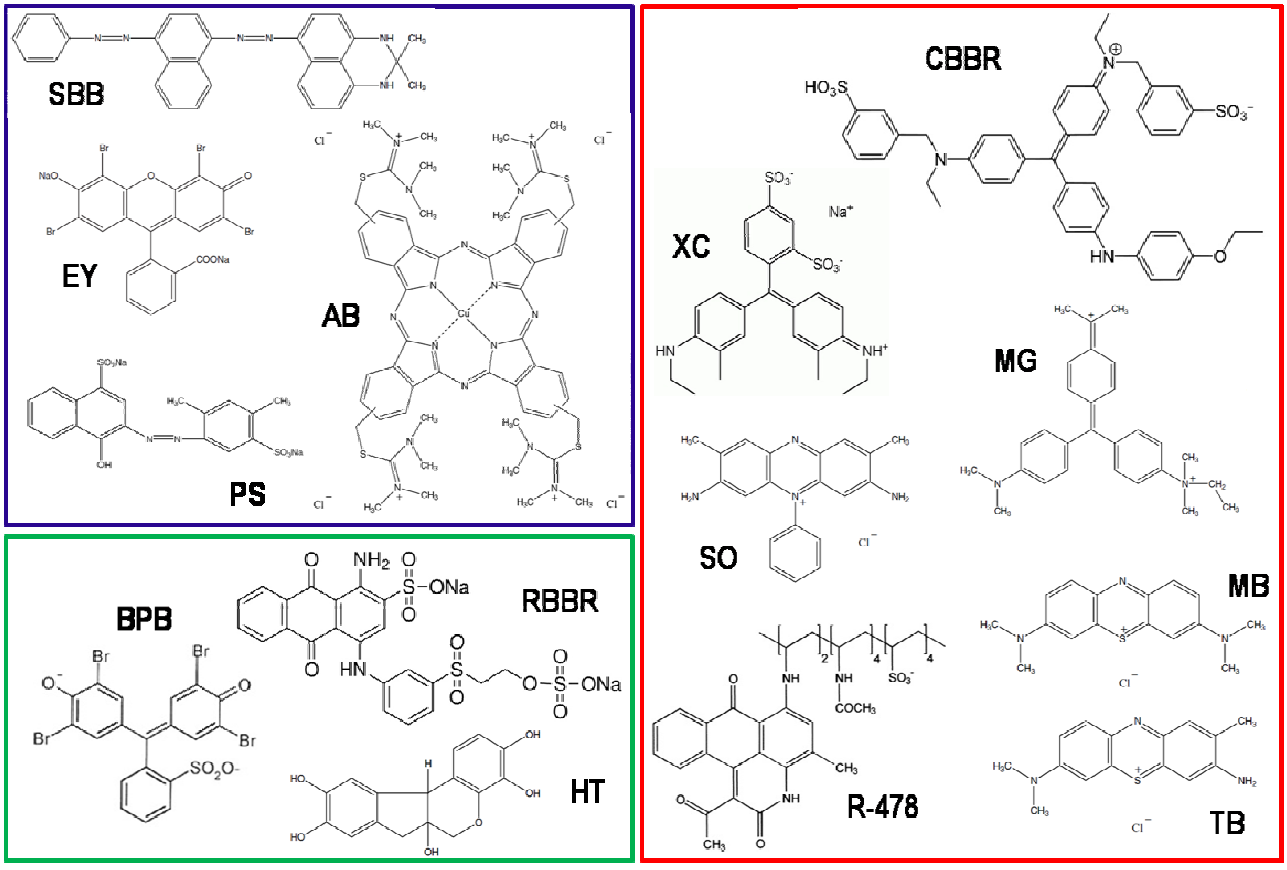

Figure 5.53: Structure of tested dyes.

Table 5.7: Decolourization activity of wild type T. versicolor laccase from Sigma-Aldrich.

Dyes	Classes	MW [g/mol]	λ [nm]	Optimal pH	Decolourization*
Sudan black B (black 6) (SBB)	Azo	456.54	600	/	No
Ponceau S (acid red 112) (PS)	Azo	760.57	500	/	No
Eosin Y (acid red 87) (EY)	Xanthene	691.85	500	/	No
Alcian blue 8G (ingrain blue 1) (AB)	Copper phthalocyanine	1298.86	580	/	No
Coomassie brilliant blue R250 (acid blue 83) (CBBR)	Triphenylmethane	825.97	600	/	B
Xylene cyanol (acid blue 147) (XC)	Triphenylmethane	538.61	600	3.0	B
Methyl green (/) (MG)	Triphenylmethane	653.24	600	4.0	B
Safranin O (basic red 2) (SO)	Phenazine	350.84	500	/	B
Toluidine blue (basic blue 17) (TB)	Phenothiazine	270.374	660	/	B
Methylene blue (basic blue 9) (MB)	Phenothiazine	319.85	675	/	B
Poly R-478 (R-478)	Polymeric	n.d.	520		B
Bromophenol blue (BPB)	Brominated phtalein	669.96	600	5.0	A, B
Hematoxylin (natural black 1) (HT)	Flavone	302.28	500	5.0	A, B, C
Remazol brilliant blue R (RBBR) (reactive blue 19)	Anthraquinone	626.54	600	4.0	A, B

*A: dye alone; B: HBT 1mM; C: Glycine 1mM

No decolourization was observed with SBB, PS, EY, and AB in none of the tested conditions. Moreover, none of the investigated mediators (HBT, Glycine, Cysteine, and Imidazole) helped the enzyme in the transformation of these dyes. Cysteine was found to be non influential, differently from what previously reported [265, 270]. Instead, the wild type laccase was able to decolorize all other tested dyes. SO, XC, TB, MB, CBBR, MG and R-478, were transformed only if 1mM HBT was present in the reaction mixture, while BPB, HT, and RBBR were also oxidized by the enzyme alone. Furthermore, HT turns from dark blue to pale yellow also if 1 mM glycine was used as a mediator

instead of HBT. The optimal pH was always in the acidic range (3.0-5.0), confirming results found by Palmieri et al. [264], whereas HBT gave better results if used as a mediator.
These results showed that the decolourization assay could be an easy and useful technique: the wild type laccase is able to transform some of the tested dyes, but some other dyes remain unaltered, also changing the pH of the reaction. The WT laccase is not able to decolourize Azo, Xanthene and Phthalocyanine dyes, neither in the presence of HBT. Decolourisation of mono-Azo and di-Azo dye has been extensively demonstrated in *Phanerochaete chrysosporium* [271] and *Trametes versicolor* [272], but these fungi are able to produce also peroxidases and other ligninolytic enzymes, and laccase alone is able to degrade these dyes only if a mediator is present [265]. Instead, the higher redox potential of lignin peroxidise make this enzyme able to oxidize almost every dye tested in this work [273]. Another parameter that could have been effectual on laccase activity toward these dyes is bioavailability and solvents in the reaction mixture: in this work, to solubilise Azo, Xanthene and Phthalocyanine dyes, ethanol and acetic acid have been used. These solvents probably interfere with the protein folding stability and enzymatic activity.
Among dyes directly oxidated by WT laccase, RBBR, an Anthraquinone dye, is one of the most important in the textile industry, that is frequently used as starting material in the production of other polymeric dyes (e.g. R-478), and represents an important class of toxic and recalcitrant organopollutants [264]. The oxidation of this dye is achieved only by laccases with a high redox potential, such as that of *Trametes* sp. [265], and not by the other isoenzymes (e.g. *M. thermophila* laccase [269]). The degradation pathway of RBBR by *T. versicolor* laccase has been recently proposed by Osma and co-workers [274] that verify the breakage of the chromophore by LC-MS. The oxidation of this dye certifies that the WT used for the subsequent protein engineering has already high metabolic properties.
The WT laccase was not able to modify Triphenylmethane, Phenazine, Phenothiazine and polymeric dyes, such as MB, MG or R-478, unless in presence of the mediator HBT. Triphenylmethane dyes are non-planar because of steric hindrance [275]. It is reasonable that these compounds are too large for the catalytic site, and consequently are not oxidized by WT laccase. Probably, the other not metabolized dyes were affected by the same problem of steric exclusion from the binding pocket. This last hypothesis is supported by the fact that HBT significantly improve the decolourization of these dyes. In a large number of papers HBT was found to significantly improve dyes and PAHs biodegradation, and was by far the best mediator tested in comparison to ABTS or other mediators [138]. At the same time, a higher potential could have been detrimental for the enzyme catalysis. It's known that small molecules, such as naphthalene, are not oxidized, although they are able to interact with the binding site, because of the high ionization potential of the compound [276].
A test with WT and mutated laccases was performed to roughly evaluate if one or more mutation positively affected the decolourization ability of the enzyme against the tested dyes. In Figure 5.54 and in Figure 5.55 the OD trends and the pictures of the plates of this experiment are reported.
The oxidation of RBBR was higher for M1 mutant, in both test conditions with HBT (Figure 5.54 B) or without HBT (Figure 5.54 A), if compared to the WT (filled star), or to the other mutants. The lower activity toward this dye was recorded for the M6 mutant. Generally, the RBBR degradation was higher for those mutants with a larger binding pocket (M1, M3 and M5), in comparison with the other that carry the D206N mutation (M2, M4 and M6). The reactivity of mutants and WT in presence of the mediator was similar, following the same order: M1>WT>M5>M2>M3>M4>M6.
The decolourization of MG was more interesting. The M1, M3 and M5mutants were more active against the dye without HBT (Figure 5.54 C) than the WT laccase, which was only partially able to degrade it. The ranking of reactivity was: M1>M5>M3>WT> M4>M6>M2. The reactivity in presence of the mediator (Figure 5.54 D) was similar to the one recorded for RBBR. Generally, as well as for

RBBR, the MG degradation was higher for those mutants with a larger binding pocket (M1, M3 and M5), in comparison with the other that carry the D206N mutation (M2, M4 and M6).
Regarding R-478, the oxidation was achieved only in presence of HBT (Figure 5.54 F), and was lower than the one reported for the other dyes. Obviously, the ranking of mutants was the same as the other two tested dyes in presence of the mediator.

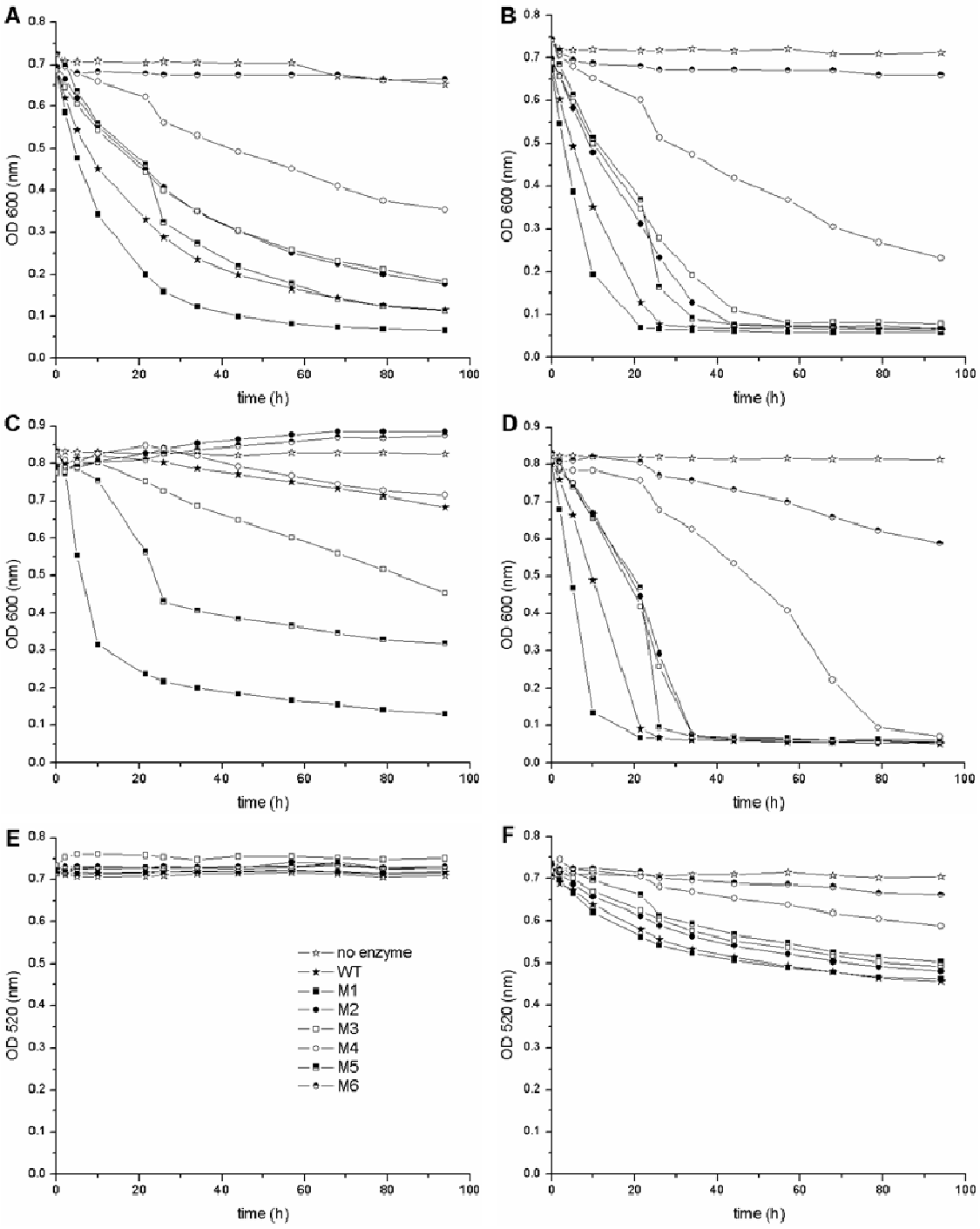

Figure 5.54: Decolourization of selected dyes: without HBT, RBBR (A), MG (C), R-478 (E), or with HBT, RBBR (B), MG (D), R-478 (F).

From the oxidation extents reported, it is evident that the bulky substrates are only partially oxidized by WT laccase, and the rationally designed mutants improved the catalytic activity of the enzyme toward dyes, but the mutations do not uniformly confer the same degradation ability. The oxidation

of RBBR, already catalyzed by the WT, was enhanced in those mutants with a larger binding pocket, and the same effect was seen for the oxidation in presence of the mediator of all three dyes. The most interesting result is that the larger binding pocket significantly increases the reactivity of the enzyme toward MG in reaction mixture without HBT. The Triphenylmethane dyes MG, usually not oxidized by WT laccase for dimensional exclusion from the binding pocket, was properly modified by mutants. More in detail, the double mutation F162A/L164A (M1) gave better results than F332A (M3), and in between, the cumulative mutant M5 (F162A/L164A/F332A) showed a midway reactivity. Galli et al. [228] tested the reactivity of F162A and F332A mutants against a set of mono- and di-substituted phenolic substrates with increasing steric hindrance, and a similar result was achieved.
Similar results have been obtained in tests with other dyes: in Figure 5.55 the tests with BPB, MB, and HT were reported. As far as the mutants have been designed in order to extend the volume of the WT laccase to the advantage of bulky substrates, these results validate the protein engineering approach.
Furthermore, laccases able to oxidize larger and more toxic compounds than the classical substrates (e.g. ABTS) without the presence of a mediator, would be of great interest. Redox mediators are toxic, poorly water soluble, expensive, and they can act as inhibitors at high concentration [138, 265]. Enzymes like the M1 mutant could be used in mediator-less reactions, such as in pulp-Kraft bleaching industry. At the same time, further protein engineering could be made starting from the M1 mutant, and by means of a directed evolution approach [277]. In this situation, the decolourization assay could be also useful as a fast and cheap screening of mutant libraries.

Figure 5.55: Decolourization assays plates.

The amount of MG decolourization at different pH was also investigated (data not shown). The values compared after 24 hours, a time length suitable to allow proper discrimination of the oxidation efficiency, demonstrate that M1 has its optimal activity at pH 5.0, with a decolourization percentage of 72.5 %, whereas M5 and M3 in the same conditions showed a decolourization % of 48.4 and 6.4, respectively.

Conclusions

Structural analysis of the computational binding modes of pollutants allowed to design a modified catalytic binding pocket of increased size, resulting in a greater ability to bind and metabolize large compounds that would not bind to the wild type enzyme.

WT and mutated laccases were successfully expressed in *P. pastoris* and purified for subsequent analysis. Both steps were optimized to reach an acceptable amount of enzyme and specific activity for the following investigations.

The activity of the enzymes was initially tested and characterized with phenolic (2,6-dymetoxyphenol or 2,6-DMP) and non phenolic substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS) at different pH (3.0-8.0). The mutated enzyme F162A/L164A (M1) showed an increased specific activity (UI/mg) for phenolic or non-phenolic compounds, in comparison to the WT. Furthermore, the rationally engineered enzymes show activity at acidic pH values where the wild type laccase has three times lower specific activity. Afterwards, the rational design approach was validated by means of biochemical characterization of wild type and mutated laccases: the specific activity against phenolic and non-phenolic compounds agree with the computational docking results (estimated free binding energy).

Stability studies have shown that purified enzymes, unless M6 mutant, are stable for at least 96 hours, under a variety of temperature conditions (4, 22 °C, room temperature).

Decolourization assays of large aromatic dyes, used as model compounds, have shown that the mutated enzymes are reactive towards molecules with chemical structure resembling that of aromatic organic pollutants. Moreover, enzyme mutants with a larger binding pocket showed higher activity against Methyl Green, especially without a mediator (HBT).

The best engineered enzyme (M1), showing higher activity against these model compounds, will be further validated with polycyclic aromatic pollutants, proving that a new useful sensing element has been created. This new sensing element will be consequently integrated on an appropriate transducer (e.g. electrode), and coupled to a wireless platform creating a new BEWS. Furthermore, laccases that have the ability to work with non-natural substrates and without mediators will have applications not only in biosensing and in PAHs bioremediation.

6 – Conclusions

The environmental pollution is still a big deal in our society. The concern about the state of the environment forced the governments to preserve the ecosystems by increasing the international policies related to environmental monitoring. To date, environmental monitoring is mainly focused on chemical analyses based on spot-sampling, characterized by several limitations.

In order to overcome these drawbacks, new biological monitoring methods are under development: these include biosensors and biological early warning system or BEWS, regarded today as a complementary tool for environmental monitoring. These kinds of devices are well-suited to cooperatively and continuously monitor the environmental conditions, particularly if they are implemented on wireless networks: if harmful toxic levels of pollutants are detected by the BEWS, this will produce an alarm that can be subsequently supported by more specific actions and analysis. A BEWS can be in the near future one of the most promising applications of environmental biosensors, only by applying the proper biological sensing element.

Nowadays, different kinds of biorecognition elements have been effectively tested in ecotoxicology and biomonitoring. Whole cell systems and enzymes are suitable for environmental monitoring by determining the bioavailable and toxic concentration of xenobiotics, especially if the source and nature of pollutants cannot be predicted. Microorganisms usually detect a broad spectrum of chemicals, and represent a good opportunity for low cost, long life use, and wide range of conditions in which they can be applied. Besides, enzymes are effective when a particular kind of pollutant would be detected because is possible to fine tune their metabolic behaviour by means of protein engineering.

In this thesis, three different biorecognition elements, associated to different kind of pollutants, were investigated for their application in biosensors for environmental monitoring based on three different principles. First of all, the broad toxicity was associated to the decrease of light emission (EC_{50} or half effective concentration) of a bioluminescent bacterium, *Vibrio fischeri*. Then, metal toxicity was connected to the siderophore production of the soil and water microorganism *Pseudomonas fluorescens* in presence of different concentration of Fe^{3+}, Cu^{2+} and Zn^{2+}. At last, EDCs toxicity, or generally, an index of toxicity given by PAHs, was related to the metabolization of these compounds by laccase of *Trametes versicolor*.

Regarding the microbial sensible elements (*V. fischeri and P. fluorescens*), both were influenced by physical-chemical parameters: temperature, pH, inoculum percentage (v/v) and carbon source. Mainly, the evaluation of the influence of physical-chemical parameters revealed the agreement with the environmental variables ranges. For example, 20 °C is the optimal temperature required to make use of the responses of these biorecognition elements.

The light emission of *V. fischeri* was highly variable, although a more stable bioluminescence was obtained by means of a glucose fed-batch. Therefore, this sensible element can be used as a whole-cell biosensor for *in-situ* application (e.g. marine pollution control), even if the influence of environmental variables on the response must be taken into account.

The interaction between *P. fluorescens* and some metals (Fe^{3+}, Cu^{2+}, and Zn^{2+}) was also studied.

Initially, the MICs of Fe^{3+}, Cu^{2+}, and Zn^{2+} were determined with a new modified Kirby-Bauer method on agar plates and in 96 well plates. The MIC and PCC (pyoverdine critical concentration; see Chapter 5) obtained values were compared to those indicated in the WHO Guidelines for drinking water quality and in European directive 98/83/EC: MICs of Fe^{3+}, Cu^{2+} and Zn^{2+} are always above the threshold specified, whilst PCCs are very near to the recommended thresholds for iron and copper. The PCC was not determined for zinc in the tested range of concentration and conditions.

These results highlighted that this sensible element should be further investigated (e.g. with other metals and differently combined), but optimistic outcomes make pyoverdine regulation useful for the development of a biosensor able to monitor metals in the environment.

PVA cryo-gels were evaluated as entrapment matrix for sensible elements. At first, the proper method of freezing/thawing casting was established. Then the compatibility of this polymer with an environmental biosensing application was estimated. PVA was not used as carbon source by *P. fluorescens*. The determined diffusivity of Methylene blue showed that the PVA porous matrix is compatible with nutrients and small molecules diffusion, even though a similar test should be repeated by substituting the dye with pyoverdine.

The behaviour of PVA gels into water and air was evaluated. In air, the gels dry to film, by evaporation of the water content, and shrink to only 6.6 % of the initial thickness. Whilst, the total release of polymer from gels kept in water, almost completed in 90 hours, was less than 10 % of the initial mass of the gels.

All these results showed that the PVA could be a suitable entrapment matrix for the development of a biosensor for environmental monitoring, although further investigation will be required (e.g. biomass leakage evaluation, trapped cells viability).

The last sensing element assessed during this work was the lccβ laccase of *T. versicolor*. This part of the work was the result of the collaboration with Dr. Luca Varani of the Institute for Research in Biomedicine (IRB, Bellinzona, CH), group leader of the Structural Biology Laboratory.

A combination of computational docking and molecular biology techniques was used to generate rationally engineered laccases with increased ability to process large and persistent aromatic compounds (PAHs and EDCs). These mutated isoforms were produced by heterologous expression in *P. pastoris*, successfully purified, and characterized by means of biochemical assays.

The activity of the enzymes was initially tested and characterized with phenolic and non phenolic substrates at different pH (3.0-8.0): the mutated enzyme F162A/L164A (M1) showed an increased specific activity (UI/mg) in comparison to the wild type, in every tested condition. Moreover, the other rationally engineered enzymes showed activity at acidic pH values where the wild type laccase has three times lower specific activity. These results agreed with those obtained by computational docking simulations (estimated free binding energy), validating the rational design approach.

Decolourization assays of large aromatic dyes, used as model compounds, have shown that the mutated enzymes are reactive towards molecules with chemical structure resembling that of aromatic organic pollutants. By means of example, enzyme mutants with a larger binding pocket showed higher activity against Methyl Green, especially without a mediator (HBT).

The best engineered enzyme (M1), showing higher activity against these model compounds, need to be further validated with polycyclic aromatic pollutants, proving that a new sensing element has been generated. Subsequently, this enzyme, that showed high stability under a variety of temperature conditions (4, 22 °C, room temperature), will be integrated on an appropriate transducer (e.g. electrode), and coupled to a wireless platform generating a BEWS for environmental monitoring.

Finally, the used rational design approach, already validated by the agreement between specific activity and computational docking results, could be applied to other enzymes, to develop new biorecognition elements.

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Annex A - Culture Media and solutions

Liquid culture broth were prepared as reported below, whereas correspondingly solid media were prepared by adding 15 g/L of agar to the following recipes. The amount of components indicated in each medium composition is for 1 L of medium.

SeaWater (SW): 10 g Beef extract, 10 g Peptone, 250 ml Tap water, 750 ml artificial Sea water, pH 7.2. Artificial sea water (1L, filter sterilized and added after thermal sterilization): 28.13 g NaCl, 0.77 g KCl, 1.60 g $CaCl_2$ x 2 H_2O, 4.80 g $MgCl_2$ x 6 H_2O, 0.11 g $NaHCO_3$, 3.50 g $MgSO_4$ x 7 H_2O.
DSMZ nutrient broth 1 (DSM1): 5g Peptone, 3g Meat extract, pH 7.0.
Meyer et al 1978 [122] (M78): 6g K_2HP0_4, 3g KH_2P0_4, 1g $(NH_4)_2S0_4$, 0.2g $MgS0_4.7H_20$, 4g succinic acid, pH 7.0.
Luria Bertani (low salt) (LB): 10g tryptone, 5g NaCl, 5g yeast extract.
Yeast extract peptone dextrose medium (YPD): 10g yeast extract, 20g peptone, 20g glucose.
Yeast extract peptone dextrose medium sorbitol (YPDS): 10g yeast extract, 20g peptone, 20g glucose, 186g sorbitol (1M).
Minimal dextrose (MD): 13.4g YNB (Yeast Nitrogen Base), 20g glucose, 0.4mg biotin.
Minimal methanol MM/Cu/ABTS agar plate: 13.4g YNB (Yeast Nitrogen Base), 0.5% (v/v) sterile methanol, 0.4mg biotin, 0.3 mM $CuSO_4*5H_2O$, 0.2 mM ABTS.
Minimal methanol (MM): 13.4g YNB (Yeast Nitrogen Base), 0.5% (v/v) sterile methanol, 0.4mg biotin.
Buffered glycerol complex medium (BMGY): 10g yeast extract, 20g peptone, 0.1 M sodium phosphate buffer pH 6.0, 10% (v/v) sterile glycerol, 0.4mg biotin.
Buffered minimal glycerol histidine (BMGH): 13.4g YNB (Yeast Nitrogen Base), 0.1 M sodium phosphate buffer pH 6.0, 10% (v/v) sterile glycerol, 0.4mg biotin, 0.04g histidine.
Buffered minimal methanol (BMM): 13.4g YNB (Yeast Nitrogen Base), 0.1 M sodium phosphate buffer pH 6.0, 0.5-2% (v/v) sterile methanol, 0.4mg biotin.

Annex B - Introduction to P. pastoris expression system

Ligninolytic fungi, such as the Basidiomycete *Trametes (Coriolus/Polyporus) versicolor*, often secretes multiple laccase enzyme isoforms with a conserved primary structure but different physical-chemical characteristics: six genes and/or cDNAs encoding these laccases isoforms have been isolated [278, 279]. This complexity makes it difficult to purify individual isozymes for analysis, or to produce mutants by protein engineering. This problem could be overcome by heterologous expression in a host that does not produce endogenous laccase [253].
Since the 1980s, yeast were adopted as an alternative eukaryotic microbial expression system for improperly folded proteins or inactive protein aggregates obtained in bacterial expression systems [230]. In fact, the yeast is able to introduce some eukaryotic-specific posttranslational modifications including glycosylation and disulfide bridge [231]. However, expression of heterologous proteins in *Saccharomyces* is not always favourable for large-scale production due to problems such as hyper-glycosylation, loss of the plasmid during scale-up, and low protein yield [229].
Among yeast, *Pichia pastoris* is a broadly used host: it is a methylotrophic yeast [230], it is easily genetically manipulated, and the protein expression system is stably maintained by the integration of the vector into the host chromosome [231]. Furthermore, the cultures readily adapt to high-biomass fermentation in minimal low-cost media.
Laccase enzymes have been already produced in yeast systems, and in Table A.1 some of these are reviewed.

Table A.1: Laccase production in Pichia expression systems.

Ref.	Strain / plasmid	Mut^+ /Mut^S	Preculture	Induction strategies	Activity [UI/L]
ferm. [169]	X33 / pPICZαA	n.d.	BMGY 10 mL 30 °C 180 rpm	Basal salt medium, 7.5 L (OD ~ 0.5); 18-25 °C; 800-1000 rpm; air (10 L/min); **0.5 %** Me-OH (every 24h)	3400 (25 °C, 12-16 days)
flask [169]	X33 / pPICZαA	n.d.	BMGY 10 mL 30 °C 180 rpm	**BMM** 50 mL (OD ~ 1); 17-30 °C; 180 rpm; **0.5 %** Me-OH (every 24h)	105 (25 °C, native sig. peptide)
flask [238]	GS115 (His^-) / pPIC9K	Mut^+	BMG 20 mL 30 °C 200 rpm	**BMM** 30 mL (OD ~ 2); 20 °C; 200 rpm; **0.5 %** Me-OH (every 24h) 0.6 % L-Ala; 0.3 mM $CuSO_4$	**8300** (13days)
flask [156]	GS115 (His^-) / pPIC9K	Mut^+	BMG 10 mL 30 °C 150 rpm 0.1 mM $CuSO_4$	**BMM** 30 mL (OD ~ 1); 20 °C; 150 rpm; **0.5 %** Me-OH (daily); 0.6 % L-Ala; 0.3 mM $CuSO_4$	32000 (13days)
flask [233]	GS115 (His^-) / pPIC9K	Mut^+	BMG 10 mL 30 °C 150 rpm 0.1 mM $CuSO_4$	**BMM** 30 mL (OD ~ 1); 20 °C; 150 rpm; **0.5 %** Me-OH (daily); 0.6 % L-Ala; 0.3 mM $CuSO_4$	5470 (13 days) 8324 (irradiated yeast)
flask [241]	SMD 1168 (pep4, His^-) / pGAPZ	/	/	BMG 50 mL n.d. °C; n.d. rpm; 0.1 mM $CuSO_4$; 0.4 % His, 0.8 % Ala	1.3
ferm. [253]	GS115 (His^-) / pPIC9 – pHIL-D2	Mut^S	Basal salt medium, 30 °C, pH 4.5, n.d. rpm	Mixed Me-OH/ glycerol fed-batch with 0.4 $CuSO_4$; 30 °C; n.d. rpm; **0.5 %** Me-OH (continuous)	1500 (5 days)
flask [234]	SMD 1168 (pep4, His^-) / pHIL-D2	Mut^+	BMGY 50 mL 30 °C 250 rpm 0.1 $CuSO_4$	**BMM** or **BMMY** 100 mL (OD ~ 1); 20-30 °C; 250 rpm; 0.1 $CuSO_4$; **0.5-2.0 %** Me-OH (daily)	110 (BMMY); 3300 (BMM); 11500 (20 °C, 0.5 % Me-OH)
ferm. [234]	SMD 1168 (pep4, His^-) / pHIL-D2	Mut^+	BMGY 50 mL 30 °C 250 rpm 0.1 $CuSO_4$	Basal salt medium 1L (OD ~ 1); 20-30 °C; air (0.5 L/min); 500-1000 rpm; **0.1-1.0%** Me-OH (continuous)	**140000** (1380 U/mg)
flask [232]	GS115 – KM71 (His^-) / pPIC 3.5	Mut^+	BMGY n.d. °C n.d. rpm	**BMM** 20 mL (OD ~ 1); 22 °C; 120 rpm; 0.6 % L-Ala; 0.2 mM $CuSO_4$; **1 %** Me-OH (daily)	20.9 (8 days)

The *P. pastoris* genome contains two copies of the alcohol oxidase gene (AOX1 and AOX2) which let the yeast growing on methanol as the sole carbon source. The 85% of the alcohol oxidase activity in the cell is regulated by the AOX1 promoter, the promoter used to drive heterologous protein expression in *Pichia* [229]. The 'AOX1 promoter-Gene of interest' expression cassette is inserted into the *Pichia* genome along with a drug resistant gene such as zeocin, or a histidinol dehydrogenase gene (HIS4), for selection of transformed cells, by mean of example in His^- host strains, i.e. GS115 (his4).

Insertion of the expression cassette into the HIS4 or AOX1 locus, by single crossover integration, generates a Mut^+ strain (methanol utilization plus), that shows an identical phenotype in comparison with the wild type *P. pastoris*. Alternatively, when the expression cassette is inserted within the AOX1 locus by double crossover gene trans-placement, the Mut^S strain (methanol utilization slow) is generated [280]. The Mut^S phenotype could be also obtained by disruption of the AOX1 gene via gene insertion i.e. KM71 (arg4 his4 aox1Δ:SARG4) [281], that grows very slowly in media containing methanol as the sole carbon source because of the defective AOX1 gene [282] .If both alcohol oxidase genes are disrupted, i.e. MC100-3 (arg4 his4 aox1Δ:SARG4 aox2Δ:Phis4) [283], a Mut^- (methanol utilization minus) strain is created. This host strain cannot utilize methanol as its sole carbon source, and requires the use of alternate carbon source for growth and recombinant protein production [229]. This last strain was not used a lot, because of the complications of the screening process: glycerol, glucose, and ethanol cannot be used since they all repress the AOX1 promoter [229].

Even though native proteases of *P. pastoris* are not secreted into the fermentation medium, cell lysis can occur, especially at high cell densities, releasing proteases. For this reason protease deficient strains of *P. pastoris* (SMD series) have been developed [284].

Another functional molecular tool useful for secretion of foreign proteins, is the insertion of a secretion signal, such as the *S. cerevisiae* α-mating factor prepro-signal sequence (αMF) [186, 285], or the *P. pastoris* acid phosphatase gene (PHO1), immediately following the AOX1 promoter in the DNA vectors used for heterologous expression.

Methylotrophic yeast possesses a general methanol utilization pathway that is highly compartmentalized in methanol-induced peroxisomes and cytoplasm [286, 287]. Methanol enters the peroxisome and is oxidized to hydrogen peroxide and formaldehyde by alcohol oxidase, utilizing oxygen as an electron acceptor. The peroxide is oxidized to water and oxygen by peroxisomal catalase. Formaldehyde in the peroxisome reacts with xylulose-5-phosphate, forming two C3 compounds, dihydroxyacetone and glyceraldehyde-3-phosphate, further metabolized in the cytosol in the assimilatory pathway: one-third of the glyceraldehyde-3-phosphate produced becomes available for central metabolism and the generation of biomass [229]. A little part of formaldehyde enters the cytosol, where it is oxidized to carbon dioxide by two subsequent dehydrogenase reactions [288].

Methylotrophic yeast are able as well to metabolize glycerol as a carbon source under aerobic condition, that results in ethanol production during the batch, fed-batch, or induction phase [186, 289, 290,]: this implies that the regulation of methanol metabolism in yeast is a very complex process. Synthesis of methanol metabolizing enzymes (including the key enzyme alcohol oxidase, AOX) is induced by methanol, formaldeyde, and formate whilst is repressed by glucose and ethanol [287, 291, 292].

Fermentation protocols for *Pichia* normally include three separate phases. First of all the glycerol batch phase (a preculture step for flask tests), in which cells are initially grown on glycerol in a batch mode. Following, it could be useful to increment the biomass amount to a desired level prior to induction with a glycerol fed-batch phase. Furthermore, the AOX1 promoter is de-repressed during this phase due to the limited amount of glycerol. The last phase is the methanol fed-batch phase (the induction phase), in which methanol is fed at a limited feed rate or maintained at some level to induce the AOX1 promoter for protein expression. The cells require 4-6 hours to adapt to methanol after switching from glycerol [189]. Sometimes a limited glycerol feed is simultaneously applied to promote protein production. Moreover, the high salts/high cell density fermentation has been invented by Wegner et al. [293] for recombinant protein production by means of suited bioreactor achieving high yields.