DISTAV DIPARTIMENTO
DI SCIENZE DELLA TERRA,
DELL'AMBIENTE E DELLA VITA

LABORATORY
OF MYCOLOGY

Università
di Genova

CORSO DI DOTTORATO IN
SCIENZE E TECNOLOGIE PER L'AMBIENTE E IL TERRITORIO

CURRICULUM
BIOLOGIA APPLICATA ALL'AGRICOLTURA E ALL'AMBIENTE

XXXIII ciclo

From collection to myco-reactor
filamentous fungi for bioremediation

CANDIDATA **Ester Rosa**

TUTOR **Prof.ssa Mirca Zotti**

CO-TUTOR **Prof. Antonio Comite**

Contents

Abstract

Riassunto

Nonostante l'acqua sia presente in grande quantità sul pianeta Terra, soltanto una piccola parte di essa può essere sfruttata dagli organismi viventi. L'uomo sfrutta questa risorsa molto di più degli altri organismi perché oltre che per la sua sopravvivenza, la utilizza per l'agricoltura, l'allevamento, l'industria e come fonte di energia. La ricerca su nuove e diverse tecniche di depurazione delle acque è ad oggi uno dei punti irrisolti per rendere meno forte l'impatto dell'uomo sul pianeta. In generale l'acqua, o una qualsiasi matrice inquinata, viene depurata con diversi metodi (chimici, fisici, biologici), ma nessuno soddisfa a pieno i criteri di efficienza e sostenibilità.

Negli ultimi trenta anni le ricerche nell'ambito di tecnologie *eco-friendly* si sono moltiplicate: tra i diversi processi di depurazione la *bioremediation* sta dando ottimi risultati. Questo sistema ha come obiettivo la riduzione delle sostanze inquinanti nelle diverse matrici ambientali, sfruttando le capacità metaboliche degli organismi viventi come batteri, funghi e piante, per la degradazione in prodotti semplici senza l'utilizzo di sostanze chimiche a loro volta inquinanti o di costosi e complessi impianti di trattamento. La *bioremediation* che impiega i funghi per la depurazione di matrici ambientali inquinate si definisce *mycoremediation*. I funghi sono organismi adatti ad essere usati nei processi di biorisanamento perché hanno un metabolismo che permette loro di crescere e sopravvivere in presenza di sostanze tossiche e in condizioni estreme non tollerabili per la maggior parte degli altri organismi.

Uno dei molteplici vantaggi dell'utilizzo dei funghi è la loro efficacia depurativa anche quando la concentrazione e la biodisponibilità degli inquinanti è molto bassa, come nel caso di farmaci o altri interferenti endocrini dispersi nelle acque reflue. Un altro vantaggio specifico dei funghi filamentosi - su cui questa tesi sarà incentrata - è la loro struttura ifale che ne aumenta di molto le capacità di distribuzione e colonizzazione. Diversi funghi filamentosi sono in grado di agire sugli inquinanti organici recalcitranti: dai clorofenoli alle diossine, dal toluene agli idrocarburi. Non sempre sono in grado di degradarli completamente, ma possono comunque trasformarli in composti meno tossici. L'attitudine a metabolizzare composti organici chimicamente molto diversi tra loro, o presenti contemporaneamente, è dovuta alla capacità dei funghi di sintetizzare enzimi anche a bassa specificità.

Le caratteristiche dei funghi sono non solo specie specifiche, ma spesso ceppo specifiche, motivo per cui la ricerca e l'individuazione di nuovi ceppi è fondamentale per la *mycoremediation.* Proprio grazie alle sperimentazioni effettuate sui ceppi conservati nelle collezioni e isolati da diversi ambienti come quelli estremi si possono mettere a punto nuovi trattamenti. Al giorno d'oggi gli ambienti definibili come estremi non sono più solo le profondità marine o i ghiacciai artici, ma sono anche gli impianti

produttivi e di trattamento dei reflui. Nonostante siano ambienti ideali per la selezione di nuovi ceppi fungini, le ricerche continuano a concentrarsi solo sulle comunità batteriche lasciando la componente fungina praticamente inesplorata.

Un altro campo inesplorato è l'applicazione di ceppi fungini dalle capacità metaboliche note in impianti di depurazione a scala laboratoriale e, soprattutto, a livello industriale. Una tipologia di impianto che può essere adatto ai funghi filamentosi, di cui oggi si trovano pochissimi esempi in letteratura, comprende i bioreattori a membrane areate (MABR). Una tecnologia promettente per il trattamento di sostanze recalcitranti in ambiente acquoso, basata sul trasferimento di un gas (es. aria o ossigeno, ma in linea di principio possono essere utilizzati anche altri gas) attraverso una membrana ad un biofilm in contatto con una fase liquida contenente i substrati da metabolizzare. L'uso di funghi filamentosi in MABR è attualmente limitato principalmente alla biotrasformazione e ai processi industriali per la produzione di enzimi e bioprodotti.

Grazie all'approccio multidisciplinare della ricerca, svolta fra il laboratorio di micologia (DiSTAV - Dipartimento di Scienze della Terra, dell'Ambiente e della Vita) e quello di chimica industriale (DCCI - Dipartimento di Chimica e Chimica Industriale) dell'Università di Genova, è stato possibile studiare i funghi filamentosi isolati da ambienti estremi e applicare i ceppi presenti nella collezione micologica del laboratorio (*ColD - Collection of DiSTAV*) per lo sviluppo di questa tipologia di impianto. In sintesi, lo scopo del lavoro è stato quello di sviluppare, ampliare e saggiare protocolli nell'ambito della *mycoremediation* come i MABR, con particolare attenzione all'isolamento, conservazione e utilizzo di ceppi fungini idonei per i trattamenti di matrici inquinate.

La tesi è strutturata in cinque capitoli. Nel primo capitolo sono trattate le tecniche con cui i laboratori di micologia isolano e identificano i funghi filamentosi ritrovati in ambienti estremi o inquinati. Nel secondo e terzo capitolo sono discussi due *case study* riguardanti l'isolamento e l'identificazione di funghi filamentosi da un percolato di discarica e da diverse fasi di maturazione di un impianto di compostaggio, come punto di partenza per future applicazioni di ceppi autoctoni per trattare reflui complessi. Nel quarto capitolo si affrontano le diversità d'impianto e di funzionamento dei trattamenti biologici aerobi, con particolare attenzione alle modalità di rifornimento di ossigeno e substrato. Il capitolo si conclude con le tipologie di bioreattore sviluppate con funghi filamentosi. Nell'ultimo capitolo si descrive come è stato sviluppato e costruito un bioreattore a membrane areate con un fungo filamentoso modello per un futuro utilizzo nel trattamento delle acque reflue.

Abstract

Despite the water is present on Earth planet in a large amount, only a small fraction of it can be exploited by living organisms. Mankind use much more water than other living beings because, in addition to its survival, it is used for agriculture, breeding, industry and as an energy source. Research on new and different water purification techniques is currently one of the unsolved points to reduce the human environmental footprint. In general, water or any polluted matrix can be purified with different methods (chemical, physical, biological), but none of them fully satisfies the efficiency and sustainability criteria.

In the last thirty years, the research in the field of eco-friendly technologies is increasing: among the various purification processes, bioremediation is giving excellent results. This system aims to reduce pollutants in the various environmental matrices, exploiting the metabolic capabilities of living organisms, such as bacteria, fungi and plants, to degrade them into simple products without using chemical substances or expensive and complex treatment plants. The bioremediation that uses fungi for the treatment of polluted environmental matrices is called mycoremediation. Fungi are organisms suitable to be used in bioremediation processes because their metabolism allows them to grow and survive in the presence of toxic substances and in extreme conditions that are not tolerated by most of the other organisms.

One of the advantages of using fungi in bioremediation processes is the possibility of using them even when the concentration and bioavailability of the pollutants is very low in the substrates, as in the case of drugs for human and veterinary use dispersed in wastewater. Another advantage related to filamentous fungi - on which this thesis will focus - is their hyphal structure which greatly increases their distribution and colonization capacities. Several filamentous fungi are able to act on recalcitrant organic pollutants: from chlorophenols to dioxins, from toluene to hydrocarbons. They are not always able to completely degrade them, but they can at least transform them into less toxic compounds. The ability to metabolize organic compounds that are very different from each other in chemical structure is due to the low specificity of many fungal enzymes.

The characteristics of the fungi are not only species specific, but often strains specific, which is why the research and identification of new strains is fundamental for mycoremediation. It is precisely from the experiments carried out on the strains kept in the collections and isolated from different environments such as extreme ones that new treatments can be developed. Nowadays, the environments that can be defined as extreme are no longer just the sea depths or Arctic glaciers, but also the production and wastewater treatment plants. Although they are ideal environments for the selection of new fungal strains, research continues to focus only on bacterial communities, leaving

the fungal component practically unexplored. Another unexplored field is the application of fungal strains with known metabolic abilities in treatment plants at laboratory scale and, above all, at industrial level. A type of plant that may be suitable for filamentous fungi, of which very few examples are found in literature today, is that of aerated membrane bioreactors (MABR). This is a promising technology for the treatment of recalcitrant substances in an aqueous environment, based on the transfer of a gas (e.g. air or oxygen, but in principle also other gases can be used) across a membrane to a biofilm in contact with a liquid phase containing the substrates to be metabolized. The use of filamentous fungi in MABR is currently limited mainly to biotransformation and industrial processes for the production of enzymes and bioproducts.

Thanks to the multidisciplinary research approach, carried out between the mycology laboratory (DiSTAV - Department of Earth, Environmental and Life Sciences) and that of industrial chemistry (DCCI - Department of Chemistry and Industrial Chemistry) of the University of Genoa, it was possible to study the filamentous fungi isolated from extreme environments and to apply the strains present in the mycological collection of the laboratory (ColD - Collection of DiSTAV) for the development of this type of implant. In summary, the purpose of the work was to develop, expand and test protocols in the field of mycoremediation such as MABR, with particular attention to the isolation, conservation and use of fungal strains suitable for the treatment of polluted matrices.

The thesis is structured in five chapters. In the first chapter the techniques used by the mycology laboratories to isolate and identify the filamentous fungi found in extreme or polluted environments are discussed. In the second and third chapters two case studies concerning isolation and identification of filamentous fungi from landfill leachate and from different ripening stages of a composting plant are discussed, as a starting point for future applications of native strains to treat complex wastewater. In the fourth chapter the plants and the diversity of aerobic biological treatments are presented, with particular attention to the methods for supplying oxygen and substrate. The chapter concludes with the types of bioreactor developed with filamentous fungi. The last chapter describes the development of an aerated membrane bioreactor with a model filamentous fungus for future use in wastewater treatment.

Chapter 1
MYCOLOGICAL CHARACTERIZATION

1. Mycological characterization

1.1. Introduction

Fungi are microscopic eukaryotic organisms that includes unicellular fungi (yeast) and pluricellular fungi (moulds and mushrooms). Filamentous fungi are able to continue their function and growth almost indefinitely. They exhibit an apical growth through the hyphae that form a network named mycelium. Fungi are heterotrophs: they need an energy source and an organic carbon source to survive. Fungi are highly plastic and most fungal cells are totipotent, so the entire organism can be regenerated not only from spores but also from hyphal fragments (Deacon 2013).

Fungi are tolerant to extreme environmental conditions: high of hydrogen ion concentration in the substrate, very acidic or highly alkaline (from 1 to 9 pH), temperatures from -5 to 60°C or with only 0,2% oxygen concentration (Harms et al. 2011).

There are very few substances present in nature that are not degradable by fungi. For example, fungi degrade simple or complex substrates, such as methane, long-chain hydrocarbons, cellulose, chitin (Deacon 2013). A wide range of organic pollutants are transformed and degraded by fungi. Fungi co-metabolize mainly organic pollutants but can also grow on some aliphatic or aromatic compounds, including volatile organic compound. Studies on the degradation and metabolism of organic substrates have shown that most of the pollutants degrading fungi belong to the phyla Ascomycota and Basidiomycota (Prasad 2018).

The ability to metabolize organic compounds that are very different from each other in chemical structure is due to the low specificity of many fungal enzymes (Noman et al. 2019). In fact, the same fungal organism can degrade very different classes of pollutants, even when mixed. A type of non-specific enzymes typical of fungi is the extracellular oxidoreductases that react with organic compounds with random structure by oxidizing them (Harms et al. 2011).

The fungi are well suited for bioremediation processes because their metabolism allows them to grow and survive in toxic and extreme conditions not tolerable for most of the organisms. Moreover, they are able to form extended mycelial networks, independent from using pollutants as a growth substrate and have low specific catabolic enzymes (Noman et al. 2019).

One of the advantages of using fungi in bioremediation processes is the possibility of using them even when the concentration and bioavailability of the pollutants is very low in the substrates, as in the case of drugs for human and veterinary use dispersed in wastewater (Harms et al. 2011).

Another advantage of filamentous fungi is due to their own structure: the network of mycelium created by the hyphae not only increases the occupied surface but it is very useful to compensate for an uneven distribution of nutrients or pollutants. Several filamentous fungi are able to act on recalcitrant organic pollutants: from chlorophenols to dioxins, from toluene to hydrocarbons. They are not always able to completely degrade them, but they can at least transform them into less toxic compounds (Singh 2006).

Humans have preserved yeasts and filamentous fungi for a long time before knowing their biology and functions, for at least 3000 years through the production of food and drinks. Since only 170 years humans have instead started to develop the sterile techniques that have allowed to preserve the microorganisms on solid soils. This method was considered fundamental for pure research and teaching. About 80 years later, humans realized the importance of fungi not only as a subject of study or for the production of food and drinks but also for the pharmaceutical industry (Ainsworth 1976). The discovery of penicillin makes the first striking result that prompted many scholars to search for substances that are also useful in medicine among the different strains preserved in the world. In the last 30 years this research process has also expanded to biotechnology with a huge amount of work on the use of fungi to obtain bioproducts, restore the environment and optimize industrial processes (Hawksworth 2013).

In 1904 the first center for the dissemination and conservation of a wide range of fungal cultures was founded: the *Centraalbureau voor Schimrnelcultures* (Baarn, The Netherlands; CBS) (Schipper 1978). Today the culture collections in the world are almost 800: they are distributed in over 70 countries in the world, with at least 250 culture collections in Europe. In addition to these, many research institutes, laboratories or universities have stocks of fungi used for teaching and research (Paton et al. 2020). This topic is explored in this chapter.

Each stored strain has a very high potential: it can be the protagonist of pure research, of applicative research, it can be used to obtain useful substances or to degrade chemical substances not yet invented. For this reason, it is essential to continue to isolate new strains: to study their characteristics, identify their DNA barcoding and share all information with the scientific community. Extreme environments are proving useful for deriving potentially useful fungal strains now and in the near future (Newsham & Boddy 2012; Chávez et al. 2015).

Nowadays, the importance of biodiversity is known by everyone as it is associated with the preservation of animal and plants. Much less known is the biodiversity linked to microorganisms, despite its enormous importance. For this reason, partnerships and best practices have only recently spread around the world to keep the biodiversity of all living things, including bacteria and micro-fungi. Acting at the habitat level to defend the biodiversity of these organisms is unmanageable, it is

possible instead to artificially keep the microbial strains isolated from different environments. Researchers, professors, and PhD students from the mycology laboratories of the world collaborate to be up to date with other laboratories with regard to isolation and conservation. Also the mycology laboratory of DiSTAV (*Department* for the Earth, Environment and Life Sciences of University of Genoa) where this PhD research was carried out is not far behind. Following a long tradition of study and research dating back to the early Nineteenth century, the laboratory goes on with isolating fungi from the environment to enrich the mycological collection and carry out pure and applied research. It has recently joined to the European cooperation program on collections of microorganisms called MIRRI, together with two other laboratories of DiSTAV dealing with environmental bacteria and yeasts. This chapter describes the main steps that every mycology laboratory has to face when dealing with pure research: the search for strains to be potentially used for bioremediation, their isolation, identification and conservation.

1.2. Isolate fungal strains

A few drops of water or a few grains of soil can show the presence of hundreds of different cultivable fungal strains. One of the mycologist's tasks is to be able to isolate them correctly in the laboratory. This paragraph deals with the importance of sampling from environmental matrices, especially extreme and polluted ones. This work is done for two main reasons: to find strains he ones suitable for use in bioremediation or other application, to expand the collections of mycology laboratories.
The serial dilution method is used (Gams et al. 1987) to quantitatively and qualitatively analyze the filamentous micro-fungi present in an environmental matrix. The method proposed by Gams allows: to have a clear vision of the present fungi and to have the possibility of isolating them in axenic culture, to quantify the concentration of microorganisms that can grow on agar media and, in this specific case, to quantify the concentration of filamentous fungi in a sample. In this case it is usual to use the log dilution, where the concentration decreases by multiples of 10.
For a quantitative analysis of the fungi present in an environmental matrix it is right to use specific media for fungal growth such as RB (Rose Bengal), which slows down rapid growth and allows easier identification, and generic media such as MEA (Malt Agar Extract), which indiscriminately grow any non-filamentous bacteria or fungi. In this case it is necessary to be very careful after the first days of growth in case genera such as *Mucor* prevent the isolation of less invasive colonies.
The incubation temperatures depends on the environment of origin of the sample from which the isolation is carried out. In general, for strains isolated in environments not particularly characterized by high or low temperatures, the thermostat at 24° C is used. Instead for samples isolated from specific

environments such as compost, that is already at higher temperatures, it is also possible to isolate at 37° or 45° C.
After incubation, plates with a concentration of 10-30 colonies are selected, from which strains in axenic culture will be isolated or identified at genera level by macromorphological and micromorphological identification directly. Furthermore, with the serial dilution method it is possible to have data on the concentration of the different strains in the starting sample. By replicating the analysis for more samples of the same matrix it is possible to have an idea of its fungal composition in percentage.

1.3. Identify fungal strains

After isolation, to determine and identify the fungal strain it is necessary to use culture media that stimulate good sporulation and whose growth results have been already used and standardized in relevant scientific papers, manuals and reviews on the subject. Unlike the culture media used for morphological identification, the media used for isolating the greatest number of fungal strains are generic, favour the growth of almost all genera, and some manage to slow their growth so that a colony is easier to follow. The fungal cultures are identified by macroscopic and microscopic characteristics. The macroscopic characteristics include colonial morphology, texture, shape, colour, diameter, and appearance of colony (Gams et al. 1987). The microscopic characteristics include shape and structure of conidia, presence of specific reproductive structures, presence of sterile mycelium and septation in mycelium (Iram et al. 2012).

Currently, the valid description of a fungal species requires the morphological characterization and the analysis of the molecular data, now widely used in the fungal systematics and in the phylogeny. Although morphology identification is in many cases a complete source of information, relying solely on morphological traits is sometimes misleading. The main reasons are: the plasticity of fungal development cycles, the cryptic species, the difficulty of identifying discriminating morphological characters, the developmental responses that fungi have depending on the growth substrate and environmental conditions. This makes identification at the species level difficult without the support of a molecular investigation (Begerow et al. 2010).

These premises are generally valid in the Fungi Kingdom. However, this work will focus on the description of the filamentous micro-fungi, protagonists of the experimental part of the thesis.

1.3.1. Morphological identification

The ability of any organism to respond to the environmental changes by altering its morphology, physiological state or behaviour is known as "phenotypic plasticity" and is defined as a pleomorphic characteristic. Also fungi are pleomorphic microorganisms and are known to explore and exploit the environment responding quickly by changing the phenotype when environmental conditions change. Their extreme phenotypic plasticity makes them suitable for laboratory studies on how and why phenotypes change when environmental conditions change. According to the study of Slepecky and Starmer there are four properties which makes fungi attractive for studies of plasticity:

- the possibility of storing the genotypes for multiple tests in different environments,
- the phenotypic reversibility of same culture over time and after sequential culture,
- the ability to use evolving fungal species studies, in comparative tests or experiments,
- the fact that many fungi have indeterminate growth, giving an opportunity to study the function of genetic mosaics and the usefulness of dynamics boundaries.

According to the same work, the phenotypic variation in colony form depends on five variables: the types of single carbon substrates used in growth average, the colony age, the incubation temperature, the light cycle and the substrate type (Slepecky & Starmer 2009).

An example of the extraordinary plasticity of the fungi is visible in Figure 1 and Figure 2: the *Aureobasidium pullulans* can take different morphologies, therefore this fungus was taken as a model in the study mentioned above.

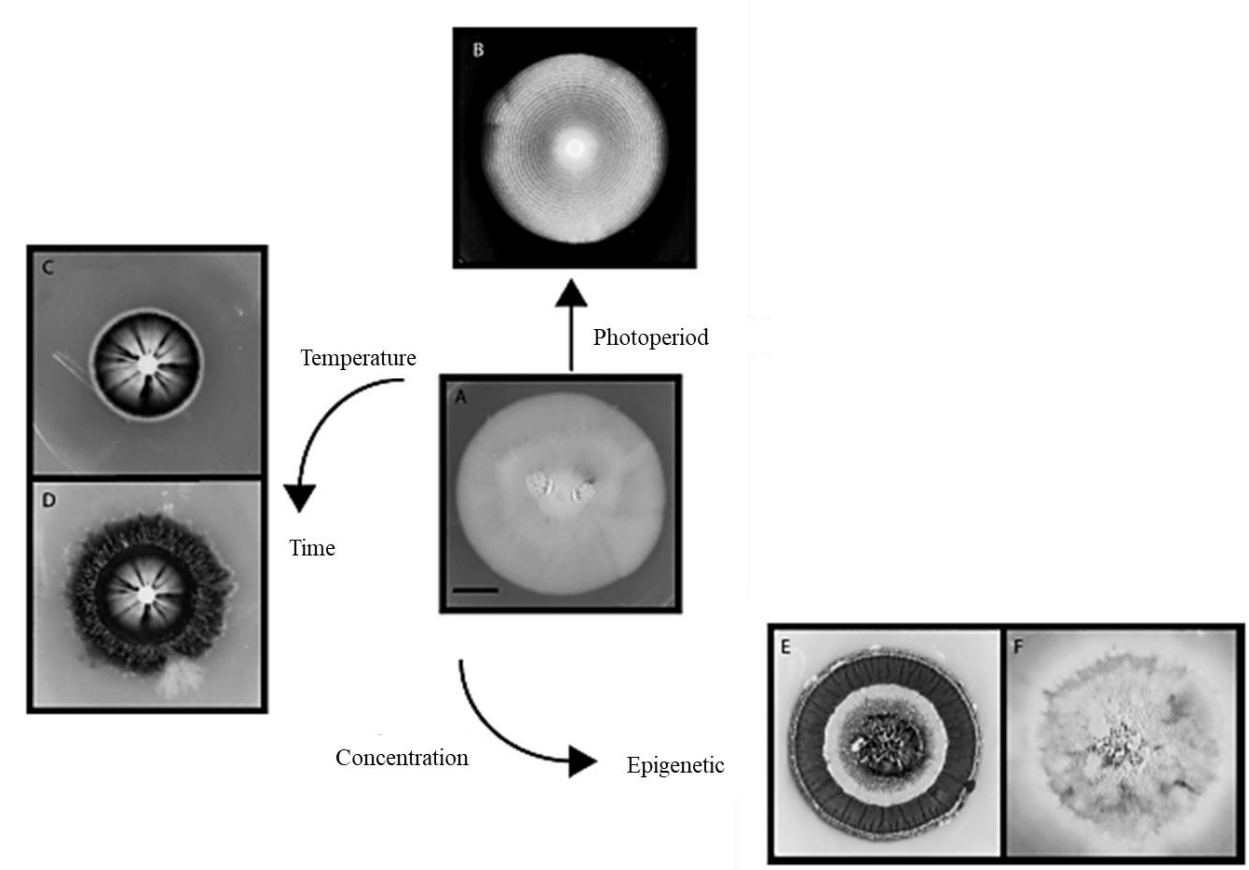


Figure 1. Colonies of A. pullulans cultured on YNB + glucose. A) 4 weeks at 25° and 0.5% glucose; B) 3 weeks at 20° C and 0.5% glucose, 12 h light : 12 h dark photoperiod (back and front illuminated). Colonies grown under continuous light or dark on the same medium appear similar to the colony in A). C) and D) 0.5% glucose at 9° C for 4 and 12 weeks respectively; E) 2.0% glucose at 25° C; F) 2.0% glucose at 25° C supplemented with 20 mM nicotinamide. Each photo is 5 cm square (Slepecky & Starmer 2009).

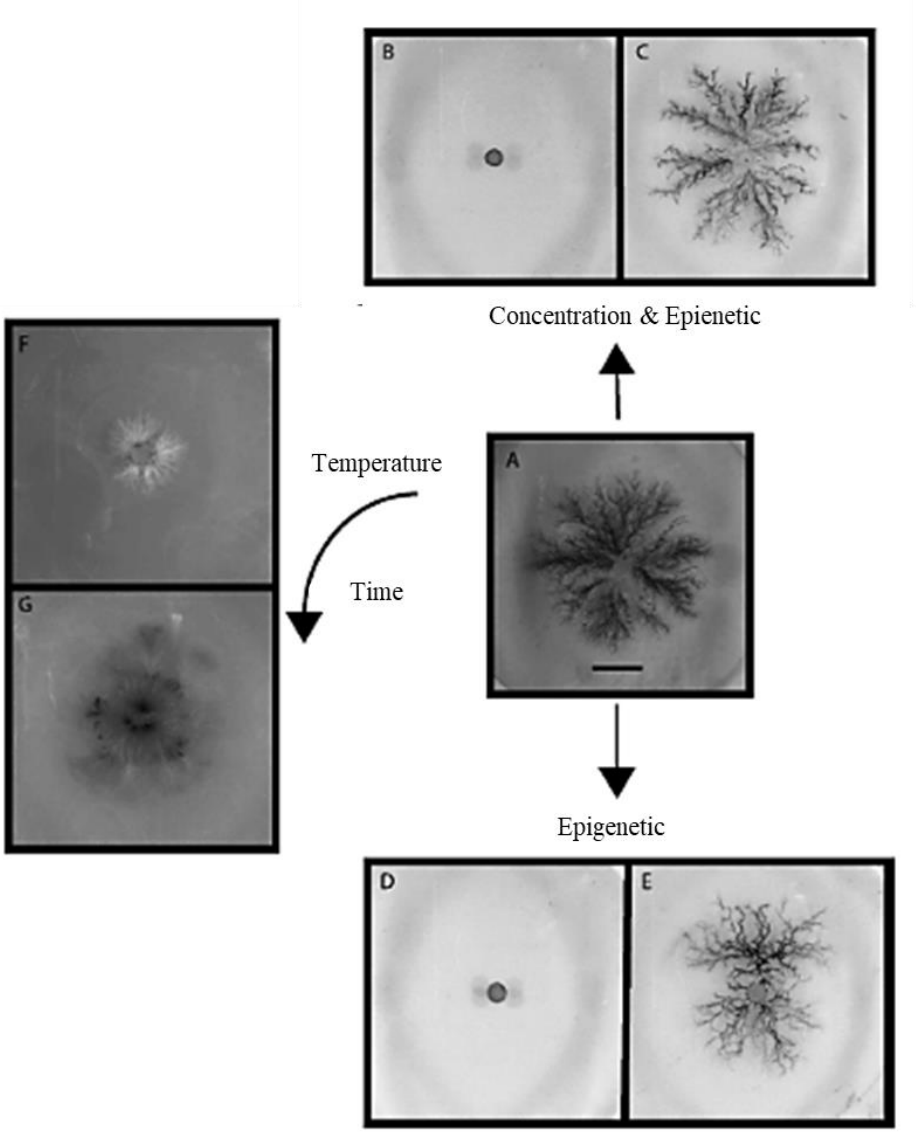


Figure 2 Colonies of A. pullulans cultured on YNB + dulcitol. A) 4 weeks at 25° C, 0.5% dulcitol; B) and C) 4 weeks at 25°C, 2.0% glucose, supplemented with 20 mM nicotinamide and 20 mM nicotinic acid respectively. Colonies grown at 25° C without supplements appear similar to the colony in A). D) and E) 4 weeks at 25° C, 0.5% glucose, supplemented with 20 mM nicotinamide and 20 Mm nicotinic acid respectively. Colonies grown at 25° C without supplements appear similar to the colony in A). F) and G) 5 0.5% glucose at 9° C for 4 and 12 weeks respectively. Each photo is 5 cm square (Slepecky & Starmer 2009).

An example of dimorphism of *Aureobasidium pullulans* was also observed in the mycology laboratory where this thesis was carried out. In two seawater samples on different media Malt Extract Agar (MEA) and Glucose Yeast Extract Agar (GYA) at the same incubation temperature it is possible to observe in the different growth strategy of the fungus.

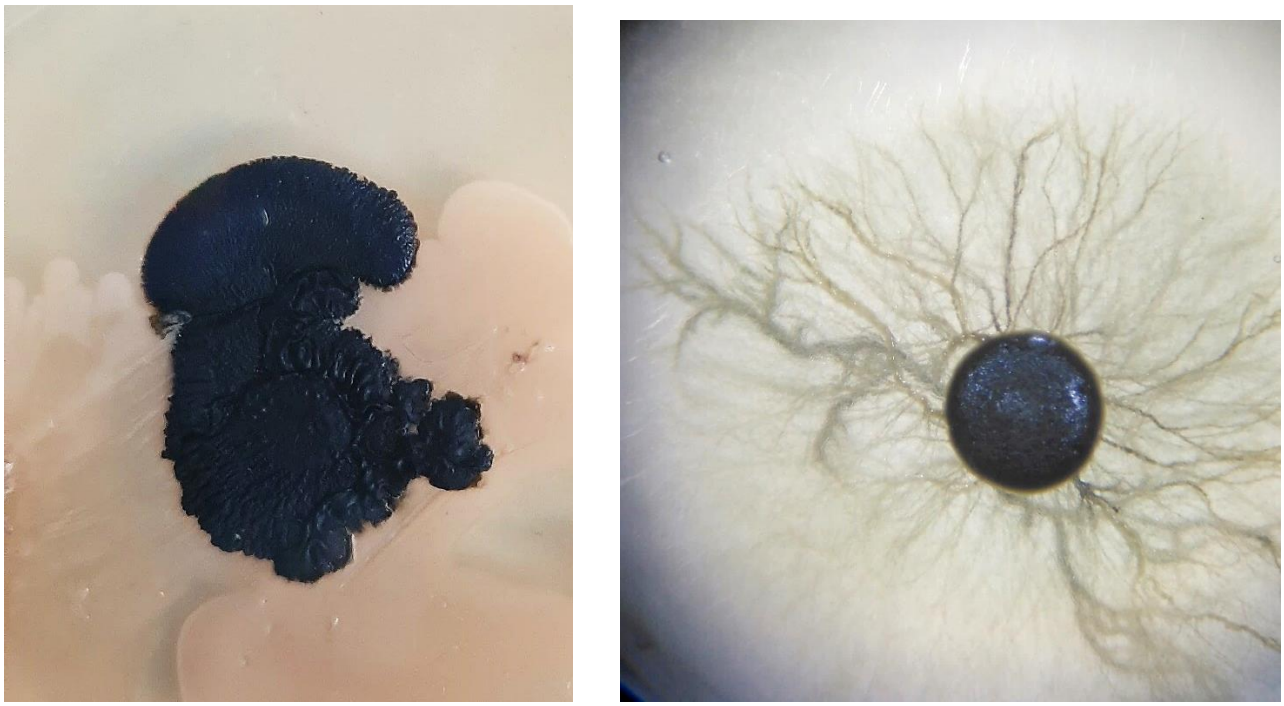


Figure 3 Growth of A. pullulans on MEA and GYA solid media isolated from seawater.

The different morphologies that characterize fungi with filamentous growth correspond to macroscopically visible aspects of microscopic differentiation processes from conidia to hyphae. The growth process of filamentous fungi follows a very complex morphological development of differentiation that involves different types of cells that lead from conidia on germ cells to hyphae. The filamentous aspect is due to two factors: the cells do not separate completely after cell division and the growth is strongly polarized. The hyphae apically differentiate into morphologically highly differentiated structures. On the hyphal tip, growth and branching take place in many different ways building morphologically diversified visible structures.

The stages of the filamentous growth process involve the cell wall and the polarity of the cell. As far as wall biosynthesis is concerned, the molecules involved are chitin and glucans linked to β (1–3) and β (1–4). Polarity, however, is influenced by a complex interaction of Ca^{2+} ionic gradients, secondary messaging systems and turgor pressure. The ions Ca^{2+} is involved in the regulation of actin. Actin filaments and microtubules make up the cytoskeleton of fungal cells. Although many processes related to the quantification and control of complex intracellular reactions that lead to morphogenesis are still poorly understood, many molecular studies are underway to prove morphogenesis, in terms of branching or growth (Grimm 2005).

The most common fungal identification method is the morphological one. The morphology and growth of fungi on solid medium is closely related to the media used and the nutrients it has. Each

media has its own composition with a specific proportion between carbohydrates and proteins, but in general each fungal strain needs a carbon source for its growth.

The type of media is different whether it is necessary to isolate or to identify a micro-fungus. To isolate micro-fungi it is necessary to have a medium that promotes rapid growth or reduction of too rapid spread. In this case, it is possible to use multiple natural media: potato dextrose agar (PDA), malt agar extract (MEA), or insoluble media like cellulose or starch. The reduced number of soluble substrates in the aforementioned media often improves sporulation, along with the acidic pH which reduces bacterial growth during isolation. Media such as MEA allow, also to a very non-selective isolation, the growth of recognizable colonies of saprotrophic fungi or common pathogens. Instead, an example of unnatural soil that can be used to isolate fungi is the Rose Bengal (RB). This media has antibacterial to promote the growth of fungal strains only and dichloran to slow down the fungal colonies growth (King et al. 1979). An example of generic isolation of three fungal strains with RB and their axenic culture transfer in a microbiology tube with MEA is reported in Figure 4 RB culture media and microbiology tube for axenic culture isolationFigure 4.

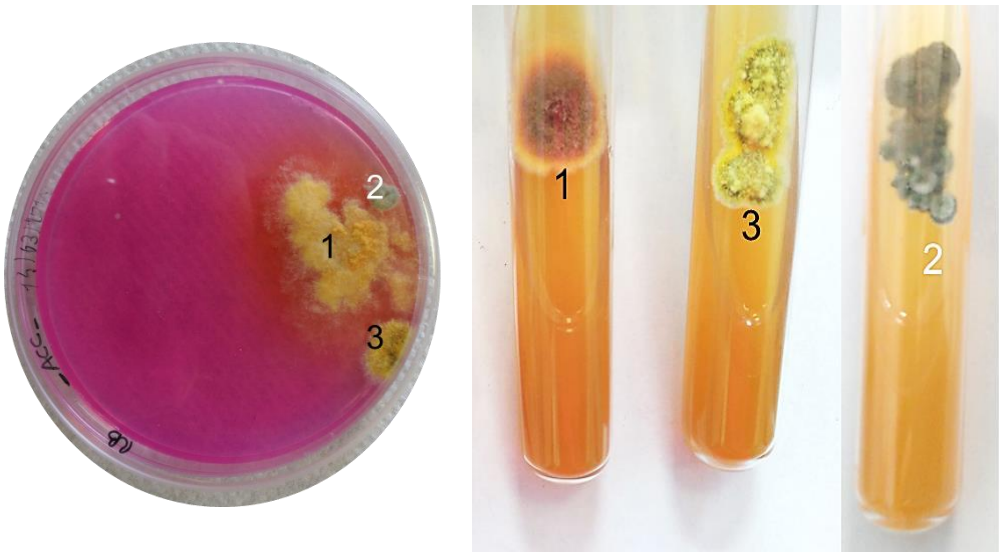


Figure 4 RB culture media and microbiology tube for axenic culture isolation of three strains.

The standard media for the identification of some of the most frequently isolated strains from environmental matrices are listed in the Table 1.

Fungi	***Media***	***References***

Alternaria	OA, MEA	(Woudenberg et al. 2013)
Aspergillus	CYA, MEA, OA	(Samson et al. 2014)
Aureobasidium	MEA, YNB	(Slepecky & Starmer 2009)
Cladosporium	PDA, MEA, SNA, OA	(Bensch et al. 2012)
Penicillium	CYA, CREA, MEA, YES	(Refai et al. 2015)

Table 1 Standard media for the identification of some taxa.

The Table 2 lists the reference works used to determine some of the most common isolable genera.

Fungi	***Main references***
Penicillium	(Pitt 1979; Samson & Houbraken 2011)
Aspergillus	(Raper & Fennel 1965; Klich 2002; Samson et al. 2014)
Trichoderma	(Rifai 1969; Bissett 1984; Bissett 1991)
Cladosporium	(Crous et al. 2007; Bensch et al. 2012)
Other taxonomical groups	(Gams 2007; Hibbett et al. 2007)

Table 2 Reference for fungal morphological identification.

The isolated strains are identified according to conventional mycology methods by observing their micromorphological and macromorphological characteristics and their different trophic and physiological requirements. The initial detection of fungal structures is carried out by means of stereomicroscope (x10-50). The strains are then identified by a micro-morphological (x40-100) analysis.

1.3.2. Molecular identification

In recent years, molecular approaches to fungal diagnostics based on polymerase chain reaction (PCR) and DNA probe technology have supported traditional identification methods based on morphology characteristics. From a practical and didactic point of view, a morphological identification certainly has interesting aspects and can be accompanied by a molecular analysis, but in many other aspects, molecular analysis has allowed investigations that were previously unthinkable.

PCR technology introduced many advances in the molecular diagnostics of fungi over the past few decades (Wright & Manos 1990). The advantages of molecular diagnostics and PCR are: the speed in obtaining the results, the minimum quantity of sample necessary for the analysis and, as regards metagenomics, which will not be discussed, the possibility of having answers even without the cultivation of the stumps on the plate (Tkins & Lark 2004).

To go ahead with the molecular identification of a fungal strain, different phases are needed: the extraction of DNA from the sample, the selection of a sequence useful for identification, the selection of the suitable primer and the interpretation of the data obtained from the sequencing.

Fungal DNA can be extracted from the mycelium of a colony using different physical methods that result in the destruction of the cells. The protocol used for the fungi isolated in this PhD thesis foresees that 100 mg of fresh fungal culture are frozen, disrupted and homogenized using a modified CTAB method (Doyle & Doyle 1987). In order to homogenize the sample, a Tissuelyser (Retsch GmbH, Haan, Germany) was used for 1 min 30 s at 18 Hz. Then, the sample processed was incubated for 1 h 30 min at 65 °C in 700 ml CTAB extraction buffer (100 mm Tris–HCl pH 8.0; 20 mm EDTA; 1.4 m NaCl; 2% PVP; 2% CTAB) supplemented with 10 µg of proteinase K (Sigma-Aldrich, St Louis, MO, USA) and 30 µg of RNase A (Sigma-Aldrich) to remove RNA from the sample. To remove cell components and to purify DNA, the sample was centrifuged at 5900 g for 10 min at room temperature and the aqueous layer was transferred to a new tube and an equal volume of a mixture of phenol, chloroform, isoamyl alcohol (25:24:1) (Sigma-Aldrich) was added.

Then, it was shaken and centrifuged at 5900 g for 5 min at room temperature. Subsequently, the aqueous layer was transferred to a new tube and an equal volume of chloroform was added. Every sample was shaken and centrifuged at 5900 g for 5 min at room temperature. Then, the aqueous layer was transferred to a new tube, and 2-propanol (two-thirds of the recovery volume) was added. The

samples were then incubated for 15 min at 4 °C, centrifuged at 9200 g for 10 min at room temperature and the aqueous layer was discarded. The samples were then washed in ethanol at 70 °C by centrifugation at 9200 g for 1 min at room temperature. The DNA pellet was air-dried and then re-suspended in 50 µl of water. The quality and quantity of DNA samples were assessed with an ND-1000 spectrophotometer NanoDrop (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Finally, DNA extracts were stored at -20 °C in the cryo-stock of the mycology laboratory in order to be available for future researches or to repeat the molecular analysis without having the strain in culture. Ten years ago, the first discussions between mycologists began to define the standards for the molecular analysis of fungi. The six DNA regions responsible for the fungal barcode were appointed and then approved by the Consortium for the Barcode of Life: ITS, RPB1, RPB2, SSU, LSU, MCM7 (Schoch et al. 2012).

The first and official region for fungal diagnostics is the ITS (internal transcribed spacer) which separates the three genes of fungal nuclear ribosomal DNA (rDNA). The ITS region and more specific primers can be used for the distinction of different taxa. ITS is also a starter for development of specific primer for fungal molecular analysis. Adjacent to the ITS region, in the ribosomal DNA there are two other parts universally recognized for fungal barcoding: the SSU region, which corresponds to the Small SubUnit ribosomal ribonucleic acid, and the LSU region, which corresponds to the Large SubUnit ribosomal ribonucleic acid (Gargas & DePriest 1996). RPB1 and RPB2 belong to the protein coding markers and correspond to the largest and second largest subunits of the RNA polymerase, respectively. The last of the six regions marker is MCM7, a sequence of a mini chromosome maintenance protein very useful to understand phylogenetic relationship between fungi. For members of the Ascomycota it is possible to use MCM7 in conjunction with the LSU gene (Raja et al. 2017).

Other secondary primers were then developed over the years to work with the ITS analysis. In particular, each primer was designed to estimate the differences between specific taxa (Stielow et al. 2015). The Table 3 lists some of the most used primers for molecular identification.

Primer	*Bp*	*Barcoding fungal marker*
ITS	~ 450-800	Official fungal barcoding
Small Subunit 18S of the rRNA (SSU)	~ 1200	All fungi
Large Subunit 18S of the rRNA (LSU)	~ 1200	All fungi

RNA polymerase II subunit 1 and 2 (RBP1-2)	~ 1200	All fungi
Translation elongation factor 1-alpha (tef1)	~ 600	All fungi
β-tubulin (Bt2a-b)	~ 500	*Penicillium*
Calmodulin (CMD5-6)	~ 580	*Aspergillus*
Mini chromosome maintenance protein (MCM7)	~ 650	All fungi
Translation elongation factor 1-alpha (tef1)	~ 1300	*Trichoderma*

Table 3 Fungal identification primers, adapted from (Raja et al. 2017).

The morphological identifications in the analyses carried out for this PhD thesis were confirmed by the amplification of the ß-tubulin gene using Bt2a and Bt2b primers (Glass & Donaldson 1995) and of the ITS region using ITS1F / ITS4 universal primers (White et al. 1990; Gardes & Bruns 1993) for amplification.

PCR allows to amplify the small selected region in order to have a qualitative analysis of the starting sequence. It is an enzymatic reaction that is based on thermal cycles: the temperature is raised and lowered to activate the Taq polymerase which reacts with the selected primers that act as a starter of the reaction. In the analyses discussed in this PhD thesis, amplification reactions were performed in a T3000 thermal cycler (Biometra biomedizinische Analytik GmbH, Goettingen, Germany) in a 50-µl aliquot of reaction mixture using the following final concentrations or total amounts: 20 ng DNA, 1 × polymerase chain reaction (PCR) buffer (20 mm Tris–HCl pH 8.4; 50 mm KCl), 1 µm of each primer, 2.5 mM MgCl, 0.25 mM of each dNTP, 0.5 unit of Taq polymerase (Promega, Madison, WI, USA). The PCR protocol was: one cycle of 5 min at 95 °C; 40 s at 94 °C; 45 s at 55 °C; thirty-five 1-min cycles at 72 °C; one 10-min cycle at 72 °C. PCR products were resolved on 1.0% agarose gel and visualized by staining with ethidium bromide.

PCR products were purified and sequenced using MACROGEN Inc. (Seoul, Republic of Korea). Sequence assembly and editing were performed using Sequencher® version 5.2 (sequence analysis software, Gene Codes Corporation, Ann Arbor, MI, USA). The taxonomic assignment of the sequenced samples was carried out using the BLASTN algorithm to compare sequences obtained in the present study against the GenBank database.

It was used a conservative approach to species-level assignment. The sequences were only assigned at species level if the query sequence matched database sequences from fungal isolates (including at least one vouchered specimen) with e-values ≤ 10−100 and percentage sequence identity ≥ 97 % and there were no contradictions among species from the same genus at the lowest e-values. The blast results were manually inspected to remove inconsistencies: the matches based on assignment of a single sequence to a genus represented by several species in the reference database were not considered, to avoid suspected misidentification in the database sequence.

1.4. Collect fungal strains

Fungi constitute a differentiated kingdom, some are pluricellular, some other are unicellular. They come in many different sizes, from a few millimetres to many kilometres. In 2014 have been described formally only 100.000 species (Heilmann-clausen et al. 2014), in 2020 the number of described species increased to almost 150.000 (Paton et al. 2020): they are estimated to exist 2.2–3.8 million fungal species in the world (Hawksworth 2017).

Among the 1 million microbial strains of The World Directory of Collections of Cultures of Microorganisms (an activity of the World Federation for Culture Collections) the 44% are fungi, the rest are bacteria, viruses, cell, or others (Paoli 2005). Microbial Biological Resource Centres (mBRCs) are dedicated to the preservation and enhancement of microbial diversity. They provide live cultures and associated data to support the development of pure and applied science in institutes from all over the world. The Biobanks are dedicated to the conservation of the live cultures of yeasts, filamentous fungi, food bacteria, human or animal pathogenic bacteria, bacteria associated with plants, entomopathogenic bacteria, etc. Biobanks represent an essential tool for the conservation of biodiversity and are the places where biological samples are collected, treated, and stored in the long term (Paoli 2005).

Whatever use will be made of a fungal strain, the first step is to isolate it correctly and keep it alive for years in the collections. In recent years, mycology laboratories around the world are trying to improve themselves year after year through protocols and good laboratory practices, and by networking with other entities. The collections of each laboratory are continuously enriched with new strains, isolated from organisms or environmental matrices. New methods are developed for isolation and cultivation of strains which are rare or difficult to grow. New protocols and guidelines are reviewed and are published to be available to mycologists around the world.

Fungi have an enormous potential to offer solutions in biotechnology, agriculture, environment, human and veterinary medicine, food industry and biofuels. There is not an individual preservation technique applicable to all fungi, but many protocols have been suggested for this purpose (Paoli 2005). In this PhD thesis work, attention will be paid to the conservation of filamentous micro-fungi, an essential practice to be able to carry out pure research, teaching projects, to share and exchange strains with other institutes to allow future studies and practical applications in biotechnology on environmental remediation.

1.4.1. Storage methods

Originally storage of fungi was kept by serial transfers into tubes from stale to fresh media (Figure 5). This method needs frequent sub-culturing and for this reason is prone to contamination and degeneration. There is the risk that the strains characteristics change during a long-term conservation because of the aging. Moreover, to keep alive in tube a large number of fungal strains in this way is time-consuming. Nowadays different methods are applied to avoid these disadvantages (Homolka 2013).

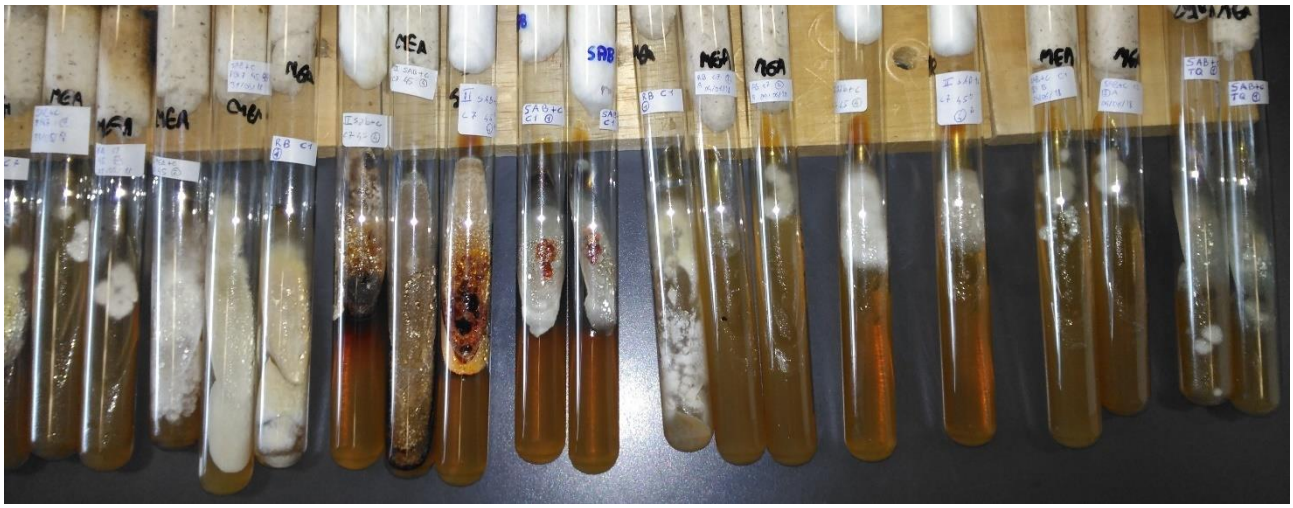


Figure 5 Microbiological tube for long-term fungal conservation.

The two most used methods for long-term storage of fungal strains, recommended by National collection of Types Cultures (NTCT) and American Type Culture Collection (ATCC), are: cryopreservation and lyophilization. The cryopreservation process includes different modes, temperatures, speeds of freezing and thawing, because different cultures show different sensitives to low-temperature (Homolka 2013; Linde et al. 2017).

Cryopreservation as a preservation technique for fungal strains has existed for seventy years, it was originally introduced by the ATCC. Later studies on cryoprotectants were carried out to improve their

long-term application (Hwang 1960; Smith 1983). It is widely used for Basidiomycota (Linde et al. 2017). In this technique it is important that the freezing process takes place in a controlled way in order to protect DNA from damage and avoid the formation of ice crystals in the cells. For this reason, a freezing rate of 1°C for minute is used and a cryoprotectant is added. The two most common cryoprotectors are dimethylsulfoxid and glycerol. Dimethylsulfoxid is used at 5% concentration and has a better penetrating ability than glycerol, but at higher concentration has toxic effect. Glycerol is used at 10-15% concentration and works better at temperatures not too low (Prakash et al. 2013).

The cryoprotectors act differently according to the freezing temperature, in fact it is possible to keep the fungi from -20 to -196°C. At low temperatures the biochemical and physiological activities of the cells are definitely slowed down, DNA and proteins are protected from denaturation and cells are protected for long periods of time.

Preservation of cells at -20°C is not recommended for long-term. Preservation with dry ice at -80 °C is adequate, while preservation at -140°C is the preferred technique in most Biobanks. In case of very low temperatures, the cultures remain stable for long periods due to the little metabolic activity occurring at that temperature. Conservation with liquid nitrogen at -196°C is considered ideal because the chances of DNA mutations are almost zero at that temperature (Prakash et al. 2013).

The second method recommended by the American Type Culture Collection for fungal store is the freeze-drying (lyophilization). This technique is most suitable for Ascomycota, Zigomycota and some Basidiomycota which can survive up to 30 years in this condition. The main advantage of this technique is that sealed ampoules offer a consistent protection against air dispersal of fungi during storage or during packaging and shipping to distant laboratories. The main disadvantage of freeze–drying is that injury and genetic damage may occur during the cooling and drying stages. Because both cryopreserved and lyophilized fungal cultures present problems of viability after reconstitution, it is very important to check viability before and after preservation independently of the technique used (Prakash et al. 2013).

1.4.2. Microbial Resource Research Infrastructure (MIRRI)

The mBRC's centers are widespread throughout Europe and are at an advanced level. Generally, they are managed by individual research laboratories or entities, they have their own identified databases and codes. The harmonization strategies of the collections can act as intermediary for the mBRC partners to share services and skills more easily. The infrastructure would support more uniform

management from the legal point of view of the data and biological resources of the various participating entities. European mBRCs work together to have a common platform (www.cabri.eu), adhering to quality standards and common best practices (www.ebrcn.eu) and evaluating the working and financial models of mBRCs (www.embarc.eu). They allow access to their products, services and skills to customers of national, regional, international academic and public service users. They take part in joint research projects, while maintaining independent lines of research on the identification and conservation of strains.

The consequence of these collaborations, based on previous evidence of a successful collaboration, was that the main European mBRCs presented the MIRRI project to the European Commission (EC). The project MIRRI aims to coordinate access to individually managed resources by developing a pan-European platform which takes the interoperability and accessibility of resources and results to a higher level (Figure 6).

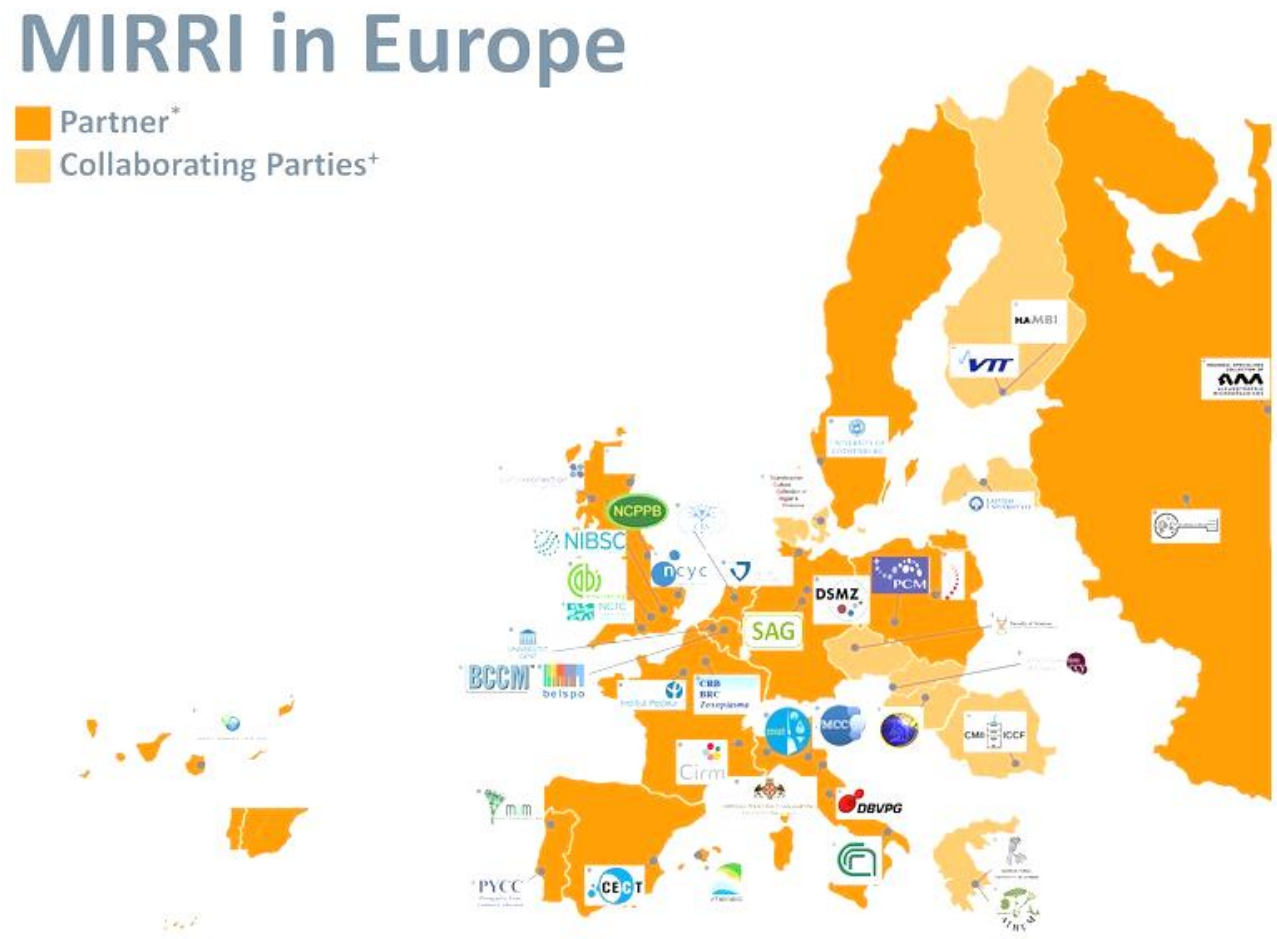


Figure 6 MIRRI in Europe.

MIRRI adds value to microbial diversity and takes care of deepening and spreading its knowledge for the innovation of bioscience and bioeconomy. The goals of MIRRI are different, such us:

- To increase knowledge of the intrinsic value of microbial resources;
- To fill gaps in data and taxonomic skills;
- To entrust and support microbial diversity services;
- To make sure that whoever isolates and conserves the strains benefits and is valued;
- To improve the quality and reproducibility of microbial science.

MIRRI will be useful to mBRC centers to allow and help access to microbial resources and associated data in the mBRC in compliance with regulations according to the Nagoya protocol. In this way the use of microbial resources for the bioeconomy will be safe. The Nagoya Protocol is an international agreement that aims to make genetic resources accessible and the benefits deriving from their use in a fair and just way shareable. The project provides experts, technical platforms and training to researchers in the various fields of modern microbiology to avoid the loss of skills by sharing and making existing information on microbial systems more accessible (Stackebrandt et al. 2015).

1.4.3. Collection of DiSTAV (ColD)

In order to extend the network to all the laboratories that have microbial collections and not only to major mBRC centers, national subgroups have been formed from the European MIRRI to coordinate the activities of each country, while maintaining the aims of the MIRRI. The main purpose of the Joint Research Unit MIRRI-IT is the implementation of the Italian node of MIRRI and the development of the Italian network of collections of microbial resources. Its mission is to overcome fragmentation in availability of resources and services, enhancing the quality management system of the collections, while focusing on needs and challenges of the stakeholders interested in the biotechnological transfer of these resources.

To network and stay updated on the state of the art of collections in Europe, European Culture Collections' Organization organize congress and meeting. I took part in the XXXVIII Annual Meeting of the European Culture Collections' Organization (ECCO 2019) in 2019 and I participated in the meeting organized by the Joint Research Unit MIRRI-IT.

In Italy about 50 collections are curated by different laboratories: institutions, universities or research centres. The majority of the strains are bacterial and fungal, about 60,000 and 50,000 strains respectively. Among the new members of the Italian MIRRI there is also the University of Genoa, with a collection of fungi, yeasts and bacteria called ColD: Collection of DiSTAV. The ColD brings

together the collection of three laboratory of University of Genoa, and host about 1900 samples of biological materials.

One of the goals of ColD is to study the strains isolated and preserved in the collection. The strains preserved are bacteria (300), yeast (40), filamentous fungi (1020), bacterial and fungal DNA (300 and 210). The strains are isolated from soils, polluted environmental matrices, and marine environments. The laboratory of mycology, where the research activities related to this PhD thesis were carried out, is one of three laboratories involved in the ColD collection. This laboratory deals with isolating fungi from the environment to enrich the mycological collection and carry out pure and applied research. In 2010, a new fungal species was isolated that causes onychomycosis. In 2015, a method was patented to recover rare earths and precious metals by recycling telephone cards. In 2017, a pathogenic fungus for corals was reported in the Mediterranean for the first time. To date, studies are underway on the cultivation of truffles and mushrooms, on the isolation of marine fungi and fungi specialized in the removal of toxic and harmful pollutants.

Figure 7 Cryo-conservation set in mycology laboratory for ColD collection

Fungi strains were collected during several research projects from the ‘70s. Preserved strains were isolated from extreme environments in particular marine environments, caves, pollution areas (such as abandoned mines), cadavers and from cutaneous or systemic human infections. These fungi are studied for their metabolites production and for their potential biotechnological applications in

bioremediation processes of marine polluted waters and contaminated soils. Moreover, focus has been put on the investigation of the synergic relationships between fungi and bacteria in particular in the marine and rhizosphere environments.

Bacterial strains were collected in the marine environment in the last 15 years. The majority belongs to the genus *Vibrio* and were isolated from several environmental matrices including seawater, sediment, plankton and marine invertebrates in the Mediterranean Sea and elsewhere. The collection includes species which are potential pathogenic for humans and marine animals (e.g. bivalves, corals, shrimps and fishes).

Dissemination activities were also carried out on the importance of fungal collections and their applications in bioremediation. The activities were carried out in person by participating in festivals and involving various schools and online by making YouTube videos and interviews on mycology.

1.5. References

Ainsworth GC. 1976. Introduction to the History of Mycology. [place unknown]: Cambridge University Press.

Begerow D, Nilsson H, Unterseher M, Maier W. 2010. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. :99–108.

Bensch K, Braun U, Groenewald JZ, Crous PW. 2012. The genus cladosporium. Stud Mycol [Internet]. 72:1–401. http://dx.doi.org/10.3114/sim0003

Bissett J. 1984. A revision of the genus Trichoderma. I. Section Longibrachiatum sect. nov. Can J Bot. 62(5):924–931.

Bissett J. 1991. A revision of the genus Trichoderma. II. Infrageneric classification. Can J Bot. 69(11):2357–2372.

Chávez R, Fierro F, García-Rico RO, Vaca I. 2015. Filamentous fungi from extreme environments as a promising source of novel bioactive secondary metabolites. Front Microbiol. 6(SEP):1–7.

Crous PW, Braun U, Schubert K, Groenewald JZ. 2007. Delimiting Cladosporium from morphologically similar genera. Stud Mycol. 58:33–56.

Deacon JW. 2013. Fungal biology. [place unknown]: John Wiley & Sons.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. [place unknown].

Gams W. 2007. Biodiversity of soil-inhabiting fungi. Biodivers Conserv. 16(1):69–72.

Gams W, Aa HA van der, Plaats-Niterink AJ van der, Samson RA, Stalpers JA. 1987. CBS course of mycology. Baarn: Centraalbureau voor Schimmelcultures.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol. 2(2):113–118.

Gargas A, DePriest PT. 1996. A nomenclature for fungal PCR primers with examples from intron-containing SSU rDNA. Mycologia. 88(5):745–748.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol. 61(4):1323–1330.

Grimm MLH. 2005. Morphology and productivity of filamentous fungi. :375–384.

Harms H, Schlosser D, Wick LY. 2011. Untapped potential: Exploiting fungi in bioremediation of hazardous chemicals. Nat Rev Microbiol [Internet]. 9(3):177–192. http://dx.doi.org/10.1038/nrmicro2519

Hawksworth DL. 2013. Fungus Culture Collections as a Biotechnological Resource Fungus Culture Collections as a Biotechnological Resource. 8725.

Hawksworth DL. 2017. The magnitude of fungal diversity : the 1 n 5 million species estimate revisited *. 2000(October).

Heilmann-clausen J, Barron ES, Boddy L, Dahlberg A, Griffith GW, Nord J, Ovaskainen O, Perini C, Senn-irlet B, Halme P. 2014. A fungal perspective on conservation biology. 29(1):61–68.

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon F, Eriksson OE, Huhndorf S, James T, Kirk PM, Matheny PB, et al. 2007. A higher-level phylogenetic classification of the Fungi. 111:509–547.

Homolka L. 2013. Methods of Cryopreservation. :9–17.

Hwang SW. 1960. Effects of ultra-low temperatures on the viability of selected fungus strains. Mycologia. 52(3):527–529.

Iram S, Arooj A, Parveen K. 2012. Tolerance potential of fungi isolated from polluted soil of. 2(10):27–34.

King AD, Hocking AD, Pitt JI. 1979. Dichloran-rose bengal medium for enumeration and isolation of molds from foods. Appl Environ Microbiol. 37(5):959–964.

Klich MA. 2002. Identification of common Aspergillus species. [place unknown]: CBS.

Linde GA, Luciani A, Lopes AD, Silveira J, Colauto NB. 2017. Long-term cryopreservation of basidiomycetes. Brazilian J Microbiol [Internet]. 49(2):220–231. https://doi.org/10.1016/j.bjm.2017.08.004

Newsham KK, Boddy L. 2012. Special Issue: Fungi in extreme environments. Fungal Ecol [Internet]. 5(4):379–479. https://www.cabdirect.org/cabdirect/abstract/20133122589

Noman E, Al-Gheethi A, Mohamed RMSR, Talip BA. 2019. Myco-Remediation of Xenobiotic Organic Compounds for a Sustainable Environment: A Critical Review. [place unknown].

Paoli P De. 2005. Biobanking in microbiology : From sample collection to epidemiology , diagnosis and research. 29:897–910.

Paton A, Antonelli A, Carine M, Forzza RC, Davies N, Demissew S, Dröge G, Fulcher T, Grall A, Holstein N, et al. 2020. Plant and fungal collections: Current status, future perspectives. Plants, People, Planet. 2(5):499–514.

Pitt JI. 1979. The genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces. genus Penicillium its teleomorphic states Eupenicillium Talaromyces.

Prakash O, Nimonkar Y, Shouche YS. 2013. Practice and prospects of microbial preservation.

Prasad R. 2018. Mycoremediation and environmental sustainability. [place unknown]: Springer.

Raja HA, Miller AN, Pearce CJ, Oberlies NH. 2017. Fungal Identi fi cation Using Molecular Tools:

A Primer for the Natural Products Research Community.

Raper KB, Fennel DI. 1965. Aspergillus terreus group. genus Aspergillus Williams Wilkins Co, Balt Md.

Refai M, El-Yazid H, Tawakkol W. 2015. Monograph on the genus Penicillium. :157.

Rifai MA. 1969. A revision of the genus Trichoderma. Mycol Pap. 116:1–56.

Samson RA, Houbraken J. 2011. Phylogenetic and taxonomic studies on the genera Penicillium and Talaromyces. Stud Mycol. 70:iii.

Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CHW, Perrone G, Seifert KA, Susca A, Tanney JB, et al. 2014. Phylogeny, identification and nomenclature of the genus Aspergillus. Stud Mycol. 78(1):141–173.

Schipper MAA. 1978. 1 . Introduction and History. 24:215–236.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. 109(16):6241–6246.

Singh H. 2006. Mycoremediation: fungal bioremediation. [place unknown]: John Wiley & Sons.

Slepecky RA, Starmer WT. 2009. Phenotypic plasticity in fungi : a review with observations on Aureobasidium pullulans Phenotypic plasticity in fungi : a review with observations on Aureobasidium pullulans. (June 2014).

Smith D. 1983. Cryoprotectants and the cryopreservation of fungi. Trans Br Mycol Soc. 80(2):360–363.

Stackebrandt E, Schüngel M, Martin D, Smith D. 2015. The Microbial Resource Research Infrastructure MIRRI: Strength through Coordination. :890–902.

Stielow JB, Lévesque CA, Seifert KA, Meyer W, Irinyi L, Smits D, Renfurm R, Verkley GJM, Groenewald M, Chaduli D, et al. 2015. One fungus , which genes ? Development and assessment of universal primers for potential secondary fungal DNA barcodes. :242–263.

Tkins SDA, Lark IMC. 2004. Fungal molecular diagnostics : a mini review. 45(1):3–15.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc a Guid to methods Appl. 18(1):315–322.

Woudenberg JHC, Groenewald JZ, Binder M, Crous PW. 2013. Alternaria redefined. Stud Mycol [Internet]. 75:171–212. http://dx.doi.org/10.3114/sim0015

Wright DK, Manos MM. 1990. PCR protocols: a guide to methods and applications. Orlando, Fla Acad Press Inc.:153–159.

Chapter 2

CASE STUDY

CHARACTERIZATION OF FILAMENTOUS FUNGI ISOLATED FROM A LANDFILL LEACHATE

2. Characterization of filamentous fungi isolated from a landfill leachate

Abstract

Currently the most widespread and economic method to dispose municipal and industrial waste is to deposit it in landfills. The large amount of leachate generated from a landfill site and the scarcity of available landfill sites are two of the main problems of municipal solid waste disposal. The aim of this study focuses on the isolation of vital filamentous fungi from two pick-up points of a landfill leachate. These fungi were characterized to potentially use them for bioremediation treatments of wastewater difficult to treat with biological methods, like leachate. Three of the fungi isolated from the samples have been tested to grow into a range of leachate incorporated with malt extract agar (MEA) and sterile water. The results of this initial screening suggest that vital fungi exist in leachate, but their survival is linked to the limiting amount of nutrients for their development.

2.1. Introduction

Landfill leachate is a complex and highly polluted effluent due to a number of biological, chemical and physical processes taking place in the landfill. Generally, leachate characteristics are influenced by external factors such as water supply (meteoric, surface and subsurface), temperature and/or internal factors such as the initial moisture content of the waste and the production and consumption of water during biodegradation. The landfill leachate is characterized by not biodegradable and hazardous compounds as phenols, phosphate, sulphite, heavy metals, dioxins, pesticides, polycyclic aromatic hydrocarbon, persistent organic pollutants, halogenated compounds, alkylating agents and ammonium (Kamaruddin et al. 2015). There are more than 200 organic compounds identified in landfill leachate, including microbes: in a landfill leachate the organic matter is biologically transformed by a variety of microorganisms with different metabolic and functional capabilities (McDonald et al. 2010). Some of these can be opportunistic pathogens and they could cause health risks, producing toxins that may cause public health problems (Schrab et al. 1993; Kjeldsen et al. 2004). The flow rate and composition of leachate depend mainly on the age of the landfill: as the waste ages, the biodegradable fraction of organic pollutant in leachate decreases, as a result of the anaerobic decomposition taking place in landfill site (Neczaj et al. 2005).

In the construction of a landfill, a system suitable for leachate must be provided: a mix of physical, chemical and biological methods should be used to get an efficient treatment. The leachate is commonly processed combining it with domestic sewage, however this union is difficult to process using conventional wastewater treatments, because of the presence of refractory or inhibitory compounds in leachate (Renou et al. 2008). The dilution of the leachate in wastewaters could create

environmental problems, but this makes it more biodegradable and therefore attachable by the microbial flora present in biological treatments for civil wastewater. Clearly the landfill leachate requires specific and complex treatment. Recently, among the biological treatments, the use of fungi has been intensively studied and has shown a great potential for removal of pollutants from water (Ellouze et al. 2008; Prigione et al. 2008; More et al. 2010; Gullotto et al. 2015).

Generally, in traditional civil wastewater plants, where leachate is often diluted, the activated sludge produced downstream of the biological treatment tank it is left in the sedimentation tank. At the end of the process the sludge precipitates to the bottom and the remaining water is then filtered (Bianucci & Bianucci 1998). This premise is important to contextualize the use of filamentous fungi in traditional biological treatments, the effect of which would be to make the sludge foamy due to mycelium production and more difficult to separate by sedimentation (Wanner 1994). This phenomenon is called bulking and can only be solved by optimizing the growth of a flora with few filamentous organisms or by modifying the system with sludge treatments, for example by filtration.

In addition to bulking, another problem related to the use of fungi in traditional treatments is that it is more expensive than bacterial treatments, in particular regarding the requirement of nutrients, low pH, sterilization and an additional treatment step to remove ammonium nitrogen (Gadd 2001). The use of fungi in wastewater and in leachate treatment is a new field of research: more studies are necessary to improve this biological treatment for wastewater (Ellouze et al. 2008; Kalčíková et al. 2014; Bardi et al. 2017).

Regardless of the wastewater examined and its specific difficulty in being treated with biological treatment, the starting point for using fungi it is certainly a mycological characterization of the wastewater. In this study, the focal point is the search of vital fungi in landfill leachate and their characterization. Samples have been taken from two leachate collection points; native fungi were isolated from both samples and were collected to test their ability to survive in the leachate wastewater and potentially be good candidates for landfill leachate bioremediation processes.

2.2. Materials and method

2.2.1. Leachate landfill

The leachate used in this research came from a municipal landfill waste in Northern Italy. This landfill is UNI EN ISO 14001 certified since 2009 and in 2011 obtained Eco-management and Audit Scheme (EMAS) registration. This landfill has only non-hazardous waste from aggregates, excavated soil,

water treatment sludge and non-recoverable waste consisting of rubber, plastic, paper, fabrics and glass, while putrescible waste is not accepted. It has been built on an impermeable geological barrier, consisting of a compact clayey marl deposit several hundred meters thick.

The samples were collected in two different sites coming from two different leachate accumulation tanks, named S1-2 and S3. The tank S1-2 is located in the lower landfill site and receives the leachate deriving from the landfill lot now closed and running out, while the tank S3 is located at the base of the new landfill lot, from which it receives the waste. The physical and chemical properties of the leachate are determined by USEPA test guideline. Standard physical and chemical parameters, including chemical oxygen demand (COD), biochemical oxygen demand (BOD), dissolved oxygen (DO), conductivity, chloride, sulphate, ammonia, and nitrate, have been determined.

2.2.2. Isolation and identification of fungi

The collected samples come from two different sampling points: the S1-2 sample is taken form a leachate generated in a closed and old landfill while the S3 sample is taken from a sampled in young and active landfill. Each sample has been immediately transported in three 1 L bottles and has been analyzed within 24 hours in the mycology laboratory and in the chemical laboratory for fungal and physical characterization, respectively.

The vital fungal strains present in the leachate samples has been examined and characterized. The modified plate dilution technique (Gams et al. 1987) was applied for the isolation of vital fungal strains from leachate samples. Three different dilutions are performed for both leachate samples: 1:10, 1:100 and 1:1000 (Figure 8). Aliquots of 100 µl of different dilutions from both samples were plated onto three different types of culture media: MEA (Malt Extract Agar), PDA (Potato Dextrose Agar) and RB (Rosa Bengala Agar). MEA and PDA media have been before enriched with chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$), a thermostable antibiotic that enhances the selectivity with respect to the majority of contaminating bacteria which inhibit fungal growth. The RB medium has not required this addition because it has already chloramphenicol in its original composition.

The inoculated plates have been incubated at 24°C in the dark for 25 days. The Colony Forming Units (CFUs) have been counted and the different fungal morphotypes have been isolated in axenic culture. The strains have been identified using trophic and physiological requirements and using macroscopic and microscopic characters.

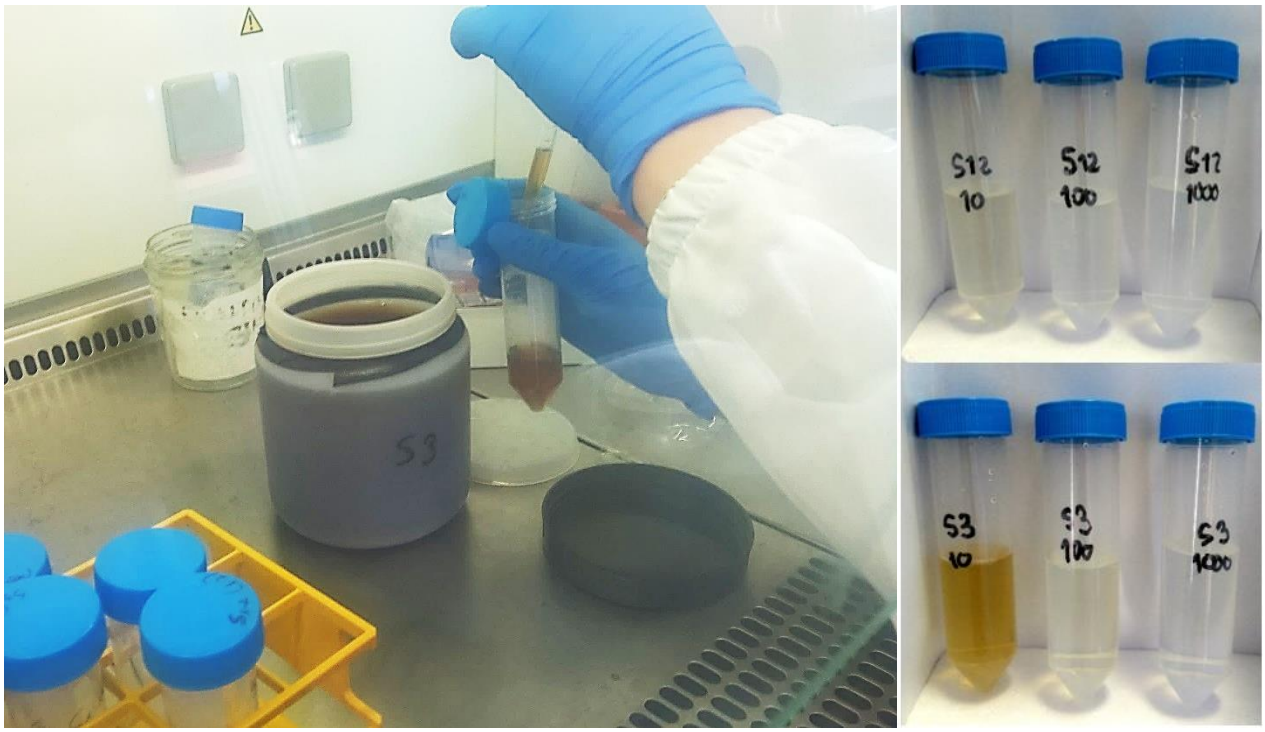


Figure 8 The leachate samples dilution techniques.

Every strain is identified also using the molecular analyses (ß-tubulin and ITS ribosomal DNA sequences) (Gams *et al.*, 1987; Samson and Houbraken, 2011; Di Piazza *et al.*, 2017). A quantity of 100 mg of fresh fungal solid culture is frozen, disrupted and homogenized using a modified CTAB method (Doyle & Doyle 1987). To homogenize the sample a Tissuelyser was used (Retsch GmbH, Haan, Germany) for 1 min 30 s at 18 Hz. Then the sample processed was incubated for 1 h 30 min at 65 °C in 700 ml CTAB extraction buffer (100 mm Tris–HCl pH 8.0; 20 mm EDTA; 1.4 m NaCl; 2% PVP; 2% CTAB) supplemented with 10 µg of proteinase K (Sigma-Aldrich, St Louis, MO, USA) and 30 µg of RNase A (Sigma-Aldrich).

Every sample was centrifuged at 5900 g for 10 min at room temperature and the aqueous layer was transferred to a new tube and an equal volume of a mixture of phenol, chloroform, isoamylic alcohol (25:24:1) (Sigma-Aldrich) was added. Then it was shaken and centrifuged at 5900 g for 5 min at room temperature and after the aqueous layer was transferred to a new tube and an equal volume of chloroform was added. Every sample was shaken and centrifuged at 5900 g for 5 min at room temperature. The aqueous layer was transferred to a new tube, and 2-propanol (two-thirds of the recovery volume) was added. The samples were then incubated for 15 min at 4 °C, centrifuged at 9200 g for 10 min at room temperature and the aqueous layer was discarded. The samples were then washed in ethanol at 70 °C by centrifugation at 9200 g for 1 min at room temperature. The DNA pellet was air-dried and then re-suspended in 50 µl of water. The quality and quantity of DNA samples were assessed with an ND-1000 spectrophotometer NanoDrop (NanoDrop Technologies, Inc.,

Wilmington, DE, USA). DNA extracts were stored at -20 °C. The morphological identifications were confirmed by amplification of the ß-tubulin gene using Bt2a and Bt2b primers (Glass & Donaldson 1995). Universal primers ITS1F/ITS4 were used for ITS region amplification (White et al. 1990; Gardes & Bruns 1993). Amplification reactions were performed in a T3000 thermal cycler (Biometra biomedizinische Analytik GmbH, Goettingen, Germany) in a 50-µl aliquot of reaction mixture using the following final concentrations or total amounts: 20 ng DNA, 1 × polymerase chain reaction (PCR) buffer (20 mm Tris–HCl pH 8.4; 50 mm KCl), 1 µm of each primer, 2.5 mm MgCl, 0.25 mm of each dNTP, 0.5 unit of Taq polymerase (Promega, Madison, WI, USA). The PCR protocol was as follows: one cycle of 5 min at 95 °C; 40 s at 94 °C; 45 s at 55 °C; thirty-five 1-min cycles at 72 °C; one 10-min cycle at 72 °C. PCR products were resolved on 1.0% agarose gel and visualized by staining with ethidium bromide. PCR products were purified and sequenced using MACROGEN Inc. (Seoul, Republic of Korea). Sequence assembly and editing were performed using Sequencher® version 5.2 (sequence analysis software, Gene Codes Corporation, Ann Arbor, MI, USA).

2.2.3. Tolerance tests

In order to understand the tolerance threshold of the different autochthonous strains to survive in the landfill leachate and to understand the possibility to use one or more of the strains in biological treatments, several tests have been carried out. To test the survival of the strains isolated in the leachate, two types of tests were carried out: the first and the second tests was made in microwells with liquid culture medium, the third test was made in petri dishes with agarized culture media. Fungal growth capacity was assayed in two multi-well toxicity tests (Figure 9) with a different leachate concentration range: between 1:1000 to 1:2 dilution in MEA, and between 1:20 to 1:2 dilution in MEA.

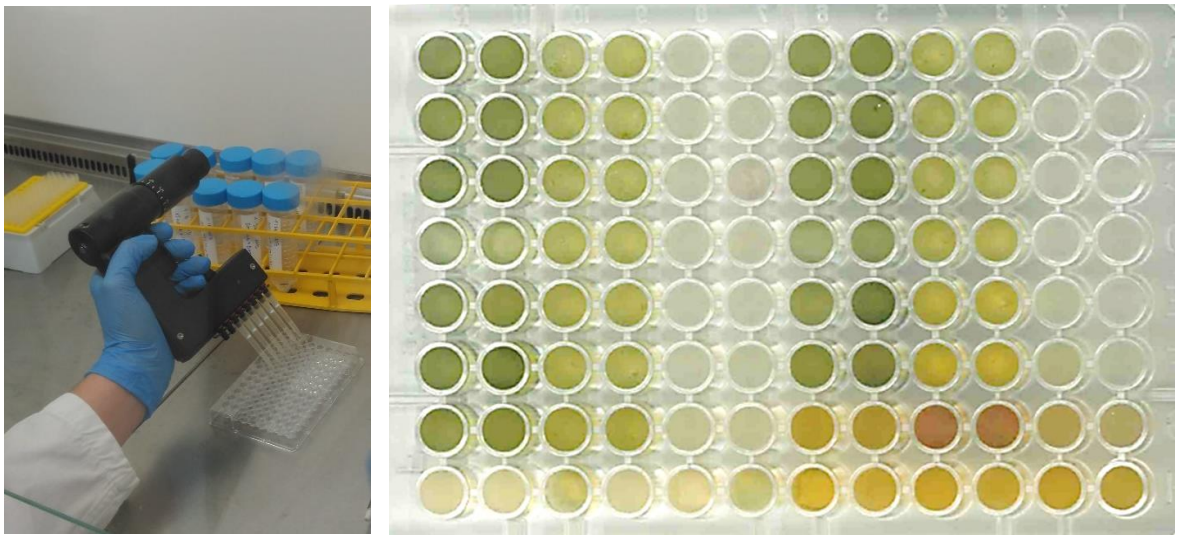


Figure 9 96-well plate used for the toxicity test: in row different leachate concentration and in column different fungal autochthonous strains.

A 96-well plate (8 x 12) was used, with a flat bottom to control easily the growth of colony in the liquid medium using the laboratory protocol modified by (Espinel-Ingroff & Kerkering 1991). Each well of the plate has a volume of 200 µl and was set up with 100 µl of leachate at a certain concentration and 100 µl of inoculum dissolved in the MEA. In the control well, the 100 µl of leachate will be replaced with 100 µl of MEA. The strains selected for the test were tested on the two types of leachate and in duplicate. Increasing concentrations of leachate combined with MEA culture medium were used in all the tests to verify if and at what concentrations the substances present in the leachate samples could become inhibitory for the growth of fungi.

Inoculum suspensions of each strain were prepared for each experiment from mature fresh (3- to 7-day-old) cultures grown at 24°C on MEA plates. Conidia were taken from the marginal portion of colonies and the cell suspensions were *prolificans* dissolved in a solution of MEA liquid culture media and TWEEN80®. Conidia were added until a liquid inoculum with a concentration of 10^5 conidia/mL was obtained with Burker chamber. The different concentrations of leachate to be combined with the inoculum were made by diluting the two samples S1-2 and S3 in distilled sterile water.

When the test is set up, the wells with the inoculum and the leachate solution are clear and transparent, but after the germination of the conidia they become opaque as in Figure 9. The different optical density is the index used to verify at what concentration the fungal strains survived and which there was no growth. The multi-well plate assay was left to incubate at 24°C for 48 hours, after which it is possible to discriminate in which wells the growth has become visible.

For the third toxicity test concerning the fungal growth media plates (diameter 40 mm) with solid medium culture were used (Figure 10). An agarized solution was made in which the leachate of the two samples S1-2 and S3 and the MEA have been added at different concentrations. Two control media have been also prepared: negative control without MEA and positive without leachate.

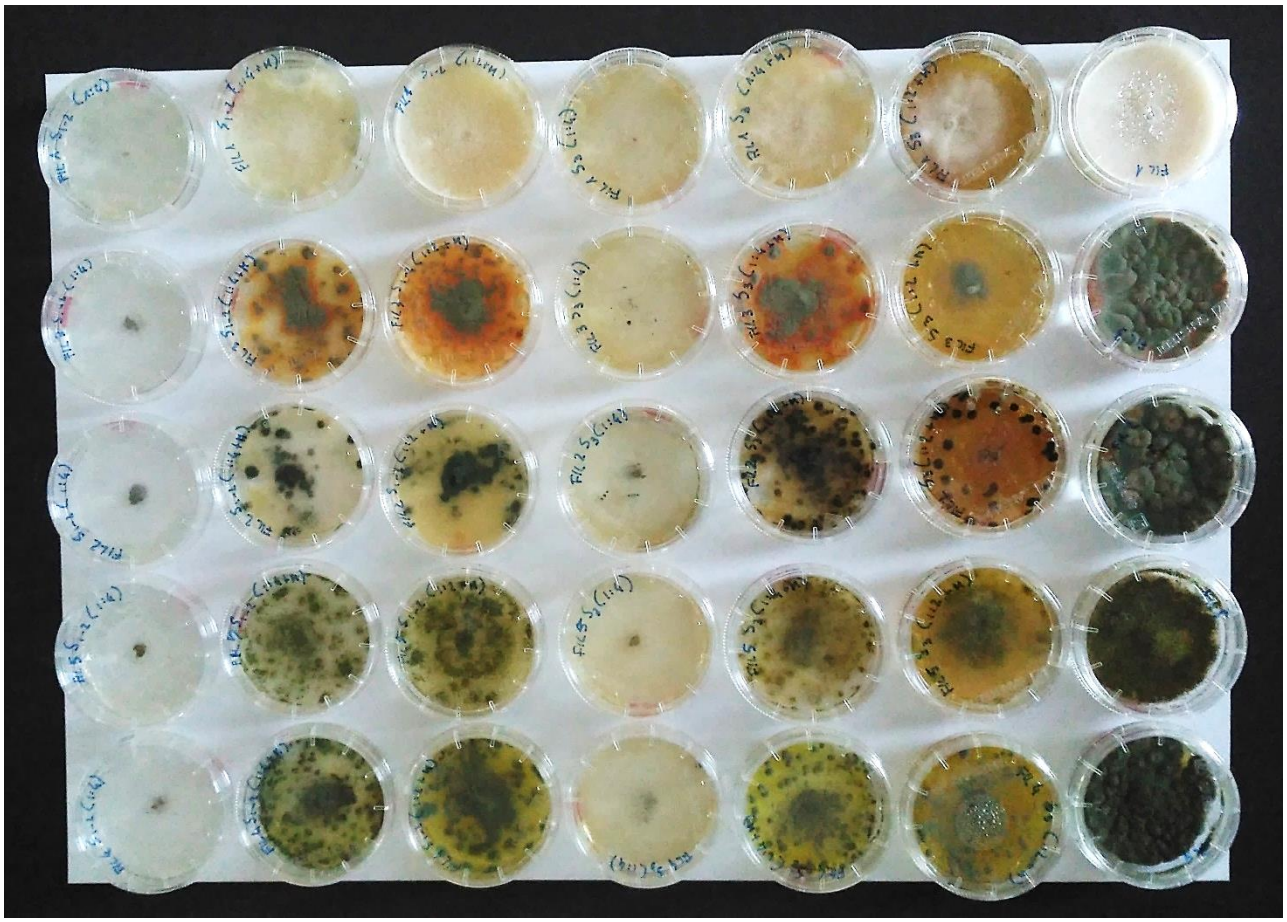


Figure 10 The third toxicity test.

The sampling was carried out from a mature colony and used on the embedded soil of leachate at different concentrations. After one week, colony growth was compared to the positive control. The absence of growth was considered a negative result, while any increase in the diameter of the colony was considered a positive result. All tests carried out in this study want to prove the possible conditions of use of the isolated fungi on the plant for the treatment of leachate.

2.3. Result and discussion

2.3.1. Landfill leachate

The different composition of the two landfill leachate samples analyzed is due to the fact that tank S1-2 receives a refluent of a batch that is running out, having an organic and inorganic component less concentrated than the sample S3. Both the samples were brown colored but S3 is dark brown and

S1-2 is orange brown (Figure 8). The smell is due mainly to the presence of organic acids and is stronger in the sample S3. Details of their chemical characteristics provided by chemical laboratory are shown in Table 4.

	Unit	*S3*	*S1-2*	*Standard deviation*
Ammonial nitrogen (NH_4^+)	mg/l	938.85	216.2	32.65
Nitric nitrogen (NO_3^-)	mg/l	< 0.1	5.1	0.9
Nitrous nitrogen (NO_2^-)	mg/l	0.022	0.11	0.01
Electric conductivity	µS/cm	29698	8339	425
Dissolved Organic Carbon (DOC)	mg/l	4445	199	10
Chloride ion (Cl^-)	mg/l	4209.1	1223.8	71
pH	pH unit	7.19	7.78	
Biological Oxygen Demand (BOD_5)	mg/l O_2	3371	272	82
Chemical Oxygen Demand (COD)	mg/l O_2	9439	816	54
Sulphates (SO_4^{2-})	mg/l	51	779.8	62.4
Total Dissolved Solids (TDS)	mg/l	821	36.4	3.6

Table 4 The chemical characteristic of landfill leachate samples.

As the data show, the concentration of the pollutants in the leachate object of this study is different in samples S1-2 and S3. In particular, as can be seen from the values of BOD, COD and DOC concentration, the dissolved organic substance is much more concentrated in sample S3: this is due to the heap from which the leachate of S3 originates is continuously supplied with new municipal waste. S3 also has higher concentration of chloride ion and so a high electrical conductivity.

In the S1-2 heap, which has been stopped for years, the residual organic matter of the waste stored in the past continues to percolate. The same reason must be attributed to the higher concentration of

chloride ion and so a high electrical conductivity. The heap S1-2 can generate leachate after closing for 30-50 years (Bhalla 2013).

In the sample S1-2 coming from the exhaustion pile, the very high value of the sulphates is opposed to the very low value of ammonia nitrogen, unlike what occurs in sample S3 coming from the still active pile. This reversal can be justified by the intense and rapid consumption of oxygen that occurs in an active heap, which reduces the oxidized compounds such as sulphates in favour of reduced species. Contrariwise in a heap in exhaustion the lower microbial activity avoids the excessive consumption of oxygen, which remains present for example in nitrates or sulphates (Adhikari et al. 2008).

2.3.2. Isolation and identification of fungi

The fungal strains isolated are all filamentous fungi ascribable to the genera *Aspergillus*, *Penicillium* and *Fusarium.* The fungi concentration in leachate samples is between 10^3 and 10^6 CFUs, and half of the found strains are present in both leachate samples. The leachate can be considered a real extreme environment, and like other extreme environments the variety of types of autochthonous fungi is restricted to a few resistant species (Newsham & Boddy 2012; Selbmann et al. 2013). Details of fungal taxa isolated are shown in Table 5.

Fungal taxa	*S3 (CFUs)*	*S1-2 (CFUs)*
Fusarium oxysporum Schltdl.	10^6	10^5
Penicillium citrinum Thom	10^3	/
Aspergillus flavus Link	10^5	10^3
Aspergilus fumigatus Fresen	10^4	10^2

Table 5 Fungal taxa isolated from landfill and their concentration in leachate samples.

The fungus most present in both leachate samples and at a higher concentration is the *Fusarium oxysporum* Schltdl. This species is normally found in the rhizosphere and soils throughout the world (Gordon 1997) and can became pathogenic for roots and plants (Nelson et al. 1994; Serig 2011). *F. oxysporum* can grow in the presence of heavy metals, producing lead and cadmium crystals (Sanyal et al. 2005). Among its enzymes of great importance there are a broad range of cellulases and

xylanases, used for bioethanol production from its biomass (Panagiotou et al. 2005). *F. oxysporum* also seems to be able to degrade the resins of crude oil that is normally resistant to microbial attack (Grace Liu et al. 2011).

Aspergillus fumigatus Fresen is present in both samples and it is often involved in degradation of leaf, plays a role in colonization of compost, but not in hard wood degradation (Tekaia & Latgé 2005). However, one aspect to consider both in the development of biotechnology and in the well-being of the landfill staff is that this species can also be pathogenic for humans and its volatile spores can cause health problems (Hohl & Feldmesser 2007). *F. oxysporum* and *A. fumigatus* were able to grow well in laboratory diesel/water or crude oil/water systems (Grace Liu et al. 2011).

Aspergillus flavus Link is the third isolated species that is present in both leachate samples. This species has already been isolated from a municipal landfill and used in a bioremediation process (Pramila & Ramesh 2011). Moreover, *A. flavus* was used to remove colour and chromium from a synthetic solution of the chromium complex dye (Ghosh et al. 2016) and as bio absorbent for removal of heavy metals from wastewater and industrial effluent (Joshi et al. 2011).

Penicillium citrinum Thom has been isolated only from the sample S3, by the way this sample has a higher concentration of all the species. *P. citrinum* is isolated from different environmental conditions, ranging from forest soils to agricultural fields (Khan et al. 2008). This strain is an alkali tolerant fungus, in fact the leachate is normally with basic pH (Dutta et al. 2008).

All isolated fungal strains have special characteristics that make them most likely able to survive in pollutant environmental and these strains are possible candidates in the development of bioremediation protocols.

2.3.3. Tolerance tests

Strains of *F. oxysporum*, *A. flavus* and *A. fumigatus* present in both leachate types were chosen to assess growth capacity using two multi-well toxicity tests with a different leachate concentration range: a large one (between 1:1000 to 1:2 dilution of leachate in MEA) in the first test to check the survival range of the strains chosen, then a more precise one (between 1:20 to 1:2 dilution in MEA) in the second test. The results of the first test show that the strains are capable of growing up to 1:20 dilution, all of them equally. Then by widening the range and increasing the concentration of leachate in the second test, the growth capacity was verified up to a dilution of 1:4 for all the strains tested. The results are shown in the Table 6 and Table 7.

Dilution	*Leachate sample added*	*Fusarium oxysporum*	*Aspergillus flavus*	*Aspergillus fumigatus*
1:2	S1-2	-	-	-
	S3	-	-	-
1:10	S1-2	-	-	-
	S3	-	-	-
1:20	S1-2	+	+	+
	S3	+	+	+
1:50	S1-2	+	+	+
	S3	+	+	+
1:100	S1-2	+	+	+
	S3	+	+	+
1:200	S1-2	+	+	+
	S3	+	+	+
1:1000	S1-2	+	+	+
	S3	+	+	+

Table 6 The first toxicity test

Dilution	*Leachate sample added*	*Fusarium oxysporum*	*Aspergillus flavus*	*Aspergillus fumigatus*
1:2	S1-2	-	-	-
	S3	-	-	-
1:4	S1-2	+	+	+
	S3	+	+	+
1:8	S1-2	+	+	+
	S3	+	+	+
1:10	S1-2	+	+	+
	S3	+	+	+
1:16	S1-2	+	+	+
	S3	+	+	+
1:20	S1-2	+	+	+
	S3	+	+	+

Table 7 The results of the second toxicity test.

In the third toxicity test plates (diameter 40 mm) with different solid medium leachate solution concentration were used: 1:2 and 1:4 dilution with MEA solid medium and 1:4 dilution with sterile deionized water, to test the fungal growth. Also in this case taxa isolated from leachate in both samples S1-2 and S3 were tested.

The result agrees with the multiwell tests. The fungi manage to grow up to a 1:4 dilution of leachate only in the presence of the MEA culture medium. It is possible to hypothesize that the recalcitrant substances present in the leachate are usable by fungi because they are found in high concentrations, but do not allow their development. The simpler nutrients provided by the culture medium allow the strains to grow in the leachate and give a positive result to the test up to a concentration in a quarter

of the leachate. The only exception is *F. oxysporum*, which manages to grow in S1-2 leachate at 1:2 dilution with water: it is known that the *F. oxysporum* is able to survive in extreme conditions such as the presence of heavy metal or hydrocarbons (Sanyal et al. 2005; Grace Liu et al. 2011). The results of this test are shown in Table 8.

Colture media plates	***Diluition***	***Leachate sample added***	***Penicillium citrinum***	***Aspergillus flavus***	***Aspergillus fumigatus***	***Fusarium oxysporum***
leachate + MEA	1:2	S1-2	+	+	+	+
		S3	+	+	+	+
leachate + MEA	1:4	S1-2	+	+	+	+
		S3	+	+	+	+
leachate	1:4	S1-2	-	-	-	+
		S3	-	-	-	-
MEA	/	S1-2	+	+	+	+
		S3	+	+	+	+

Table 8 The results of the third toxicity test.

2.4. Conclusion

Landfill leachate is a complex polluted liquid matrix due to the diversity of substances present inside it and the diversity that occurs between one sample and another. In the case of the study of this work, the samples S1-2 and S3 contain high concentrations of ammonium nitrogenum, organic substances and chloride ion. The mycological analysis allowed to isolate four strains, three of them are present in both samples. The isolated fungi were identified with morphological and molecular techniques. To move towards research that aims to isolate and select fungi from wastewater that can be used in bioremediation, it is important, after having isolated and conserved the strains, to test them in different toxicity tests. In this way it is possible to do a first screening that verifies the growth in the wastewater

of origin. To assess their ability to grow in leachate toxicity tests were carried out. The results of the tests suggest the possible use of these fungi in leachate bioremediation protocols, probably in the presence of simpler nutrients and with a dilution of the wastewater. In any case the fungi could have a role in the degradation of the matrix before it is introduced into the wastewater system. In particular *F. oxysporum*, already present in high concentrations in the leachate, could be subject to further studies on the bioremediation of the leachate.

2.5. References

Adhikari B, Dahal KR, Nath Khanal S. 2008. A Review of Factors Affecting the Composition of Municipal Solid Waste Landfill Leachate 1*. Certif Int J Eng Sci Innov Technol. 9001(5):2319–5967.

Bardi A, Siracusa G, Chicca I, Islam M, Tigini V, Di Gregorio S, Petroni G, Yuan Q, Munz G. 2017. Landfill Leachate Treatment through Fungi in an Attached Growth System. 10th Int Conf Biofilm React 2017 [Internet].:1–4. https://iris.unito.it/handle/2318/1641135#.XJ8MBdgnat8

Bhalla B. 2013. Effect of Age and Seasonal Variations on Leachate Characteristics of Municipal Solid Waste Landfill. Int J Res Eng Technol. 02(08):223–232.

Bianucci G, Bianucci ER. 1998. Il trattamento delle acque inquinate. [place unknown]: HOEPLI EDITORE.

Cecchi G, Vagge G, Cutroneo L, Greco G, Di Piazza S, Faga M, Zotti M, Capello M. 2019. Fungi as potential tool for polluted port sediment remediation. Environ Sci Pollut Res.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. [place unknown].

Dutta T, Sahoo R, Sengupta R, Ray SS, Bhattacharjee A, Ghosh S. 2008. Novel cellulases from an extremophilic filamentous fungi Penicillium citrinum: production and characterization. J Ind Microbiol Biotechnol. 35(4):275–282.

Ellouze M, Aloui F, Sayadi S. 2008. Detoxification of Tunisian landfill leachates by selected fungi. J Hazard Mater. 150(3):642–648.

Espinel-Ingroff A, Kerkering TM. 1991. Spectrophotometric method of inoculum preparation for the in vitro susceptibility testing of filamentous fungi. J Clin Microbiol. 29(2):393–394.

Gadd GM. 2001. Fungi in Bioremediation. Fungi in Bioremediation.

Gams W, Aa HA van der, Plaats-Niterink AJ van der, Samson RA, Stalpers JA. 1987. CBS course of mycology. Baarn: Centraalbureau voor Schimmelcultures.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol. 2(2):113–118.

Ghosh A, Dastidar MG, Sreekrishnan TR. 2016. Bioremediation of a Chromium Complex Dye Using Aspergillus flavus and Aspergillus tamarii. Chem Eng Technol. 39(9):1636–1644.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol. 61(4):1323–1330.

Gordon TR. 1997. the Evolutionary Biology. Annu Rev Phytopathol. 35:111–128.

Grace Liu PW, Chang TC, Whang LM, Kao CH, Pan PT, Cheng SS. 2011. Bioremediation of petroleum hydrocarbon contaminated soil: Effects of strategies and microbial community shift. Int Biodeterior Biodegrad [Internet]. 65(8):1119–1127. http://dx.doi.org/10.1016/j.ibiod.2011.09.002

Gullotto A, Lubello C, Mannucci A, Gori R, Munz G, Briganti F. 2015. Biodegradation of naphthalenesulphonate polymers: The potential of a combined application of fungi and bacteria. Environ Technol (United Kingdom). 36(4):538–545.

Hohl TM, Feldmesser M. 2007. Aspergillus fumigatus: Principles of pathogenesis and host defense. Eukaryot Cell. 6(11):1953–1963.

Joshi PK, Swarup A, Maheshwari S, Kumar R, Singh N. 2011. Bioremediation of Heavy Metals in Liquid Media Through Fungi Isolated from Contaminated Sources. Indian J Microbiol. 51(4):482–487.

Kalčíková G, Babič J, Pavko A, Gotvajn AŽ. 2014. Fungal and enzymatic treatment of mature municipal landfill leachate. Waste Manag. 34(4):798–803.

Kamaruddin MA, Yusoff MS, Aziz HA, Hung Y-T. 2015. Sustainable treatment of landfill leachate. Appl Water Sci. 5(2):113–126.

Khan SA, Hamayun M, Yoon H, Kim HY, Suh SJ, Hwang SK, Kim JM, Lee IJ, Choo YS, Yoon UH,

et al. 2008. Plant growth promotion and Penicillium citrinum. BMC Microbiol. 8:1–10.

Kjeldsen P, Barlaz MA, Rooker AP, Baun A, Ledin A, Christensen TH, Schlumberger Water Services, Zheng, Chunmiao; Hill, Mary C.; Cao, Guoliang; Ma R, Simon F, Meggyes T, et al. 2004. Principles Contaminat Transport. Ground Water Monit Remediat [Internet]. 32(4):65. http://www.elpais.com.co/elpais/archivos/informe-cvc-2001.pdf%0Ahttp://bibliotecavirtual.unl.edu.ar:8080/tesis/handle/11185/335%0Awww.elsevier.com/locate/apgeochem%0Ahttps://repositorio.unican.es/xmlui/handle/10902/1286%0Ahttp://doi.wiley.com/10.1111/j.17

McDonald JE, Allison HE, McCarthy AJ. 2010. Composition of the landfill microbial community as determined by application of domain- And group-specific 16S and 18S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol. 76(4):1301–1306.

More TT, Yan S, Tyagi RD, Surampalli RY. 2010. Potential use of filamentous fungi for wastewater sludge treatment. Bioresour Technol [Internet]. 101(20):7691–7700. http://dx.doi.org/10.1016/j.biortech.2010.05.033

Neczaj E, Okoniewska E, Kacprzak M. 2005. Treatment of landfill leachate by sequencing batch reactor. Desalination. 185(1–3):357–362.

Nelson PE, Dignani MC, Anaissie EJ. 1994. Taxonomy, biology, and clinical aspects of Fusarium species. Clin Microbiol Rev. 7(4):479–504.

Newsham KK, Boddy L. 2012. Special Issue: Fungi in extreme environments. Fungal Ecol [Internet]. 5(4):379–479. https://www.cabdirect.org/cabdirect/abstract/20133122589

Panagiotou G, Villas-Bôas SG, Christakopoulos P, Nielsen J, Olsson L. 2005. Intracellular metabolite profiling of Fusarium oxysporum converting glucose to ethanol. J Biotechnol. 115(4):425–434.

Di Piazza S, Isaia M, Vizzini A, Badino G, Voyron S, Zotti M. 2017. First mycological assessment in hydrothermal caves of Monte Kronio (Sicily, southern Italy). Webbia [Internet]. 72(2):277–285. http://doi.org/10.1080/00837792.2017.1347368

Pramila R, Ramesh KV. 2011. Biodegradation of low density polyethylene (LDPE) by fungi isolated from municipal landfill area. J Microbiol Biotechnol Res [Internet]. 1(4):131–136. https://pdfs.semanticscholar.org/7451/c723b8ce9c3bfd0c9e0eafcb69f9376c7365.pdf

Prigione V, Varese GC, Casieri L, Marchisio VF, Anastasi A, Spina F, Romagnolo A, Tigini V, Prigione V, Varese GC, et al. 2008. Scale-up of a bioprocess for textile wastewater treatment using Bjerkandera adusta. Bioresour Technol [Internet]. 123(9):3559–3567. http://dx.doi.org/10.1016/j.biortech.2009.12.067

Renou S, Givaudan JG, Poulain S, Dirassouyan F, Moulin P. 2008. Landfill leachate treatment: Review and opportunity. J Hazard Mater. 150(3):468–493.

Samson RA, Houbraken J. 2011. Phylogenetic and taxonomic studies on the genera Penicillium and Talaromyces. Stud Mycol. 70:iii.

Sanyal A, Rautaray D, Bansal V, Ahmad A, Sastry M. 2005. Heavy-metal remediation by a fungus as a means of production of lead and cadmium carbonate crystals. Langmuir. 21(16):7220–7224.

Schrab GE, Brown KW, Donnelly KC. 1993. Acute and genetic toxicity of municipal landfill leachate. Water, Air, Soil Pollut. 69(1–2):99–112.

Selbmann L, Egidi E, Isola D, Onofri S, Zucconi L, de Hoog GS, Chinaglia S, Testa L, Tosi S, Balestrazzi A, et al. 2013. Biodiversity, evolution and adaptation of fungi in extreme environments. Plant Biosyst - An Int J Deal with all Asp Plant Biol [Internet]. 147(1):237–246. https://doi.org/10.1080/11263504.2012.753134

Serig D. 2011. Research review. Teach Artist J. 9(3):193–198.

Tekaia F, Latgé JP. 2005. Aspergillus fumigatus: Saprophyte or pathogen? Curr Opin Microbiol. 8(4):385–392.

Wanner J. 1994. Activated sludge: bulking and foaming control. [place unknown]: CRC Press.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc a Guid to methods Appl. 18(1):315–322.

Chapter 3

CASE STUDY

CHARACTERIZATION OF FILAMENTOUS FUNGI ISOLATED FROM DIFFERENT STEPS OF COMPOST MATURATION

3. Characterization of filamentous fungi isolated from different steps of compost maturation

The study reported is part of a larger research published recently: Thermotolerant and thermophilic mycobiota in different steps of compost maturation (Di Piazza et al. 2020). Performing this research, I have applied the techniques of identification and conservation of fungal strains isolated from extreme environments. The strains are currently stored in the mycology laboratory stock.

Abstract

Composting is a complex process in which micro-organisms are involved, mainly fungi and bacteria. This process depends on a large number of factors (biological, chemical and physical) among which microbial populations play a fundamental role. The high temperatures reached during the composting process indicate the presence of thermotolerant and thermophilic microorganisms, which are the key for optimization of the process. However, the same microorganisms can be harmful (allergenic, pathogenic) for workers in the plant and end-users. For this reason, an accurate knowledge of the thermotolerant and thermophilic component present during the composting stages is required.

At first, the key organisms that can improve the composting process need to be identified and this data can be used to estimate the potential health risk. Fungi were isolated at four different temperatures (25, 37, 45 and 50 °C) from compost samples collected in five different steps through a 21-days compost maturation in an active composting plant in Liguria (north-western Italy). The samples were subsequently plated on three different media. Our results show a high presence of fungi with an order of magnitude ranging from 1 to 10 x 10^4 CFU/g. The isolated strains, identified by means of specific molecular tools (ITS, β-tubulin, calmodulin, elongation factor 1-alpha), belong to 48 different species. Several thermophilic species belonging to the genera *Thermoascus* and *Thermomyces* were detected. Moreover, the presence of several potential harmful fungal species such as *Aspergillus fumigatus*, *A. terreus*, *Scedosporium apiospermum* were found during the whole process, including the final product.

Among the isolated fungi, two new strains have been identified: the *Penicillium valleboromidaense* Houbraken & Di Piazza is described in detail in the Fungal Planet description sheets (Crous et al. 2020) and the other is currently the subject of further research. This confirms the incredible potential that environments with extreme characteristics have to isolate new strains.

2.1. Introduction

Composting represents one of the most efficient methods for sustainable waste management. As already highlighted by other authors, this methodology depends on many factors, the most important being the nature, composition, sizing and quality of the substrate (Cerda et al. 2018; Reyes-Torres et al. 2018), environmental parameters during the process (Partanen et al. 2010; Reyes-Torres et al. 2018) and the microbial populations present during composting (Wei et al. 2012; Song et al. 2014; Onwosi et al. 2017; Liu et al. 2018). Different methodologies and technologies were applied on the basis of processed materials (Cerda et al. 2018; Reyes-Torres et al. 2018; Etesami et al. 2019).

Regardless of the used technology, all composting processes reach high temperatures of about 40–55 °C degrees; these temperatures are limiting and even lethal for most living organisms. The high temperatures of the composting process create strong selection on the biological components: on one hand, they eliminate many mesophilic organisms that are threatening for plants but, on the other hand, they select for extremophilic and extremotolerant organisms particularly adapted to high temperatures. These thermophilic and thermotolerant micro-organisms are fundamental in biochemical processes since several species have key roles in lignin degradation and thermoresistant- or thermostable-enzyme production. Moreover, potentially dangerous propagules of the same organisms can be inhaled, becoming harmful for workers who handle daily large quantities of compost. Therefore, knowledge of the microbiological components of compost is essential to optimize the process and to make a product of excellent quality and free of hazards for workers, consumers and the environment. In the last few decades, some authors studied the microbial components of compost, mainly focusing on the bacterial components (Franke-Whittle et al. 2005; Wang et al. 2016; Wang et al. 2017; Liu et al. 2018) and other studies listed potentially dangerous species for humans (Fuchs 2010; Franceschini et al. 2016).

Nowadays, commercial products with special bacterial starter cultures, fungi and chemical activators (in particular nitrogen) were developed and are useful both on the domestic and industrial scale, resulting in the improvement of the composting process and lowering production costs. With the advent of new high-throughput sequencing technologies, nonculture-based approaches were applied to characterize microbial communities, giving a wider picture on them than classical culture-based approaches (Bonito et al. 2010; Fuchs 2010; Antunes et al. 2016; Franceschini et al. 2016).

These methods provided excellent indications of the community but did not allow the isolation of organisms involved in the process. For these reasons, a culture-dependent method is still essential to isolate vital strains present in the matrix. In fact, isolation of the organisms into an axenic culture allows:

- to identify organisms, safely discriminating potentially dangerous from harmless species;

- verification of the effective viability of strains present in the investigated matrix;
- isolation and preservation of living organisms;
- study and exploitation of the organisms' biochemical characteristics.

In the last decade, studies investigated the fungal communities in compost, confirming that fungi are a significant part of the microbial community, in particular during the maturation phase (Kane & Mullins 1973; Anastasi et al. 2005; Langarica-Fuentes et al. 2014; López-González et al. 2015; Haas et al. 2016; Laich & Andrade 2016; Tian et al. 2017; Thanh et al. 2019). Moreover, some of these studies detected fungal species that are useful during stages of maturation and/or potentially harmful (Anastasi et al. 2005; Haas et al. 2016; Atif et al. 2020).

For these reasons, it is fundamental to conduct studies on fungal communities involved in the composting processes in order to select both useful populations to improve the process and to investigate the presence of potentially harmful species. This work contributes to the knowledge of thermotolerant and thermophilic fungal communities in compost. The fungal community of compost samples taken during the ripening of the product at five different stages in a composting plant in northwestern Italy was characterized. The strains in axenic culture were identified at the species level using the latest taxonomic insights.

3.2. Materials and Methods

3.2.1. Compost

Samples were collected from a compost-production plant in Liguria (Cairo Montenotte, northwestern Italy). The plant produces biogas through anaerobic digestion in a batch system from the organic fraction of urban solid waste collected from a municipality in the area. After the anaerobic-digestion phase, a new mixture is made composed of 1:1 (w:w) by the anaerobic digestate (which has a pH ranging between 6,5 and 7,5, and a temperature of 55°C) and green and brown waste from municipal green planting and pruning. This new mixture is composted in chambers measuring 4 m × 8 m × 2 m. Ripening time is, on average, 21 days after which the product is ready to leave the plant. As part of this study, the pile was sampled at five different times during the ripening process to analyze the mycological components present and their evolution over time. More precisely, the biodigestate (sample TQ) was analyzed, and samples were collected after 1, 7, 14, and 21 days of maturation (samples 1D, 7D, 14D, 21D). Given the size of the chambers, each sample consisted of 10 randomly taken composite samples of the biopile and biodigestate at various stages of maturation.

3.2.2. Isolation and identification of fungi

Fungi were isolated by a modified plate dilution method (Gams et al. 1987). Each solid sample was initially diluted 1:10 in sterile water, and the suspension was shaken at room temperature for 1 min. Later, the suspension was further diluted 1:100 and 1:1000. The three dilutions were plated out on three different media in triplicate: malt-extract agar supplemented with penicillin and streptomycin (MEA/PS), oatmeal agar with penicillin and streptomycin (OA/PS) and dichloran 18% glycerol agar (DG18). The plates were incubated at four temperatures (25, 37, 45 and 50°C) and checked daily between 3 and 15 days. Colonies were counted, and these data were used to determine the concentration of fungi (number of colony-forming units per gram, CFU/g) in the compost. For longer-term studies, the isolates were stored at 20°C in the Laboratory of Mycology DISTAV University of Genoa (ColD) collection. An experimental set for fungal isolation in shown in Figure 11.

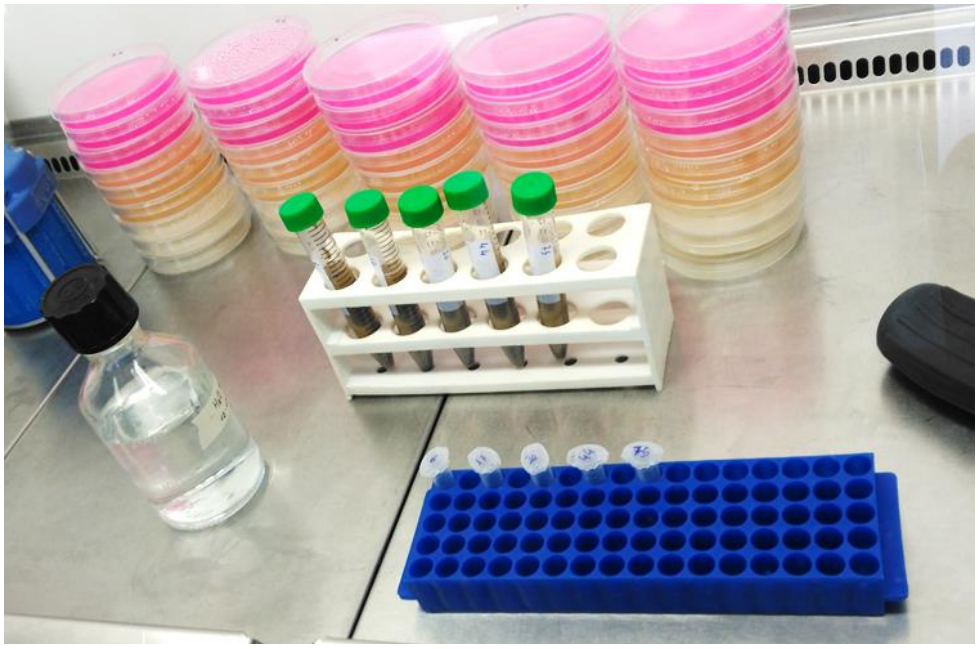


Figure 11 Fungal isolation set.

A preliminary sorting of the isolated strains was carried out on the basis of phenotypic characters. DNA barcoding of the isolated strains was carried out by amplification of the internal-transcribed-spacer (ITS) region (Samson et al. 2019). Later, in order to obtain more precise identification, other fragments were amplified on the basis of the (preliminary) identification result based on ITS sequencing (β-tubulin for *Penicillium*, calmodulin for *Aspergillus*, elongation factor 1-alpha and LSU for others) according to Samson et al. method (Samson & Houbraken 2011; Samson et al. 2014).

A quantity of 100 mg of fresh fungal culture is frozen, disrupted and homogenized using a modified CTAB method (Doyle & Doyle 1987). To homogenize the sample is used a Tissuelyser (Retsch

GmbH, Haan, Germany) for 1 min 30 s at 18 Hz. Then the sample processed is incubated for 1 h 30 min at 65 °C in 700 ml CTAB extraction buffer (100 mm Tris–HCl pH 8.0; 20 mm EDTA; 1.4 m NaCl; 2% PVP; 2% CTAB) supplemented with 10 µg of proteinase K (Sigma-Aldrich, St Louis, MO, USA) and 30 µg of RNase A (Sigma-Aldrich).

Each sample was centrifuged at 5900 g for 10 min at room temperature and the aqueous layer was transferred to a new tube and an equal volume of a mixture of phenol, chloroform, isoamylic alcohol (25:24:1) (Sigma-Aldrich) was added. Then it was shaken and centrifuged at 5900 g for 5 min at room temperature and after the aqueous layer was transferred to a new tube and an equal volume of chloroform was added. Each sample was shaken and centrifuged at 5900 g for 5 min at room temperature. The aqueous layer was transferred to a new tube, and 2-propanol (two-thirds of the recovery volume) was added. The samples were then incubated for 15 min at 4 °C, centrifuged at 9200 g for 10 min at room temperature and the aqueous layer was discarded. The samples were then washed in ethanol at 70 °C by centrifugation at 9200 g for 1 min at room temperature. The DNA pellet was air-dried and then re-suspended in 50 µl of water. The quality and quantity of DNA samples were assessed with an ND-1000 spectrophotometer NanoDrop (NanoDrop Technologies, Inc., Wilmington, DE, USA). DNA extracts were stored at -20 °C. The morphological identifications were confirmed by amplification of the β-tubulin gene using Bt2a and Bt2b primers (Glass & Donaldson 1995). Universal primers ITS1F/ITS4 were used for ITS region amplification (White et al. 1990; Gardes & Bruns 1993). Amplification reactions were performed in a T3000 thermal cycler (Biometra biomedizinische Analytik GmbH, Goettingen, Germany) in a 50 µl aliquot of reaction mixture using the following final concentrations or total amounts: 20 ng DNA, 1 × polymerase chain reaction (PCR) buffer (20 mm Tris–HCl pH 8.4; 50 mm KCl), 1 µm of each primer, 2.5 mm MgCl, 0.25 mm of each dNTP, 0.5 unit of Taq polymerase (Promega, Madison, WI, USA). The PCR protocol was as follows: one cycle of 5 min at 95 °C; 40 s at 94 °C; 45 s at 55 °C; thirty-five 1-min cycles at 72 °C; one 10-min cycle at 72 °C. PCR products were resolved on 1.0% agarose gel and visualized by staining with ethidium bromide. PCR products were purified and sequenced using MACROGEN Inc. (Seoul, Republic of Korea). Sequence assembly and editing were performed using Sequencher® version 5.2 (sequence analysis software, Gene Codes Corporation, Ann Arbor, MI, USA).

3.3. Result and discussion

3.3.1. Isolation and identification of compost fungi

The heat map in Table 9 represents the amount of CFUs observed during the monitoring period at different incubation temperatures. The order of magnitude observed of CFUs per gram of compost rose from 1×10^4 CFU/g to 3×10^5 CFU/g during a maturation period of 21 days.

Samples		TQ	1D	7D	14D	21D
Incubation temperature	25 °C					
	37 °C					
	45 °C					
	50 °C					

Table 9 Heat map of fungal concentration at different temperatures.

The isolated strains belong to 45 different species ascribable to 25 genera. The Table 10 gives an overview of the occurrence of different strains during the maturation period, highlighting the presence of some species in several steps of maturation. The most frequently occurring fungal species (isolated in all five samples) were *Scedosporium apiospermum* (Sacc.) Sacc. ex Castell. & Chalm., *Thermomyces dupontii* Griffon & Maublanc (=*Talaromyces thermophilus*) and *Thermomyces lanuginosus* Tsikl. Strains of *Aspergillus chevalieri* (Mangin) Thom. & Church, *A. terreus* Thom, and *Talaromyces trachyspermus* (Shear) Stolk & Samson were isolated in 4 of the 5 samples. They were followed by six species that were isolated in three of the five samples: *Aspergillus fumigatus* Fresen., *Malbranchea cinnamomea* (Lib.) Oorschot & de Hoog, *Rasamsonia emersonii* (Stolk) Houbraken & Frisvad (=*Talaromyces emersonii*), *Scedosporium aurantiacum* Gilgado, Cano, Gené & Guarro and *Scopulariopsis brevicaulis* (Sacc.) Bainier.

Species	*TQ*	*1D*	*7D*	*14D*	*21D*
Acaulium acremonium			+		
Aspergillus chevalieri	+	+	+	+	
Aspergillus fumigatus			+	+	+

Aspergillus nidulans				+	+
Aspergillus niger				+	+
Aspergillus pseudoglaucus	+		+		
Aspergillus quadrilineatus					+
Aspergillus terreus	+		+	+	+
Chaetomium thermophilum				+	
Chaetomium uniapiculatum		+	+		
Enterocarpus grenotii		+			
Lichtheimia corymbifera				+	+
Malbranchea cinnamomea			+	+	+
Melanocarpus albomyces			+		
Microascus paisii		+			
Microascus restrictus	+				
Monascus ruber	+	+			
Mortierella wolfii					+
Mycothermus thermophilus		+			+
Paecilomyces variotii	+		+		
Penicillium crustosum				+	+
Rasamsonia emersonii	+	+	+		
Rhizomucor miehei				+	+
Rhizopus microsporus				+	
Rhizopus oryzae		+			
Scedosporium apiospermum	+	+	+	+	+
Scedosporium aurantiacum		+	+	+	
Scedosporium dehoogii		+		+	

Scedosporium minutisporum	+				
Scedosporium prolificans			+		
Scopulariopsis brevicaulis		+	+	+	
Talaromyces trachyspermus	+	+		+	+
Talaromyces tratensis		+			
Talaromyces wortmannii		+			
Thermoascus aurantiacus	+		+		
Thermomyces dupontii	+	+	+	+	+
Thermomyces lanuginosus	+	+	+	+	+
Trichoderma longibrachiatum					+

Table 10 Fungal taxa isolated from compost samples.

Our data, collected in an active plant, confirmed the presence of thermotolerant and thermophilic fungi. During the maturation period, the number of CFUs increased, biodiversity rose, while mycobiota evolved from unequal distribution among species at the beginning of maturation to fairer distribution between species at maturity. Despite this evolution, we confirmed that the mycobiota were rather stable from a qualitative point of view. However, several potentially pathogenic species emerged in all phases of the cycle, and, for this reason, the periodic monitoring of the concentrations of certain species during the production cycle is necessary. For example, in our case, the opportunistic pathogenic fungal species *A. fumigatus*, *A. terreus* and *S. apiospermum* were found in high concentrations. Analyzing our data, we observed an increase in the number of CFUs during the maturation period up to 10 times in the final product compared to the digestate and the mixed product sampled after one day of maturation (Table 9). The high temperatures at the beginning of the process probably temporarily inhibited germination that restarted once the pile reached a temperature of around 45°C. Results concerning the isolated fungi are consistent with previously published data on compost samples (Anastasi et al. 2005; Jurado et al. 2014; Haas et al. 2016). We isolated several thermophilic fungi capable of producing enzymes such as amylase, xylanase, phytase and chitinase, among which were *Thermomyces dupontii* (=*Talaromyces thermophilus*), *T. lanuginosus*, and *Thermoascus aurantiacus* that degrade the woody components in the compost. However, opportunistic pathogenic species were also found in the samples. The most recurrent genus was *Scedosporium*. In particular, *Scedosporium apiospermum* is found in all samples, including in the

mature compost. Five other species, *S. aurantiacum, S. brevicaulis, S. dehoogii, S. minutisporum,* and *S. prolificans,* were found in decreasing numbers. Moreover, a strong presence of *Aspergillus fumigatus* was detected in the mature samples. The high temperatures, therefore, shaped the mycobiota during compost maturation. Only some specialized species can survive and proliferate in these particularly hostile conditions. Our results agreed with those of López González et al. (Jurado et al. 2014). In fact, we found some predominant species throughout the process (resident mycobiota) in contrast to others occasionally found (transient mycobiota).

3.4. Conclusion

The fungal community of compost samples taken during the ripening of the product at five different stages in a composting plant. The strains in axenic culture were identified at the species level using molecular analysis. Results confirmed high biodiversity of the fungal component that tends to stabilize during the compost-maturation process. Results showed a high presence of thermophilic and thermotolerant species that, if properly managed, could improve the composting process. However, a significant presence of species potentially harmful to the health of workers and end-users should also be noted.

3.5. References

Anastasi A, Varese GC, Filipello Marchisio V. 2005. Isolation and identification of fungal communities in compost and vermicompost. Mycologia. 97(1):33–44.

Antunes LP, Martins LF, Pereira RV, Thomas AM, Barbosa D, Lemos LN, Silva GMM, Moura LMS, Epamino GWC, Digiampietri LA. 2016. Microbial community structure and dynamics in thermophilic composting viewed through metagenomics and metatranscriptomics. Sci Rep. 6(1):1–13.

Atif K, Haouas A, Aziz F, Jamali MY, Tallou A, Amir S. 2020. Pathogens evolution during the composting of the household waste mixture enriched with phosphate residues and olive oil mill wastewater. Waste and Biomass Valorization. 11(5):1789–1797.

Bonito G, Isikhuemhen OS, Vilgalys R. 2010. Identification of fungi associated with municipal compost using DNA-based techniques. Bioresour Technol. 101(3):1021–1027.

Cerda A, Artola A, Font X, Barrena R, Gea T, Sánchez A. 2018. Composting of food wastes: Status and challenges. Bioresour Technol [Internet]. 248:57–67. https://doi.org/10.1016/j.biortech.2017.06.133

Crous PW, Cowan DA, Maggs-Kölling G, Yilmaz N, Larsson E, Angelini C, Brandrud TE, Dearnaley JDW, Dima B, Dovana F. 2020. Fungal Planet description sheets: 1112–1181. Persoonia-Molecular Phylogeny Evol Fungi.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. [place unknown].

Etesami H, Hemati A, Alikhani HA. 2019. Microbial Bioconversion of Agricultural Wastes for Rural Sanitation and Soil Carbon Enrichment. In: Microb Interv Agric Environ. [place unknown]: Springer; p. 179–204.

Franceschini S, Chitarra W, Pugliese M, Gisi U, Garibaldi A, Lodovica Gullino M. 2016. Quantification of Aspergillus fumigatus and enteric bacteria in European compost and biochar. Compost Sci Util. 24(1):20–29.

Franke-Whittle IH, Klammer SH, Insam H. 2005. Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. J Microbiol Methods. 62(1):37–56.

Fuchs JG. 2010. Interactions between beneficial and harmful microorganisms: from the composting process to compost application. In: Microbes Work. [place unknown]: Springer; p. 213–229.

Gams W, Aa HA van der, Plaats-Niterink AJ van der, Samson RA, Stalpers JA. 1987. CBS course of mycology. Baarn: Centraalbureau voor Schimmelcultures.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol. 2(2):113–118.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol. 61(4):1323–1330.

Haas D, Lesch S, Buzina W, Galler H, Gutschi AM, Habib J, Pfeifer B, Luxner J, Reinthaler FF. 2016. Culturable fungi in potting soils and compost. Sabouraudia. 54(8):825–834.

Jurado MM, Suárez-Estrella F, Vargas-García MC, López MJ, López-González JA, Moreno J. 2014. Increasing native microbiota in lignocellulosic waste composting: Effects on process efficiency and final product maturity. Process Biochem [Internet]. 49(11):1958–1969. http://dx.doi.org/10.1016/j.procbio.2014.08.003

Kane BE, Mullins JT. 1973. Thermophilic fungi in a municipal waste compost system. Mycologia. 65(5):1087–1100.

Laich F, Andrade J. 2016. Penicillium pedernalense sp. nov., isolated from whiteleg shrimp heads waste compost. Int J Syst Evol Microbiol. 66(11):4382–4388.

Langarica-Fuentes A, Handley PS, Houlden A, Fox G, Robson GD. 2014. An investigation of the biodiversity of thermophilic and thermotolerant fungal species in composts using culture-based and molecular techniques. Fungal Ecol. 11:132–144.

Liu L, Wang S, Guo X, Zhao T, Zhang B. 2018. Succession and diversity of microorganisms and their association with physicochemical properties during green waste thermophilic composting. Waste Manag. 73:101–112.

López-González JA, Vargas-García M del C, López MJ, Suárez-Estrella F, Jurado M del M, Moreno J. 2015. Biodiversity and succession of mycobiota associated to agricultural lignocellulosic waste-based composting. Bioresour Technol. 187:305–313.

Onwosi CO, Igbokwe VC, Odimba JN, Eke IE, Nwankwoala MO, Iroh IN, Ezeogu LI. 2017. Composting technology in waste stabilization: On the methods, challenges and future prospects. J Environ Manage. 190:140–157.

Partanen P, Hultman J, Paulin L, Auvinen P, Romantschuk M. 2010. Bacterial diversity at different stages of the composting process. BMC Microbiol. 10(1):1–11.

Di Piazza S, Houbraken J, Meijer M, Cecchi G, Kraak B, Rosa E, Zotti M. 2020. Thermotolerant and thermophilic mycobiota in different steps of compost maturation. Microorganisms. 8(6):1–9.

Reyes-Torres M, Oviedo-Ocaña ER, Dominguez I, Komilis D, Sánchez A. 2018. A systematic review on the composting of green waste: Feedstock quality and optimization strategies. Waste Manag. 77:486–499.

Samson RA, Houbraken J. 2011. Phylogenetic and taxonomic studies on the genera Penicillium and Talaromyces. Stud Mycol. 70:iii.

Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B. 2019. Food and indoor fungi. [place unknown]: Westerdijk Fungal Biodiversity Institute.

Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CHW, Perrone G, Seifert KA,

Susca A, Tanney JB, et al. 2014. Phylogeny, identification and nomenclature of the genus Aspergillus. Stud Mycol. 78(1):141–173.

Song C, Li M, Jia X, Wei Z, Zhao Y, Xi B, Zhu C, Liu D. 2014. Comparison of bacterial community structure and dynamics during the thermophilic composting of different types of solid wastes: anaerobic digestion residue, pig manure and chicken manure. Microb Biotechnol. 7(5):424–433.

Thanh VN, Thuy NT, Huong HTT, Hien DD, Hang DTM, Anh DTK, Hüttner S, Larsbrink J, Olsson L. 2019. Surveying of acid-tolerant thermophilic lignocellulolytic fungi in Vietnam reveals surprisingly high genetic diversity, Sci. Rep. 9 (2019) 1–12.

Tian X, Yang T, He J, Chu Q, Jia X, Huang J. 2017. Fungal community and cellulose-degrading genes in the composting process of Chinese medicinal herbal residues. Bioresour Technol [Internet]. 241:374–383. http://dx.doi.org/10.1016/j.biortech.2017.05.116

Wang C, Dong D, Wang Haoshu, Müller K, Qin Y, Wang Hailong, Wu W. 2016. Metagenomic analysis of microbial consortia enriched from compost: new insights into the role of Actinobacteria in lignocellulose decomposition. Biotechnol Biofuels. 9(1):1–17.

Wang X, Pan S, Zhang Z, Lin X, Zhang Y, Chen S. 2017. Effects of the feeding ratio of food waste on fed-batch aerobic composting and its microbial community. Bioresour Technol. 224:397–404.

Wei H, Tucker MP, Baker JO, Harris M, Luo Y, Xu Q, Himmel ME, Ding S-Y. 2012. Tracking dynamics of plant biomass composting by changes in substrate structure, microbial community, and enzyme activity. Biotechnol Biofuels. 5(1):1–14.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc a Guid to methods Appl. 18(1):315–322.

Chapter 4
BIOREACTORS AND MYCOREACTORS

4. Bioreactors and mycoreactors

4.1. Introduction

Nowadays, extreme environments are not only natural ones, like volcanoes, sea depths, anoxic lakes or Antarctica. Also treatment or production plants built by human are extreme: toxic substances, temperature conditions and pH low or high, competition with other microorganisms, absence of oxygen, sudden changes in humidity or concentration of nutrients, presence of pharmaceutical residues like antibacterial or antifungal, etc.

Searching for new microorganisms in plants could greatly expand the collections of extremely resistant strains. In recent years, more and more studies have been performed looking for bacterial communities in plants (Bramucci & Nagarajan 2000). The most common procedure involves the inoculation of a sample of wastewater into a solid minimal culture medium containing a simple carbon source and salts, the PCR amplification of 16S rRNA of the strains obtained by comparing the sequences obtained with the data present in GenBank (Singh 2006).

The plants are perfect environments also to look for new fungal strains: in the near future it is foreseen that studies in this area will increase. Two case studies reported in this thesis concern the isolation of fungal strains from wastewater: from a landfill leachate treatment plant and a composting plant. The disparity between the studies carried out on other microorganisms compared to filamentous fungi also concerns the construction of plants: live or immobilized biomasses used in the biological treatments of wastewater are generally not constituted only by filamentous fungi.

For this reason, in this thesis, plants will be treated not only as a source of new potentially useful fungi but also as technologies to be tested on these organisms. Thanks to the collaboration with the industrial chemistry laboratory of the University of Genoa, part of this thesis work concerned the first stages of construction of a bioreactor with filamentous micro-fungi. This kind of reactors has not been developed yet on a large scale with micro-fungi.

This chapter will provide an overview of the bioreactors that currently use aerobic organisms to treat wastewater. These systems are also called aerobic biological treatments and they are designed in such a way to optimally control the growth of biomass by providing it with oxygen and wastewater (substrate) to consume. To do this, technology and research have been running and chasing each other for over a hundred years, and there is still a lot to do. Nowadays, most of the aerobic biological treatments to treat wastewater use a pool of microorganisms that includes bacteria, filamentous fungi, yeasts, protozoa, etc. Only few systems use fungi alone on a large scale. An overview on myco-

reactors, reported in the last part, is useful to contextualize the case study reported in the next chapter, which can be framed as a pilot study for the development of a new type of myco-reactors.

Despite the water is present on Earth planet in a large amount, only a small fraction of it can be exploited by living organisms. Mankind use much more water than other living beings because in addition to its survival, it is used for agriculture, breeding, industry and as an energy source. Environmental scientists from all over the world are working to solve one of the main problems of the modern civilization: recover and purify wastewaters.

The biological treatment processes are part of the wastewater purification process chain that begins with the sewage system and ends with the release of purified water into the environment (Vesilind 2003). These processes, often coupled with physical/chemical treatments, allow to reduce the content of many organic substances by simply using the metabolic capabilities of a pool of microorganisms selected over the years (Bashaar 2004).

Each biological treatment must necessarily supply the microorganisms involved in the treatment with oxygen and nutrients: this can be done with different methods and systems.

For a hundred years, activated sludge is used in civil wastewater treatment plants. The microorganisms, thanks to the supplied oxygen and agitation, form initially flakes and then a sludge. Biomass of the sludge actively removes organic substances consuming them (Wanner 1994). At the end of the process, the sludge is separated by filtration or sedimentation to be eventually reused. Among the used microorganisms there are certainly also filamentous fungi: the mycelium can influence the formation of the flake acting as a network for bacteria and protozoa (Ni & Yu 2012).

More recently the adhered biomass system has been development: in this technology the microorganisms form a biofilm on an external support, instead of forming flakes. Also in this case the oxygen supply can take place in different ways (Downing & Nerenberg 2008).

Different types of bioreactors have also been tested on fungal cultures, both yeast and filamentous. In this chapter, the classic bioreactors used in wastewater treatment are presented together with the ones that are using filamentous fungi. The study of fungi to treat wastewater is fairly recent, in fact the first studies concerned the negative effect of fungi in wastewater plants -for example about the excessive development of mycelium in activated sludge- rather than their exploitation (Kyzas & Matis 2014). Generally, the filamentous fungi are linked in most cases to bulking or foaming phenomena because the mycelium creates a remarkable, and sometimes difficult to manage, biomass in aqueous environments. The problem is linked above all to filamentous fungi and concerns the different structure that the mycelium can develop in the aqueous environment (Zhang & Zhang 2016).

All the literature related to the negative aspects of fungi in purification plants has also indirectly investigated the morphology, metabolism and kinetics of growth and development of these organisms. This is the reason why it has been an excellent basis for more recent studies that are instead investigating the possibility of using fungi in bioreactors. It is certainly a starting point to know some of the organisms with incredible degradation capacity in all types of wastewater: these organisms can be exploited to build more suitable bioreactors. For this reason, the first part of the chapter will focus on traditional treatments that involve the use of bacteria or mixed microbial communities, such as those of activated sludge. The bioreactors that have been created and used for fungi will then be described, with particular attention to the filamentous fungi, object of the research reported in the case study below.

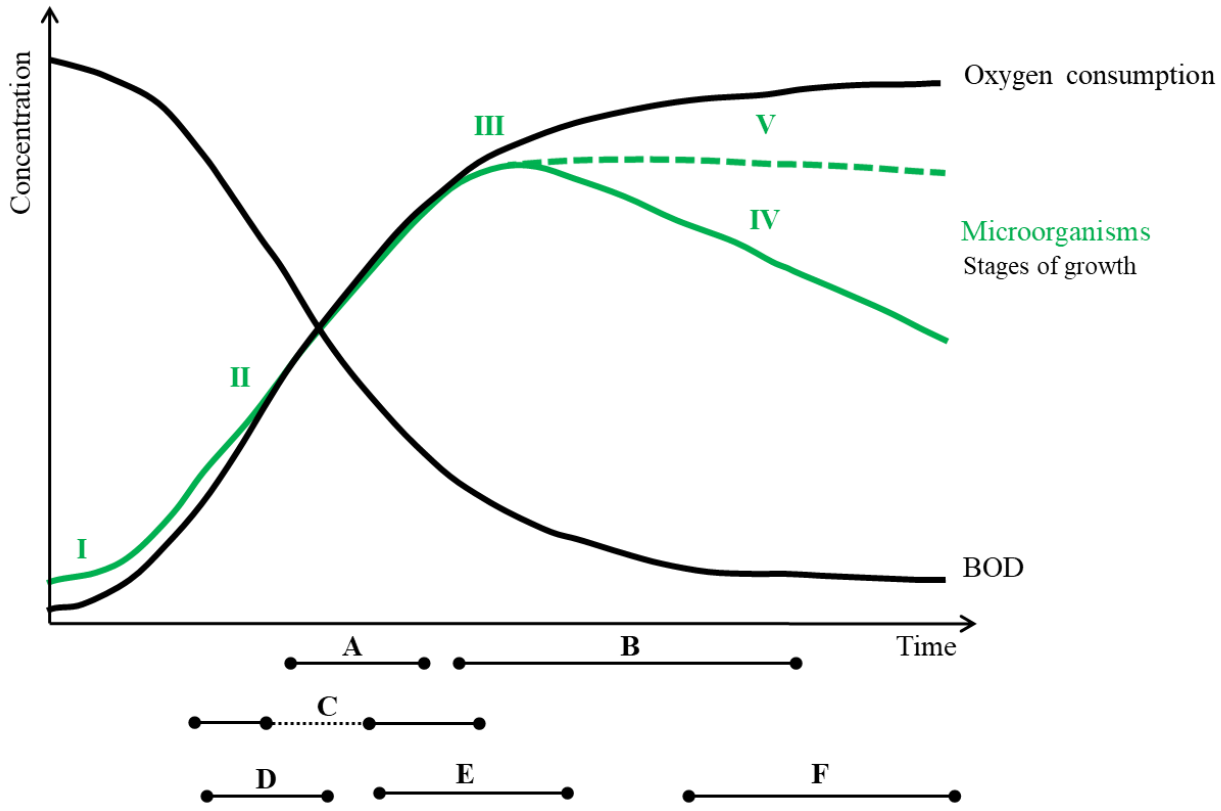
4.2. Wastewater biological treatment

In biological processes, the ability of various microorganisms to assimilate the complex organic substances present in the wastewater is exploited. The substances are separated from the liquid medium and degraded to simpler and less dangerous ones. About 100 years ago, the first biological purification systems were built, they operated thanks to the microfauna of activated sludge. Only 50 years later the first bioreactors were developed as a valid alternative to the now widespread activated sludge system, which however remain widely used for civil waste even today (Sehar & Naz 2016). In order to be able to create a biological treatment system, the pollutants must be biodegraded or at least retained by microorganisms, which can be more or less easily separable from the purified effluent after the process.

In conventional biological wastewater treatment plants, the microbial energy source determines the dominant microorganism in the mixed liquor (Sankaran et al. 2010). The microorganisms exploited to degrade organic matter can be anaerobic or aerobic, consequently one can intervene on the development of one of the two types by acting on the environmental conditions that can favor the action of one type or the other. For example, if an aeration system is active, the aerobic microfauna will be dominant, while in plants without aeration systems and closed the anaerobic microfauna will be favored.

The most efficient and fastest biological treatments are the aerobic ones, but they also require more energy. Several technologies are available, they differ in the methods of aeration, mixing and growth of the biomass in the wastewater. This thesis will include only aerobic biological treatments. Graph 1 shows the trend of the growth curve of microorganisms in an aerobic biological treatment with respect to oxygen consumption and substrate degradation, expressed as BOD (biological oxygen demand). Each type of treatment system exploits a particular interval of the growth curve and is

therefore different in terms of BOD consumption rate, oxygen consumption and sludge residence times.



Graph 1 Growth curve of microorganisms in an aerobic biological treatment. The letters indicate the phases generally used by the different purification systems: A) percolating filters or double alternating filtration, B) conventional percolating filters, C) stabilization by contact, D) rapid activated sludge; E) conventional activated sludge, F) total oxidation. Adapted from (Bianucci & Bianucci 1998)

The growth curve shown in the *Graph 1* represent the average of the growth curves of the different species that make up the biomass. It can be divided in the following five stages:

I) Lag phase: in which there is no increase in the number of cells. The growth rate at this stage is very low. It is also known as induction, stationary or acclimatization phase.

II) Log phase: in which there is an exponential and unlimited growth, where the substrate or oxygen presence are not limiting factors. This situation is similar to the theoretical condition. It is also known as logarithmic or exponential phase.

III) Stationary growth phase: in which a balance is established between cells and substrate supply. In this phase the cells are multiplying, but the growth rate remains stable. It is also known as limited growth or steady phase.

IV) Death phase: in which the number of cells is reduced due to the lack of substrate or oxygen. Is also known as decline phase (Bianucci & Bianucci 1998; Guo & Ngo 2008).

V) Equilibrium phase: if nutrients are being constantly fed through the reactor, the biomass will reach a state of equilibrium in which the rate of growth of cells will be proportional to the rate at which substrates are being fed into the system.

The Graph 1 gives another fundamental information: in aerobic processes it is necessary to supply the microorganisms with a substrate, indicated by the BOD and oxygen. BOD and COD together allows to observe how and at which speed the supplied substrate is consumed.

Aerobic biological treatments are usually divided into two large groups: suspended biomass systems and attached biomass systems. This division is based also on the different ways in which oxygen and substrate are made available to microorganisms (Sehar & Naz 2016).

4.2.1. Suspended biomass growth system

The suspended or dispersed biomass systems are secondary treatments characterized by the development of the microfauna directly in the liquid phase, where suitable aeration and mixing systems keep the concentrations of oxygen and nutrients fairly uniform (Bianucci & Bianucci 1998). A real ecosystem develops, in which different organisms prevail over others, forming a biomass known as activated sludge. In general, activated sludge must meet two quality criteria: maximum removal of organic substances in the shortest possible time and production of flakes with good sedimentation characteristics for traditional plants.
Downstream of the activated sludge treatment there is a clarifying system, traditionally based on sedimentation or more recently on filtration, to separate the produced sludge from the purified wastewater.
In this system, the substrate and oxygen are supplied in the same direction, as shown in the Figure 12. The concentration of oxygen and substrate in an activated sludge tank, therefore in a suspended biomass system, is maximum in the wastewater and decreases when it goes towards the center of the floc which is the point that is most likely to be anoxic. Oxygen can be supplied to the liquid wastewater by mechanical or diffused aeration means, using pure oxygen or air. Oxygen will be transferred from the gas phase to the liquid phase in variable quantities, until it reaches equilibrium, according to Henry's law (Ghaly & Kok 1988). The concentration of oxygen available to the microorganisms present on the surface of the sludge floc in contact with the liquid depends on the amount of oxygen that has dissolved. The limiting factor of dispersed biomass systems is the size of the plant: in order to have a microbial population capable of continuing to degrade the organic matter of the wastewater, the hydraulic retention time (HRT) must be low enough to allow microorganisms to multiply (Sehar & Naz 2016).

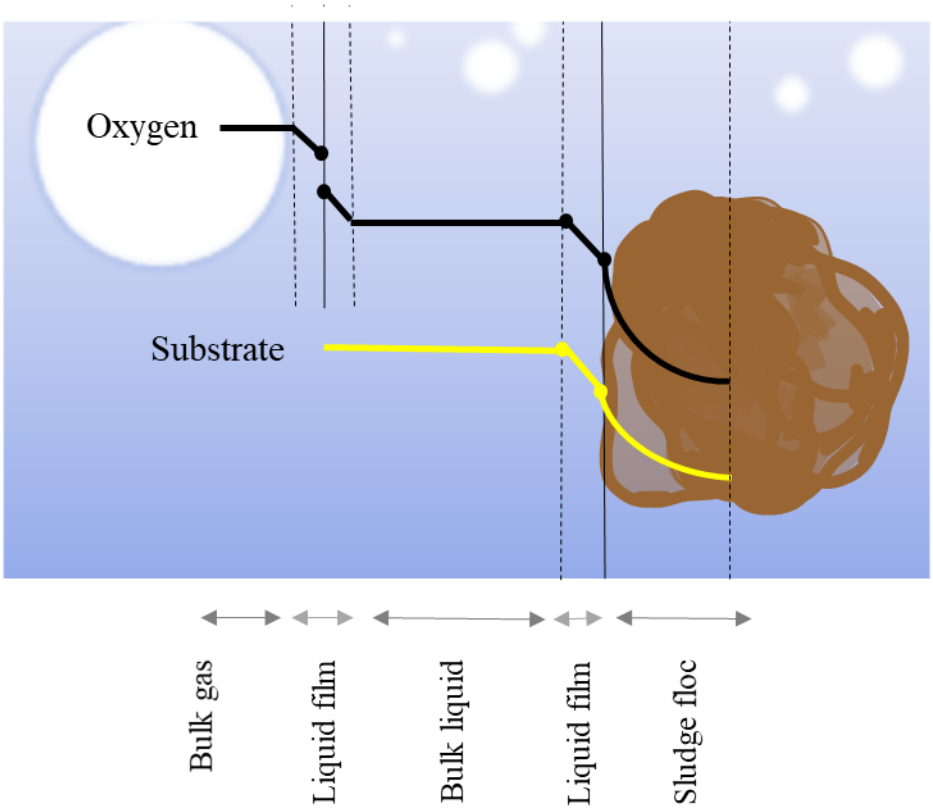


Figure 12 Activated sludge oxygen and substrate transfer.

4.2.2. Attached biomass growth system

In attached or fixed biomass systems the microorganisms do not develop and aggregate with each other, but an inert support is used for the growth of the biomass. The most widespread biomass system is the percolating filter: the sewage is percolated through a perforated surface made by a thick layer of pebbles and gravel. In this case the aeration takes place from below by natural or forced draft. These systems use inert substrates as a starting point for the development of the microfauna, which will no longer produce sludge flakes but a biological film. The biofilm attached to the discs assimilates the organic materials and nutrients in the wastewater. The concentration of oxygen is minimal in the biofilm layer closest to the support. An anaerobic process is triggered which results in the development of waste gas and ultimately the decrease in adhesion to the support, as shown in the Figure 13.

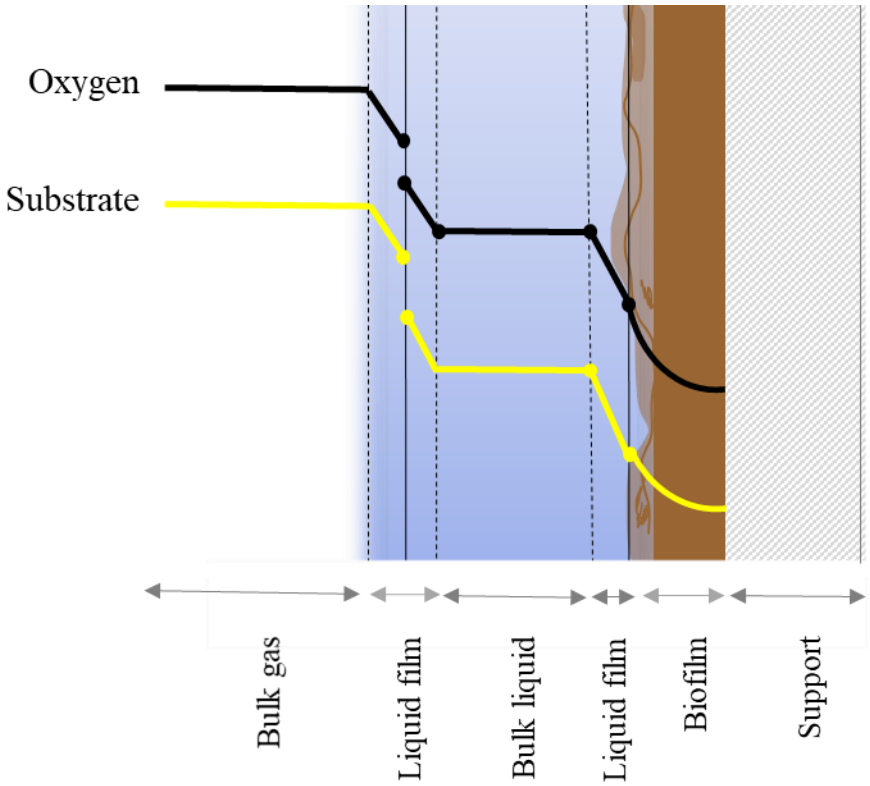


Figure 13 Trickling filters oxygen and substrate transfer.

The trickling filter was the first biomass treatment system used, followed by different systems such as rotating biological contactor or biodiscs. These systems rely on the growth of biofilms on an inert medium (Tchobanoglous et al. 2003). The supports for the growth of the biofilm are inert in traditional plants, their structure and roughness influence the biofilm development (Donlan 2002). Biotrickling filters are based on heterotrophic microorganisms. Since 30 years, biotrickling filters are used to treat complex wastewater containing H_2S, organo-sulfur compounds or NH_3, or to remove high concentrations of organic compounds, because pH and other reaction conditions can be controlled very well (Groenestijn 2005).

The rotating biological contactor is a fixed biomass system with a mobile support consisting of a series of discs that slowly rotate around a tree. The discs are in polystyrene or polyethylene and are partially immersed in the sewage, with the rotation the surface of the biodiscs is alternately in contact with the air and the liquid phase. Fluidized bed bioreactors (FBR) consist of a solid and a liquid phase: the bed of solid particles to which the biofilm adheres is fluidized by recirculating the liquid waste. They are used both for industrial applications and for the treatment of wastewater, the advantages of this system lie in the compact dimensions and in the ease with which it is possible to homogenize the wastewater, aerate it and enrich the biomass (Özkaya et al. 2019).

4.2.3. Membrane biofilm reactor (MBfR)

The Membrane Biofilm Reactor (MBfR) is an innovative wastewater treatment based on the transfer of a gas through a membrane to a biofilm. This biological treatment system uses attached biomass, but the support has an active function in supplying the biofilm as well as allowing it to adhere. The outer surface of the biofilm is in contact with a liquid phase containing the substrates to be metabolized. In a MBfR:

- the membrane separates the gaseous phase from the liquid phase;
- the biofilm, intended as an aggregation of living microorganisms, is adhered to the surface of the membrane which is in contact with the liquid phase;
- the compounds present in the liquid phase and the compounds that cross the membrane from the gas phase are biochemically transformed by the biofilm;
- the goal is the degradation of the pollutants present in the liquid phase.

Three types of MBfR reactors can be defined on the basis of the communities of microorganisms that are useful for biological treatment: air or oxygen MBfR, hydrogen MBfR, methane MBfR (Basile 2013). They have the same function: to create a flow of methane, oxygen or hydrogen through the membrane colonized by microorganisms to allow the biodegradation of the compounds present in the liquid. As shown in Figure 14, the relationship between biofilm, substrate and supplied gas is very different from the configuration of other systems. In this case the substrate and the gas are supplied from two opposite directions with respect to the membranous support and only the substrate gradient has the same trend. In fact, oxygen, methane or hydrogen is supplied to the biofilm directly from the gaseous phase, and Henry's law no longer regulates its transfer: the amount of gas available to the biomass depends on the transfer capacity of the membranous support and on the speed consumption by microorganisms.

MBfR technology is applicable to the removal of nitrogen and carbon compounds when oxygen is supplied, to the reduction of oxidized compounds when hydrogen is supplied. It also boasts high efficiency in the use of gases, low energy consumption and a reduced volume of reactors compared to traditional methods (Martin & Nerenberg 2012). The membranes used for MBfRs can be microporous, dense or composite.

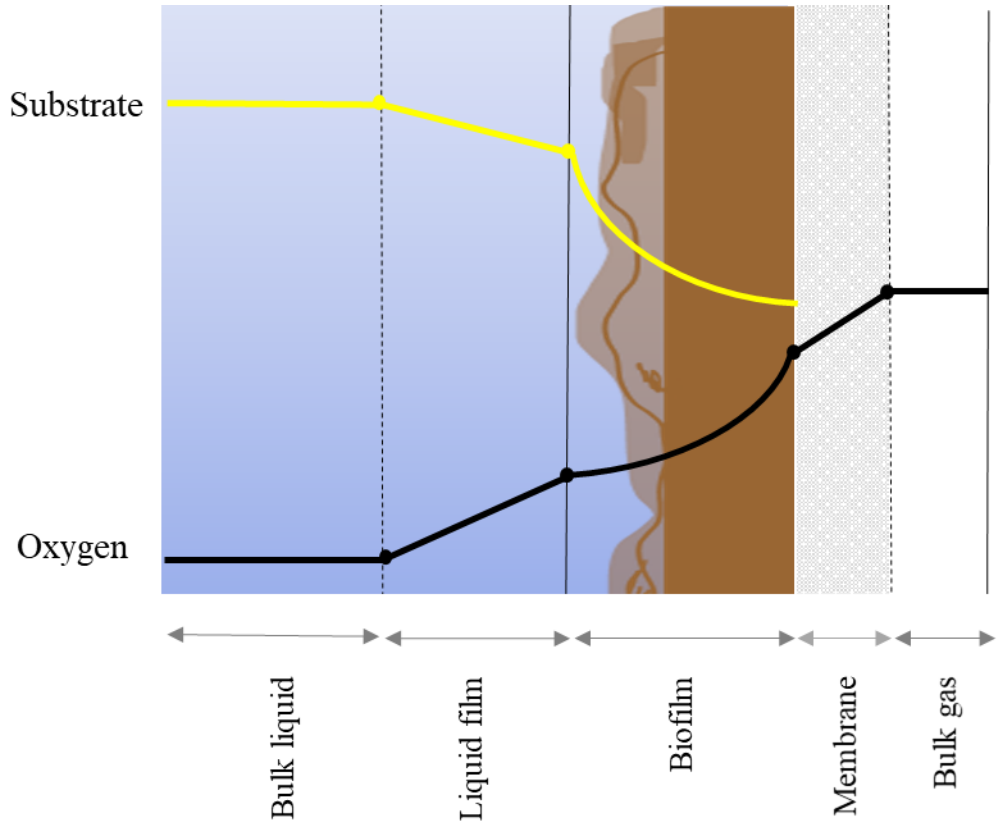


Figure 14 The operating principle of a MBfR-oxygen based system.

In the microporous hydrophobic membranes, the gas is distributed on the surface of the membrane in contact with the wastewater through the pores in which the gases can easily diffuse. If the gas pressure exceeds a certain threshold value, the membrane tends to release bubbles from its surface. Using bubble-less pressure allows to increase a stable biofilm on the membrane surface. In MBfRs-oxygen based system the oxygen transfer capacity of microporous membranes is higher than dense membranes (Martin & Nerenberg 2012).

In dense membranes without pores the oxygen passes through the membrane thanks to a solution-diffusion mechanism. Oxygen is able to dissolve in suitable materials much better than nitrogen so it can diffuse towards the surface of the membrane in contact with water. Increasing the air pressure increases the amount of oxygen solubilized in the membrane material and therefore a greater amount is transferred (Basile 2013). A popular dense membranes material is the silicone (Casey et al. 1999a).

It is possible to choose to use also composite membranes: they can be in polyethylene or polyurethane. The dense layer can be found only inside or between an internal and external layer of microporous membrane (Downing & Nerenberg 2008).

4.2.4. Membrane Areated Biofilm Reactor (MABR)

MBfR is often referred to as Membrane Areated Biofilm Reactor (MABR) when the gas supplied is air or pure oxygen, in order to promote an aerobic biological process. The MABR technology combines efficient aeration with a huge surface for biofilm growth. This aeration system is based on the transfer of air/oxygen through a membrane to a superficial biofilm in contact with a liquid phase. The substrates are contained in the liquid phase and the membranes become a support for the growth of the biofilm (Casey et al. 1999b; Casey et al. 1999a). In Table 11 the most common porous materials used in the last 20 years in MABR are described.

Membrane materials	***Pore size range (µm)***
Polypropylene (PP)	0,1 – 0,36
Polyvinylidene fluoride (PVDF)	0,2 - 3
Coal	0,1 - 10

Table 11 Microporous membranes for MABR (Casey et al. 1999b; Rittman 2006; Lu et al. 2020).

In the MABR based on microporous membrane the aerobic conditions are maintained by bubble-less air flow through the membranes: the oxygen is supplied in opposite direction among the substrates of the liquid phase. Moreover, the energy costs to aerate the tank and the sludge production are reduced and consequently the disposal costs because there is the possibility to provide oxygen directly to the biofilm without passing from the liquid phase.

In a MABR, the aim is to encourage the development of aerobic biofilming microorganisms on the surface of airy membranes immersed in a liquid to be purified. In many studies concerning MABR, non-specific membranes, normally they are used for microfiltration or ultrafiltration, are used for the adhesion of a biomass. A membrane can be used in a MABR if inside the module the pressure loss is minimal, the gas is distributed evenly across the membrane, the membrane resists degradation by microorganisms and has pores that always maintain good hydrophobicity to prevent flooding by waste water.

MABRs have only been studied in the last two decades, in particular regarding the gas passage efficiency, the biofilm surface/volume ratio and the performance over time. However, numerous problems remain unsolved, which have prevented their large-scale use (Muffler et al. 2014).

In the traditional attached biomass system, the microorganisms take root on an inert surface and receive oxygen and nutrients only from the aqueous matrix, until the thickness of the biofilm is such that it no longer allows an efficient passage of oxygen. Consequently, in the deeper layer they trigger anaerobic phenomena and the accumulation of the gaseous products of fermentation causes the detachment of the biofilm from the support. Instead, in a MABR, the biofilm receives the nutrients from the aqueous matrix and oxygen from the opposite side, through the membrane, so any development of anaerobic microorganisms will be on the outermost layer with respect to the support. The biofilm will never be lacking in oxygen and will not detach. Furthermore, the development of stratified populations of microorganisms will be favored, based on metabolic needs. The concentration of oxygen will be maximum in the biofilm in contact with the membrane and will reduce going towards the liquid phase, while the concentration of substrate will decrease in the opposite direction. Consequently, the species of microorganisms that make up the attached biomass will develop in layers: in contact with the liquid there will be anaerobic or facultative aerobic species, such as denitrifying agents, while in contact with the membrane there will be aerobic microorganisms, such as nitrifying agents (Downing et al. 2000).

MABRs have been developed to purify wastewaters that are difficult to treat with traditional biological processes. Most studies at laboratory scale have involved the use of synthetic wastewater by making activated sludge taken from traditional treatment plants take root on the membranes.

For this reason, companies that produce MABRs are starting to spread today, mainly to deal with the treatment of municipal sewage and wastewater by reducing the concentration of COD and nutrients. Few companies sell and produce large-scale MABR plants, all relatively young, among which we can mention *OxyMem Limited, Suez Technologies & Solutions* and *Fluence Corporation*.

In the Table 12 below some real recalcitrant wastewater tested in a MABR (Lu et al. 2020).

Refractory pollutants wastewater	***Main references***
Acetonitrile wastewater	(Li et al. 2008)
Industrial wastewater	(Wei et al. 2012)
High-concentration pharmarmaceutical intermediate wastewater	(Peng et al. 2015)
Oil-field wastewater	(Li et al. 2015)
Formaldehyde wastewater	(Mei et al. 2019)

O-aminophenol wastewater	(Tian et al. 2019)
Phenolic compounds in high saline wastewater	(Tian et al. 2020)

Table 12 Real wastewater tested on MABR.

Currently there are no membranes specifically created for MABRs: during the development of this technology, materials and modules of other systems were used, for example porous membranes normally used in filtration systems.

The biological reactions that are exploited in this type of plant are nitrification and denitrification. The oxygen concentration gradient created favours the development of specific microbial communities from an activated sludge starter formed in a traditional plant. Each of these studies has different microorganisms, mostly bacterial communities, that adhere to the biofilm. Often as starter for the biofilm development an activated sludge in which are present bacteria and fungi is used. At the moment they have not yet been developed on myco-MABR.

The development of MABR reactor starts from the assumption that the microorganisms blocked in the biofilm have a greater resistance to toxic substances and to stress, as shown for bacteria and for active sludge organisms (Cole et al. 2002; Murphy & Casey 2013; Pellicer-Nàcher & Smets 2014).

Several studies concern the performances of the reactors, where the degradation capacity is improved compared to traditional biological treatments. The study by Wei et al. (2012) can be considered a reference to describe the development of a pilot scale MABR for the treatment of complex wastewater. The membranes used are made of dense and hydrophobic polypropylene and the experimentation lasted less than a year. As often happens in MABR trials, the organisms used as biodegraders were taken from the activated sludge of another already functioning plant and adapted in the new pilot plant. The inoculation of activated sludge generated the biofilm on the membranes in a month. Initially a nutritional solution of carbohydrates and proteins was provided, subsequently this solution was gradually replaced by a real mixed pharmaceutical wastewater. The removal efficiency of COD and nitrogen - generally considered as functional parameters of a biological treatment - has decreased with the change of nutrient solution, but slowly a new balance has been established. Finally, the study showed a biofilm thickness of 0,5 mm stable in two month and a removal capacity of 90% of COD and 98% of ammonia, despite the complexity of the organic substances present in the mixed pharmaceutical wastewater (Wei et al. 2012).

The development of the biofilm in a MABR and its stability over time depend on several factors: the amount of oxygen, the structure of the plant and the membranes used, the type of wastewater the biofilm is in contact with and the ability to adapt of the starting inoculum (Syron & Casey 2008). It is also influenced by the flow rate of the wastewater, as explained in the Downing et al. (2000) study which used a bacterium as a model obligate aerobic organism and acetate as a carbon source. A laboratory-scale MABR with silicone membranes was constructed (Casey et al. 1999a). In the initial phase, the batch condition was maintained and a nutrient solution was provided several times in order to stimulate the development of the biofilm. The batch condition allows to not wash away the bacteria that are in the early stages of biofilm development with the flow of the liquid. In less than a day, the irregular biofilm completely invaded the surface of the membranes and then gradually increased throughout the experiment (two weeks). Tests with variable flow rates have shown that the thickness of the biofilm tends to be greater when the flow rate is lower (Downing et al. 2000). To develop a new type of bioreactor, it is necessary to pass at least four study phases ranging from the realization of a laboratory-scale system to the production of a large-scale reproducible inoculum.

4.3. Wasterwater mycoreactor

A bioreactor refers to any manufactured system that supports a biological treatment, it is possible to develops a bioreactor using a specific microorganism, like bacteria or fungi (Muffler et al. 2014). Generally wastewater can be treated using a community of microorganisms like protozoa, bacteria and fungi, but it is also possible to build a fungi-based bioreactor to treat wastewater using only fungi as biodegrading agents (Kumar et al. 2011). Fungal bioreactors are also known as myco-reactors. Every year new waste and wastewater are being generated: probably in the future fungi will play a greater role in the transformation and detoxification of hazardous wastewater. Wastewaters from different processes (fine chemical, agrochemical, pharmaceutical and textile industries) contain chemical mixtures that are minimally held back by conventional wastewater treatment. Many of these contaminants are not even compatible with bacterial degradation because of xenobiotic structures, so waiting for the evolution of specific catabolic pathways in bacteria seems like a less realistic option than using the nonspecific nature of fungal metabolism (Harms et al. 2011).

The main applications of fungi in myco-reactors are: myco-filtration, use of active substances and myco-sorption (Noman et al. 2019). The use of fungi in filtration refers to gray water or water used for agriculture, mycofilm is able to filter bacteria and pollutants and release antibacterial substances. The most used fungi for this type of treatment are basidiomycetes. A study was done on the synthetic storm water to remove *E. coli* using myco-filtration with the mycelium of *Stropharia rugosa-annulata* with an efficiency of 90% (Taylor et al. 2015),

Application of active substances is defined when in myco-reactors it is not the mycelium or an enzyme that is responsible for the action, but another substance. As an example, the study by Hussain et al. has demonstrated how the use of some fungi in wastewater affected by algal bloom can solve the problem, releasing substances that act as algicides into the water (Hussain et al. 2015).

Myco-sorption is a type of treatment that involves the use of fungal biomass. In particular, the functional groups of fungal cells act on a specific pollutant, blocking it as a physical-chemical process or degrading it as a biological process. In this case chitin, mannans, glucans and other fungal functional group allow the remotion of azo-dyes and heavy metals. The most commons genera of fungi used in this biological treatment include *Trichoderma*, *Alternaria*, *Aspergillus*, *Rhizopus* and *Penicillium* (Noman et al. 2019).

Like other biological reactors, myco-reactors can operate in aerobic or anaerobic conditions and the operation can be batch, semi-batch, sequencing batch or continuous. Myco-reactors can be classified by the fungal growth system: biofilmed (or immobilized) and suspended biomass.

4.3.1. Attached fungal growth system

Myco-reactors based on attached growth include trickling filters, rotating biological contactors, up flow fixed-film reactors, fluidized-bed reactors.

Fungal-based trickling filters take up hydrophobic compounds from the gas phase more easily than bacterial biofilm and are more resistant to dry and acid conditions (Groenestijn 2005). Many researches demonstrated the removal of complex substance. Fungal-based trickling filters can be used to remove hydrophobic VOC from sludge such as toluene, xylene, ethylbenzene ethane, 1,3-butadiene (Kennes et al. 1996; Van Groenestijn et al. 2001), styrene (Cox 1995), chlorobenzene, hexane and methanol (Cheng et al. 2016). Fungal biofilm can create clogging problems in biofilters, it can be treated by:

- physical methods, by modifying the injected air pressure or the wastewater rate;
- chemical methods, by regulating the amount of nutrients supplied or by modifying the humidity;
- biological methods, by inoculating fungi predators. In this case, there will be a reduction in biofilm and a consequent decrease in clogging (Zhang et al. 2020).

The biodiscs of the trickling filter are mounted on a fixed support: the wastewater flows through them. While in the rotating biological contactor, the biodiscs leave part of the biofilm exposed to the air by rotating alternately. They have been developed with a fungal biological component to treat different types of wastewater: micropollutants (Cruz del Álamo et al. 2020), surfactant solubilized polycyclic

aromatic hydrocarbons (Zheng & Obbard 2002), bleach or colored industrial waste (Van Driessel & Christov 2001).

Fungal fluidized bed reactors are often made with white rot fungi and, in general, may have advantages for the treatment of industrial wastewater. This has been demonstrated in fluidized bed bioreactor based on filamentous fungi in terms of removal of recalcitrant compound like micropollutant, textile dye with a high decolorization efficiency, pharmaceutical active compounds and endocrine disruptors from hospital wastewater (Özkaya et al. 2019). Fungal based fluidized bed bioreactor works even when conditions of sterility are not maintained: fungi are able to compete efficiently with indigenous microorganisms, resisting more easily to extreme conditions (Espinosa-Ortiz et al. 2016).

4.3.2. Suspended fungal growth system

Unlike traditional waste biomass waste treatments which are widely used and give excellent results especially for civil waste, the fungal based suspended bioreactors are difficult to manage. The mycelium, as explained above, when it is excessive in the sludge tanks, creates problems of foaming and bulking, drecreasing the treatment efficiency of the plant (Wanner 1994).

Mycelium can growth in three different forms in suspended growth bioreactors. The fungal cells can be free, aggregated dense or dispersed. This three different morphological forms can be defined suspended mycelia, pellets or clumps, respectively (Espinosa-Ortiz et al. 2016). However, the created biomass has a very different viscosity from the starting fluid and is difficult to separate from it. Furthermore, it is a complex system to be uniformly aerated and fungi tend to grow in the tubes and on the walls of the bioreactors. The advantages of slurry fungal reactor include better reuse of the biomass, low clogging and easy scale up. The disadvantages include limited transport of oxygen and nutrients, mostly inside the pellets or into fungal clumps aggregates, resulting in the formation of dead zones, and the difficulty in managing the variability of mycelium growth forms on the base of plant conditions (Pazouki & Panda 2000).

Better efficiency than fixed bed reactors was obtained for the removal of aromatic compounds and color from wastewater, compared with studies that used the same fungi but on different types of plants (Font et al. 2003; Font et al. 2006). The different forms in which the mycelium develops also influence quantitatively and qualitatively the production of the enzymes. Almost all the bioreactors of this type have remained at the laboratory scale for all the aforementioned limits. At laboratory level, the slurry fungal bioreactors have been shown to work for the treatment of olive-mill wastewater (Yesilada et

al. 1997), metal-containing effluents (Joshi et al. 2011), hospital and pharmaceutical effluents (Cruz-Morató et al. 2014), agricultural activities (Mir-Tutusaus et al. 2014).

4.3.3. MABR based on filamentous fungi

In the literature few examples are found of MABRs based on filamentous fungi and tested to treat pollutants in wastewater. The use of filamentous fungi in MABR is currently mainly limited to biotransformation and industrial processes for the production of enzymes and bioproducts.

In the study by Onken et al. the basidiomycete *Cystoderma carcharias* (Pers.) Fayod was used in an MABR for the gas phase biotransformation of citronellol into rose oxide, which is a fragrance chemical present in traces in essential plants oils like rose. In this study, the biomass was kept in agitation while oxygen was supplied from the membranes. The nutrient substrate was slowly replaced by citronellol which for the first time resulted in oxide rose, usually produced by photochemical, as biotransformation products. Hydrophobic, polypropylene and porous membranes (*Accurel PP/S6)* were used. Silicone membranes were also tested but had worse results due to the different ability to transfer gas in their dense and non-porous structure (Onken & Berger 1999).

Silicone membranes were used instead in the study by Palmerín-Carreño et al. (2014) for the growth of the mycofilm of *Botryodiplodia theobromae* (current name *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.) in a MABR developed for the biotransformation of (+)-valencene to (+)-nootkatone. Also in this case the product is a flavour of industrial interest, present in the orange essential oil. Nootkatone typically is producted through the biochemical or chemical oxidation of valencene from grapefruit. Again, MABR proved to be a good method to reduce the loss of volatile substances during a biotransformation process. In order to allow a mycofilm development, firstly a mineral medium based on sucrose was added, 7 days later orange essential oil was added as a source of (+)-valencene in liquid phase which is recirculated (Palmerín-Carreño et al. 2014).

The use of silicone membranes in MABRs was also investigated using the white rot fungus *Phanerochaete chrysosporium* Burds. In this work the production of high levels of lignin peroxidase has been proved using a repeated batch technique with *P. chrysosporium* (Venkatadri & Irvine 1993).

In the study by Esquivel-Viveros (2009) a synthetic wastewater containing hexafluorophosphate (PF_6) was treated in a MABR with *Fusarium sp.* mycofilm. PF_6 can be present in industrial wastewater because it is one of the components used for the construction of secondary batteries as lithium salt (lithium hexafluorophosphate). The nutrient medium based on sucrose and glucose as carbon source has been replaced with synthetic wastewaters after two weeks, in order to allow the

mycofilm development around the aerated membrane. After 28 days of incubation using a repeated batch technique 80% biodegradation was observed, this was the first time that biodegradation of PF_6 was demonstrated (Esquivel-Viveros et al. 2009).

4.4. References

Bashaar YA. 2004. Nutrients requirements in biological industrial wastewater treatment. African J Biotechnol. 3(4):236–238.

Basile A. 2013. Handbook of membrane reactors: fundamental materials science, design and optimisation. [place unknown]: Elsevier.

Bianucci G, Bianucci ER. 1998. Il trattamento delle acque inquinate. [place unknown]: HOEPLI EDITORE.

Casey E, Glennon B, Hamer G. 1999a. Oxygen mass transfer characteristics in a membrane-aerated biofilm reactor. Biotechnol Bioeng. 62(2):183–192.

Casey E, Glennon B, Hamer G. 1999b. Review of membrane aerated biofilm reactors. Resour Conserv Recycl. 27(1–2):203–215.

Cheng Y, He H, Yang C, Zeng G, Li X, Chen H, Yu G. 2016. Challenges and solutions for biofiltration of hydrophobic volatile organic compounds. Biotechnol Adv [Internet]. 34(6):1091–1102. https://www.sciencedirect.com/science/article/pii/S0734975016300751

Cole AC, Shanahan JW, Semmens MJ, LaPara TM. 2002. Preliminary studies on the microbial community structure of membrane-aerated biofilms treating municipal wastewater. Desalination. 146(1–3):421–426.

Cox HHJ. 1995. Styrene Removal from Waster Gas by the Fungus Exophiala Jeanselmei in a Biofilter. [place unknown]: Rijksuniversiteit te Groningen.

Cruz-Morató C, Lucas D, Llorca M, Rodriguez-Mozaz S, Gorga M, Petrovic M, Barceló D, Vicent T, Sarrà M, Marco-Urrea E. 2014. Hospital wastewater treatment by fungal bioreactor: removal efficiency for pharmaceuticals and endocrine disruptor compounds. Sci Total Environ. 493:365–376.

Cruz del Álamo A, Pariente MI, Martínez F, Molina R. 2020. Trametes versicolor immobilized on rotating biological contactors as alternative biological treatment for the removal of emerging concern micropollutants. Water Res [Internet]. 170:115313. https://www.sciencedirect.com/science/article/pii/S0043135419310875

Donlan RM. 2002. Biofilms: Microbial life on surfaces. Emerg Infect Dis. 8(9):881–890.

Downing LS, Nerenberg R. 2008. Effect of oxygen gradients on the activity and microbial community structure of a nitrifying, membrane-aerated biofilm. Biotechnol Bioeng. 101(6):1193–1204.

Downing LS, Nerenberg R, Casey E, Glennon B, Hamer G, Pellicer-Nàcher C, Smets BF, Cole AC, Shanahan JW, Semmens MJ, LaPara TM. 2000. Biofilm development in a membrane-aerated biofilm reactor: Effect of flow velocity on performance. Biotechnol Bioeng. 101(1–3):476–486.

Van Driessel B, Christov L. 2001. Decolorization of bleach plant effluent by mucoralean and white-rot fungi in a rotating biological contactor reactor. J Biosci Bioeng [Internet]. 92(3):271–276. https://www.sciencedirect.com/science/article/pii/S1389172301802619

Espinosa-Ortiz EJ, Rene ER, Pakshirajan K, van Hullebusch ED, Lens PNL. 2016. Fungal pelleted reactors in wastewater treatment: applications and perspectives. Chem Eng J. 283:553–571.

Esquivel-Viveros A, Ponce-Vargas F, Esponda-Aguilar P, Prado-Barragán LA, Gutiérrez-Rojas M, Lye GJ, Huerta-Ochoa S. 2009. Biodegradation of [bmim][PF6] using Fusarium sp. Rev Mex Ing Quim. 8(2):163–168.

Font X, Caminal G, Gabarrell X, Romero S, Vicent MT. 2003. Black liquor detoxification by laccase of Trametes versicolor pellets. J Chem Technol Biotechnol Int Res Process Environ Clean Technol. 78(5):548–554.

Font X, Caminal G, Gabarrell X, Vicent T. 2006. Treatment of toxic industrial wastewater in fluidized and fixed-bed batch reactors with Trametes versicolor: influence of immobilisation. Environ Technol. 27(8):845–854.

Ghaly AE, Kok R. 1988. The effect of sodium sulfite and cobalt chloride on the oxygen transfer coefficient. Appl Biochem Biotechnol. 19(3):259–270.

Groenestijn JW Van. 2005. Biotechniques for air pollution control : past , present and future trends. Proc Biotech Air Pollut Control [Internet]. 1978:3–12. https://core.ac.uk/download/pdf/61908782.pdf

Van Groenestijn JW, Van Heiningen WNM, Kraakman NJR. 2001. Biofilters based on the action of fungi. Water Sci Technol. 44(9):227–232.

Guo W, Ngo H-H. 2008. Specific Options in Biological Wastewater Treatment for Reclamation and Reuse. Encycl Life Support Syst.

Harms H, Schlosser D, Wick LY. 2011. Untapped potential: Exploiting fungi in bioremediation of hazardous chemicals. Nat Rev Microbiol [Internet]. 9(3):177–192.

http://dx.doi.org/10.1038/nrmicro2519

Hussain H, Jabeen F, Krohn K, Schulz B. 2015. CHEMISTRY Antimicrobial activity of two mellein derivatives isolated from an endophytic fungus. Med Chem Res [Internet].:2111–2114. http://dx.doi.org/10.1007/s00044-014-1250-3

Joshi PK, Swarup A, Maheshwari S, Kumar R, Singh N. 2011. Bioremediation of Heavy Metals in Liquid Media Through Fungi Isolated from Contaminated Sources. Indian J Microbiol. 51(4):482–487.

Kennes C, Cox HHJ, Doddema HJ, Harder W. 1996. Design and performance of biofilters for the removal of alkylbenzene vapors. J Chem Technol Biotechnol Int Res Process Environ Clean Technol. 66(3):300–304.

Kumar A, Bisht B., Joshi V., Dhewa T. 2011. Review on Bioremediation of Polluted Environment : A Management Tool. Int J Environ Sci. 1(6):1079–1093.

Kyzas GZ, Matis KA. 2014. Flotation of biological materials. Processes. 2(1):293–310.

Li P, Zhao D, Zhang Y, Sun L, Zhang H, Lian M, Li B. 2015. Oil-field wastewater treatment by hybrid membrane-aerated biofilm reactor (MABR) system. Chem Eng J [Internet]. 264:595–602. http://dx.doi.org/10.1016/j.cej.2014.11.131

Li T, Liu J, Bai R, Wong FS. 2008. Membrane-aerated biofilm reactor for the treatment of acetonitrile wastewater. Environ Sci Technol. 42(6):2099–2104.

Lu D, Bai H, Kong F, Liss SN, Liao B. 2020. Recent advances in membrane aerated biofilm reactors. Crit Rev Environ Sci Technol [Internet]. 0(0):1–55. https://doi.org/10.1080/10643389.2020.1734432

Martin KJ, Nerenberg R. 2012. The membrane biofilm reactor (MBfR) for water and wastewater treatment: Principles, applications, and recent developments. Bioresour Technol [Internet]. 122:83–94. http://dx.doi.org/10.1016/j.biortech.2012.02.110

Mei X, Guo Z, Liu J, Bi S, Li P, Wang Yong, Shen W, Yang Y, Wang Yihan, Xiao Y, et al. 2019. Treatment of formaldehyde wastewater by a membrane-aerated biofilm reactor (MABR): The degradation of formaldehyde in the presence of the cosubstrate methanol. Chem Eng J [Internet]. 372(January):673–683. https://doi.org/10.1016/j.cej.2019.04.184

Mir-Tutusaus JA, Masís-Mora M, Corcellas C, Eljarrat E, Barceló D, Sarrà M, Caminal G, Vicent T, Rodríguez-Rodríguez CE. 2014. Degradation of selected agrochemicals by the white rot fungus Trametes versicolor. Sci Total Environ. 500:235–242.

Muffler K, Lakatos M, Schlegel C, Strieth D, Kuhne S, Ulber R. 2014. Application of biofilm bioreactors in white biotechnology. Product biofilms.:123–161.

Murphy CD, Casey E. 2013. Biofi lm-catalysed transformation of organofl uorine compounds. 31(June).

Ni BJ, Yu HQ. 2012. Microbial products of activated sludge in biological wastewater treatment systems: A critical review. Crit Rev Environ Sci Technol. 42(2):187–223.

Noman E, Al-Gheethi A, Mohamed RMSR, Talip BA. 2019. Myco-Remediation of Xenobiotic Organic Compounds for a Sustainable Environment: A Critical Review. [place unknown].

Onken J, Berger RG. 1999. Biotransformation of citronellol by the basidiomycete Cystoderma carcharias in an aerated-membrane bioreactor. Appl Microbiol Biotechnol. 51(2):158–163.

Özkaya B, Kaksonen AH, Sahinkaya E, Puhakka JA. 2019. Fluidized bed bioreactor for multiple environmental engineering solutions. Water Res. 150:452–465.

Palmerín-Carreño DM, Verde-Calvo JR, Huerta-Ochoa S, Rutiaga-Quiñones OM. 2014. Bioconversion of (+)-nootkatone by botryodiplodia theobromae using a membrane aerated biofilm reactor. Rev Mex Ing Quim. 13(3):757–764.

Pazouki M, Panda T. 2000. Understanding the morphology of fungi. 22.

Pellicer-Nàcher C, Smets BF. 2014. Structure, composition, and strength of nitrifying membrane-aerated biofilms. Water Res. 57:151–161.

Peng L, Chen X, Xu Y, Liu Y, Gao SH, Ni BJ. 2015. Biodegradation of pharmaceuticals in membrane aerated biofilm reactor for autotrophic nitrogen removal: A model-based evaluation. J Memb Sci [Internet]. 494:39–47. http://dx.doi.org/10.1016/j.memsci.2015.07.043

Rittman BE. 2006. The membrane biofilm reactor: The natural partnership of membranes and biofilm. Water Sci Technol. 53(3):219–225.

Sankaran S, Khanal SK, Jasti N, Jin B, Pometto AL, Van Leeuwen JH. 2010. Use of filamentous fungi for wastewater treatment and production of high value fungal byproducts: A review. Crit Rev Environ Sci Technol. 40(5):400–449.

Sehar S, Naz I. 2016. Role of the Biofilms in Wastewater Treatment. Microb Biofilms - Importance Appl.

Syron E, Casey E. 2008. Membrane-aerated biofilms for high rate biotreatment: Performance

appraisal, engineering principles, scale-up, and development requirements. Environ Sci Technol. 42(6):1833–1844.

Taylor A, Flatt A, Beutel M, Wolff M, Brownson K, Stamets P. 2015. Removal of Escherichia coli from synthetic stormwater using mycofiltration. Ecol Eng [Internet]. 78:79–86. http://dx.doi.org/10.1016/j.ecoleng.2014.05.016

Tchobanoglous G, Burton FL, Stensel HD. 2003. Metcalf & Eddy wastewater engineering: treatment and reuse. Int Ed McGrawHill. 4:361–411.

Tian H, Hu Yanzhuo, Xu X, Hui M, Hu Yuansen, Qi W, Xu H, Li B. 2019. Enhanced wastewater treatment with high o-aminophenol concentration by two-stage MABR and its biodegradation mechanism. Bioresour Technol [Internet]. 289(May):121649. https://doi.org/10.1016/j.biortech.2019.121649

Tian H, Xu X, Qu J, Li H, Hu Y, Huang L, He W, Li B. 2020. Biodegradation of phenolic compounds in high saline wastewater by biofilms adhering on aerated membranes. J Hazard Mater [Internet]. 392(December 2019):122463. https://doi.org/10.1016/j.jhazmat.2020.122463

Venkatadri R, Irvine RL. 1993. Cultivation of Phanerochaete chrysosporium and production of lignin peroxidase in novel biofilm reactor systems: Hollow fiber reactor and silicone membrane reactor. Water Res [Internet]. 27(4):591–596. https://www.sciencedirect.com/science/article/pii/004313549390168H

Vesilind P. 2003. Wastewater treatment plant design. [place unknown]: IWA publishing.

Wanner J. 1994. Activated sludge: bulking and foaming control. [place unknown]: CRC Press.

Wei X, Li B, Zhao S, Wang L, Zhang H, Li C, Wang S. 2012. Mixed pharmaceutical wastewater treatment by integrated membrane-aerated biofilm reactor (MABR) system - A pilot-scale study. Bioresour Technol [Internet]. 122:189–195. http://dx.doi.org/10.1016/j.biortech.2012.06.041

Yesilada O, Sik S, Sam M. 1997. Biodegradation of olive oil mill wastewater by Coriolus versicolor and Funalia trogii: Effects of agitation, initial COD concentration, inoculum size and immobilization. World J Microbiol Biotechnol. 14(1):37–42.

Zhang Jianguo, Zhang Jining. 2016. The filamentous fungal pellet and forces driving its formation. Crit Rev Biotechnol. 36(6):1066–1077.

Zhang Y, Liu J, Li J. 2020. Comparison of four methods to solve clogging issues in a fungi-based bio-trickling filter. Biochem Eng J. 153:107401.

Zheng Z, Obbard JP. 2002. Removal of surfactant solubilized polycyclic aromatic hydrocarbons by Phanerochaete chrysosporium in a rotating biological contactor reactor. J Biotechnol [Internet]. 96(3):241–249. https://www.sciencedirect.com/science/article/pii/S0168165602000500

Chapter 5
CASE STUDY
DEVELOPMENT OF NEW MYCO-REACTOR

5. Development of mycoreactor

5.1. Introduction

The biological treatment processes are part of the wastewater purification process chain that begins with the sewage system and ends with the release into the environment (Vesilind 2003). Civil and industrial wastewaters are often mixed and then processed using physical/chemical treatments that allow to reduce the content of many organic substances by simply exploiting the metabolic capabilities of a pool of microorganisms selected over the years (Bashaar 2004). Each biological treatment must necessarily supply the microorganisms involved in the treatment with oxygen and nutrients: this can be done with different methods and systems.

The traditional process used for about a century in civil wastewater treatment plants employs activated sludges. Thanks to the supplied oxygen and agitation, the microorganisms form flakes and then a sludge that actively removes organic substances (Wanner 1994). This sludge is then separated by filtration or sedimentation to be eventually reused. Among the commonly used microorganisms there are certainly also filamentous fungi: the mycelium can act as a network for bacteria and protozoa and can influence the formation of the flakes (Ni & Yu 2012).

A relatively recent solution is the adhered biomass system, in which the microorganisms do not accumulate in flakes but creates a biofilm on an external support. Also in this case oxygen can be supplied in different ways (Downing & Nerenberg 2008). Both types of systems were described in the chapter four.

This case study exploits aerated biological membrane reactors (MABR): a system that takes the advantages of both modalities supplying the microorganisms with oxygen in an efficient way, while keeping them attached to a support (Martin & Nerenberg 2012). In particular, the possibility of using only fungal microorganisms in MABRs was tested, in place of the known pool of bacteria and other microorganisms commonly described in the literature. The capacity of a fiber, designed for microfiltration treatments, to act as a support for the microorganisms and as an oxygen distributor, was tested, finally realizing a bioreactor with aerated membranes.

This technology is based on the transfer of a gas (e.g. air or oxygen, but in principle also other gases can be used) through a membrane to a biofilm that is attached to the membranes surface and is in contact with a liquid phase that contains the substrates to be metabolized (Nerenberg 2016). The large specific surface area of the membrane may permit to obtain a large active surface for the fungal

biofilm growth in a compact reactor volume. The bioreactor has been tested with biofilming fungal communities. Among the bioreactors not yet tested with fungi, MABRs have great interest in water treatment. In this case study, the first of the four-phase strategy for implementation of mycoremediation was carried out: the bench-scale treatability. The laboratory-scale myco-MABR developed, was operated in batch and under aerobic condition (Prasad 2018). The use and construction of this myco-MABR will be discussed in this chapter.

5.2. Materials and method

5.2.1. Membranes

The choice of the membranes to build the modules is a fundamental part of the bioreactor plant project. The membranes used in this work are the *Accurel PP S6/2* produced by Membrana GmbH (Germany), their characteristics are summarized in Table 13 (Bienati et al. 2008).

Polymer	Polypropylene
Wall thickness (mm)	0,45
Inner diameter (mm)	1,8
Outer diameter (mm)	2,7
Inner pore diameter (μm)	0,2
Outer pore diameter (μm)	~ 40
Nominal porosity (%)	60-70
Specific outer surface (mm^2/mm)	8,164

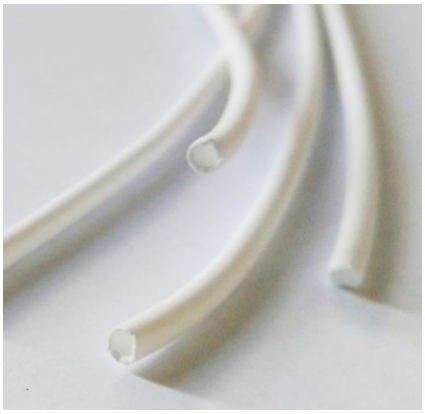


Table 13 Accurel PP S6/02 membranes technical details.

These membranes were already tested in MABR bioreactors with filamentous fungi, showing a better oxygen transfer efficiency than dense silicone membranes (Onken & Berger 1999). Furthermore, polypropylene, of which these membranes are made, is a material on which it has been shown that the fungus selected for the tests is capable of forming biofilms (Siqueira & Lima 2012). The porous commercial hollow fiber membranes assembled in modules are used in order to study the growth of the *P. expansum* mycofilm in a liquid medium. When using microporous membranes, it is necessary to operate at low gas pressure inside the fibers to avoid bubbling and the consequent loss of biofilm from the surface.

The asymmetry of the membranes cross section allows the hyphae of the fungus to adhere better to the surface and resist to any stress due to the tangential flow of wastewater in the bioreactor or caused by an excessive air pressure.

5.2.2. Modules

Several modules were prepared by using these hollow fiber membranes: the fibers ends were fixed with epoxy resin in order to build membrane modules adapt for the size of the reactor. A gap of 2-3 mm between each membrane was left to allow the growth of thicker mycofilm and a more effective water flow around the membranes. To build a module about four days are necessary because the resin used takes 24 hours to dry and both ends were fixed by keeping it vertical for 24 hours on each side. The resin provides the fixing of the membranes in the geometry we have chosen and the connection of the module to the air inlet. The resin adheres to the polypropylene of the membranes making the passage inside the tubes the only way for the entry of oxygen: this allows to regulate the pressure of the air flow, so that no bubbles can form on the membrane surface. The pressure depends on the number and length of fibers.

Membrane modules were used for tests to measure the ability to transfer oxygen, and positioned in a transparent vessel, where it was possible to accurately observe the absence of bubbles, the growth of mycofilm and possible damage to the membranes. The terminals of the modules were connected with plastic pipes to the compressed air line. The module housed in the transparent cylinder and the terminal during the assembly are shown in Figure 15. Many modules with different numbers of fibers were used to grow the biofilm and were housed in a cylinder connected with the feed tank. In case the module or a single membrane has defects or breaks, bubbles will soon appear just near them already at very low pressures: this test allow to identify and reject bad modules. If the module has no defects, the bubbles will form uniformly along the entire surface of the module when the bubble pressure is exceeded.

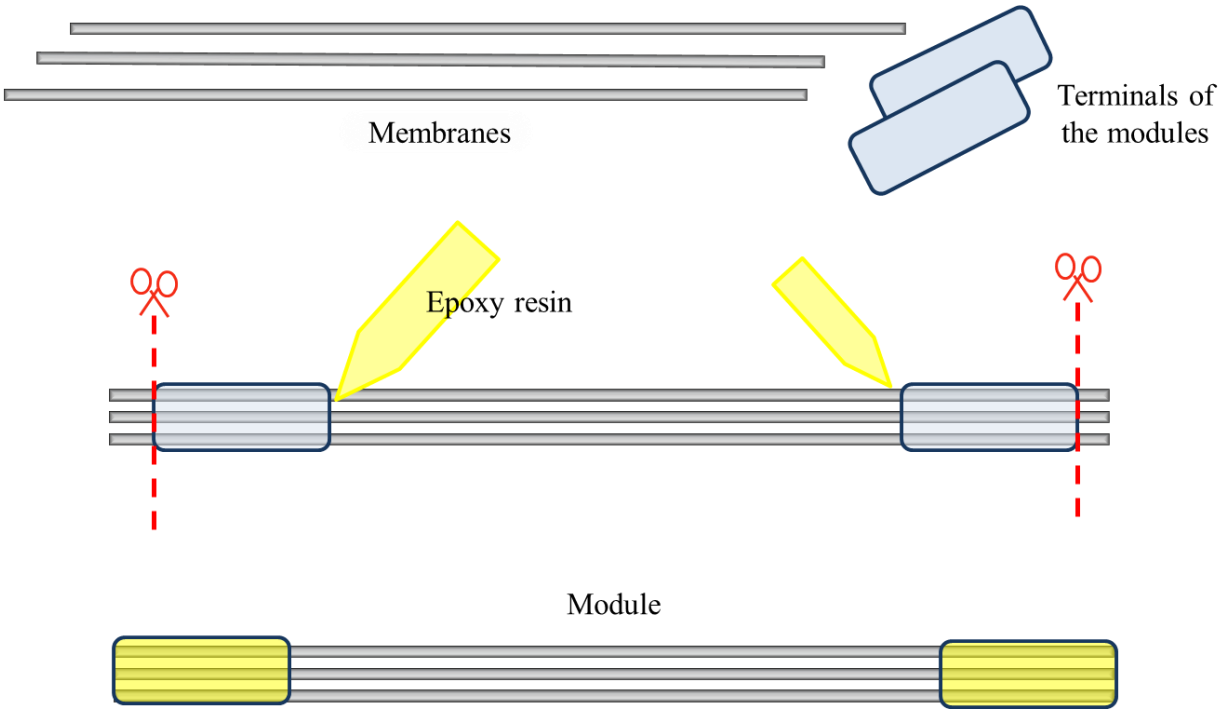


Figure 15 Module assembly.

4.2.3. Oxygen transfer rate measurements

The capability to transfer oxygen through the membranes depends on the gas and liquid flows velocity, on the gas used in the bioreactor as well as on the type of the membranes (Casey et al. 1999). In this study an air flow through the membrane lumen was used and its pressure was controlled in order to avoid bubbling. A batch system was built to perform the measurement of the capability of the membranes to transfer oxygen. A membrane module was put inside a closed case filled with water and was connected to a compressed air cylinder as shown in Figure 16.

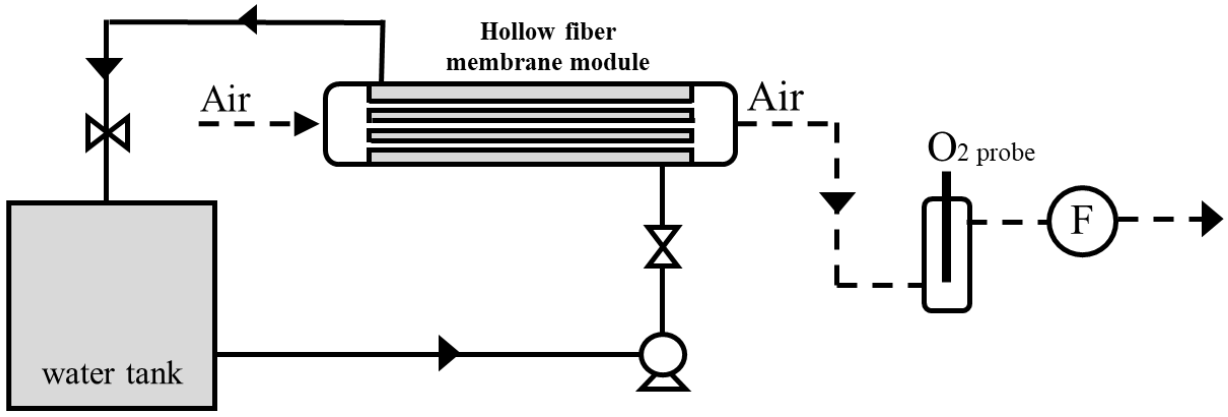


Figure 16 Oxygen transfer rate experimental setup.

The dissolved oxygen (DO) was removed from the water according to the sodium sulfite method (Ghaly & Kok 1988). This method is based on the reaction of sodium sulfite, a reducing agent, with DO to produce sulfate, in the presence of a catalyst, usually a divalent cation, such as cupric sulphate (Lewis & Whitman 1924). The DO concentration was measured with a portable dissolved oxygen meter (HANNA instruments HI 9146) at fixed intervals. Sodium sulphite was added to the water in a concentration between 185 and 280 mg/L from the air through the membranes before starting the oxygen transfer test in order to zero the oxygen concentration in the water. In the tests carried out, the air flow in the modules was always within 3 L/min. Using a portable digital oximeter, the growth of the oxygen concentration in the water was measured as a function of the time until a water saturation condition was reached.

When there was no DO left in the water, the air was flown inside the membrane lumen and the DO concentration was measured until the system reached a steady state. For the data analysis, the MABR reactor was considered as a gas-liquid contactor in order to calculate the liquid mass transfer coefficient ($K_L a$) that makes the transfer capacity comparable under different conditions (Lewis & Whitman 1924; Mishra et al. 2005).

The purpose of these experiments was to obtain the volumetric mass transfer coefficient $K_L a$ by applying the double film theory of Lewis and Whitman, which is the simplest and most consolidated model to explain the mechanism of transfer of a substance across the interface between a gas and a liquid. The theory is based on the hypothesis that next to the interface there are two films in a quiet state which determine a resistance to the motion of the molecules towards one or the other direction. It is also assumed that the gas and liquid beyond the interface films are in conditions of continuous mixing, therefore with uniform pressure and concentration (Figure 17).

To better understand the case study reported in this thesis, it is necessary to introduce the membrane which acts as an interface between the two phases and adds an additional resistance to the mass transfer. In the case of a hydrophobic membrane, the pores will be invaded only by the gas phase and the resistance will be dictated by the porosity of the membrane and the type of diffusion of the gas molecules through the pores.

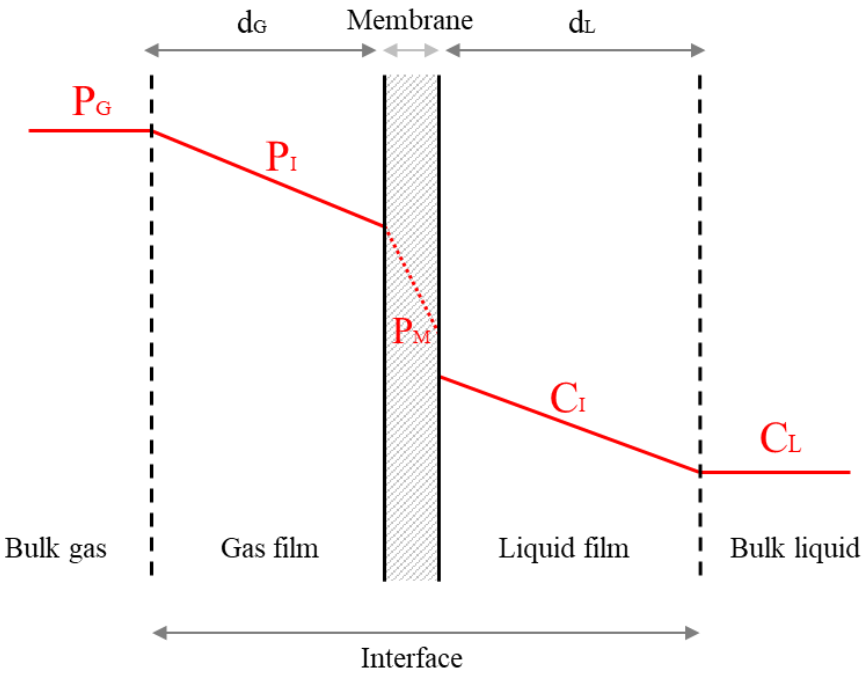


Figure 17 Double film theory: P indicates the partial pressure of the gas phase (G), at the interface (I) and in the membrane (M); C indicates the concentration at the interface (I) and in the liquid phase (L); d_G and d_L indicate the thickness of the two films at the exchange interface.

The discontinuity that is observed between the pressure of the gas near the surface of the membrane on the liquid side and the concentration of gas at the membrane-liquid interface is described by Henry's law:

$$p=HC$$

where p is the partial pressure of the gas (bar), C is the concentration of the gas in the liquid phase (mol/L) and H is the Henry's constant (bar L/mol).

One way to simplify the treatment of the phase transfer is to consider all the resistances on the gas side, liquid side and membrane as being combined in a single resistance, for example on the liquid side. In these conditions the gas flow can be represented by the following expression:

$$\frac{1}{V}\left(\frac{dn}{dt}\right) = K_L\, a\, (C_s - C_L)$$

V (m^3) is the volume of the liquid;

dn/dt (mol/s) are the moles of gas transferred in the unit of time;

K_L ($m^3/m^2/s$) is the global transfer coefficient on the liquid side of the gas;

a (m^2/m^3) is the interfacial surface defined in this case study as the ratio between the surface of the module and the volume of liquid;

C_s (mol/m^3) is the saturated concentration of oxygen;

C_L is the concentration of oxygen in the liquid phase.

From the resolution of the differential equation reported, the following expression can be obtained:

$$log(c_s - c_t) = log(c_s - c_0) - K_L a \left(\frac{t}{2{,}303}\right)$$

c_s is the saturated concentration of oxygen;

c_t is the concentration of oxygen at different time intervals;

c_0 is the initial time concentration.

If the hypothesized model is valid, the data will be well represented by a straight line in a graphical representation in which the abscissa is time (t) and the ordinate $log(c_s - c_t)$. From the intercept of the line it will be possible to obtain the value of $K_L a$ and, if the value of a is known, also that of K_L.

4.2.4. Cultivation

To start developing a new type of plant, in this case a MABR, it is preferable to start using a model fungus. For this reason a well-known fungus was selected, on which studies have already been made and whose metabolic potential for the treatment of wastewater has been already confirmed. It was decided to start from *Penicillium*, a very common genus of fungi and whose presence in the sludge has been confirmed by many researches (Cooke & Pipes 1970; Diener et al. 1976; Fleury 2007) with an abundance until the 50% compared to other microorganisms present in the sludge (Kacprzak et al. 2005).

Many wastewaters, such as that produced in landfills (treated in this thesis) or in other industrial production activities, have the characteristic of being hypersaline environments. Many species of *Penicillium* genus are generally halotolerant, which is why they can be used in environments that contain a high percentage of salts, in addition to various organic substances that are able to degrade (Leitão 2009). Capacities of *Penicillium* strain as degraders are shown in Table 14.

Xenobiotics		*Main references*
Main heavy metal adsorption	Cd (II)	(Niu et al. 1993; Holan & Volesky 1995; Skowroński et al. 2001; Say et al. 2003; Fan et al. 2008)
	Zn (II)	(Townsley & Ross 1985; Biomedical 1986; Niu et al. 1993; Skowroński et al. 2001; Fan et al. 2008)
	Pb (II)	(Biomedical 1986; Niu et al. 1993; Skowroński et al. 2001; Say et al. 2003)
	Cu (II)	(Skowroński et al. 2001; Say et al. 2003; Ianis et al. 2006; Mendil et al. 2008)
Main transformation of polycyclic hydrocarbons (PAH)	Fluorene	(Garon et al. 2002; Garon & Sage 2004)
	Pyrene	(Ravelet et al. 2000; Saraswathy & Hallberg 2002; Saraswathy & Hallberg 2005)
	Benz(a)pyrene	(Launen et al. 1995; Boonchan et al. 2000)
Main degradation of phenols and its derivatives	Phenol	(Marr et al. 1996; Leitão et al. 2007)
	Chlorophenol	(Hofrichter et al. 1994; Marr et al. 1996)
	Fluorophenol	(Hofrichter et al. 1994; Marr et al. 1996)
	Dichlorophenol	(Hofrichter et al. 1994)

Waste and wastewater	Olive Mill WasteWater (OMWW)	(Robles et al. 2000)
	Vinasse	(Jimenez et al. 2005; Jiménez et al. 2006)
	Coffee residual	(Schwimmer & Kurtzman 1972; Roussos et al. 1994; Roussos et al. 1995; Dantigny et al. 2006)

Table 14 Capacities of Penicillium strains as degraders.

The enzymes involved in the degradation, adsorption and transformation of xenobiotic by the species of *Penicillium* genus are various. The enzymes involved in the degradation of hydrocarbons and phenolic compounds are the laccases. Laccases are a very robust enzyme for degrading and transforming endocrine disruptors, pesticides, PAH, aromatic diamines, polyphenols, methoxy-substituted phenols and other organic compounds. Laccases are produced from fungi and bacteria, their properties and characteristics depend on the substrate used, the environmental conditions and fungal species, but in general fungi have higher oxidative enzyme productivity than bacteria (Noman et al. 2019).

Laccases activity is often synergical with cellulases enzymes (Gusakov & Sinitsyn 2012). Cellulases are complex enzymes that can be divided into three classes (endoglucanases, cellobiohydrolase and glucohydrolase). They allow fungi and other organisms to degrade cellulose, hydrolyze solid agricultural residues and reduce industrial pollutants.

Peroxidases work by cleaving the carbon-carbon and carbon-oxygen bonds of the xenobiotics by a free radical mechanism, which makes these enzymes non-specific and useful for a variety of structurally different pollutants. Peroxidases enzymes are involved in oxidation and mineralization of PAH, phenols and its derivatives, industrial and dyes wastewater (Noman et al. 2019).

Among the numerous *Penicillium* strains isolated from wastewater sludge which have turned out to be excellent biodegraders, it has been selected the *Penicillium expansum* Link. The *P. expansum* was chosen because it is spread all over the world and, used in mycoremediation, it is able to degradate complex compounds with low co-substrate requirements (Leitão 2009). *P. expansum* has been found in many studies between the fungi present in the activated sludge of traditional biological treatments, where it plays the function of reducing pathogens and organic substances thanks to its mycelium and its metabolic properties (More et al. 2010). It has also been identified as a floc-forming fungi in

activated sludge plants, so it is the ideal candidate to be tested in a new wastewater treatment plant (Subramanian et al. 2008). Moreover, it is able to form a biofilm in an aqueous environment and on a hydrophobic support (Siqueira & Lima 2012).

The culture medium Malt Extract Agar (MEA) were used to allow the fungal growth. The *P. expansum* used in this study was inoculated in a 9 cm petri dish containing MEA and was incubated at 24 °C for seven days. The inocula suspension was prepared by scraping the surface of 7-days-old cultures with a loop (Figure 18) to reach a concentration of 10^5 conidia/mL, measured using a Burker Chamber (Figure 19).

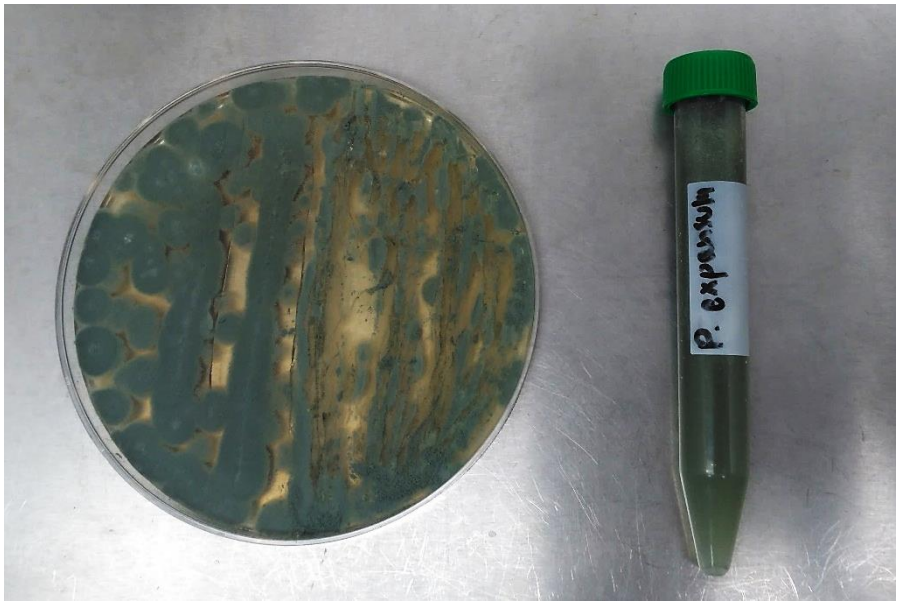


Figure 18 Conidial inoculum.

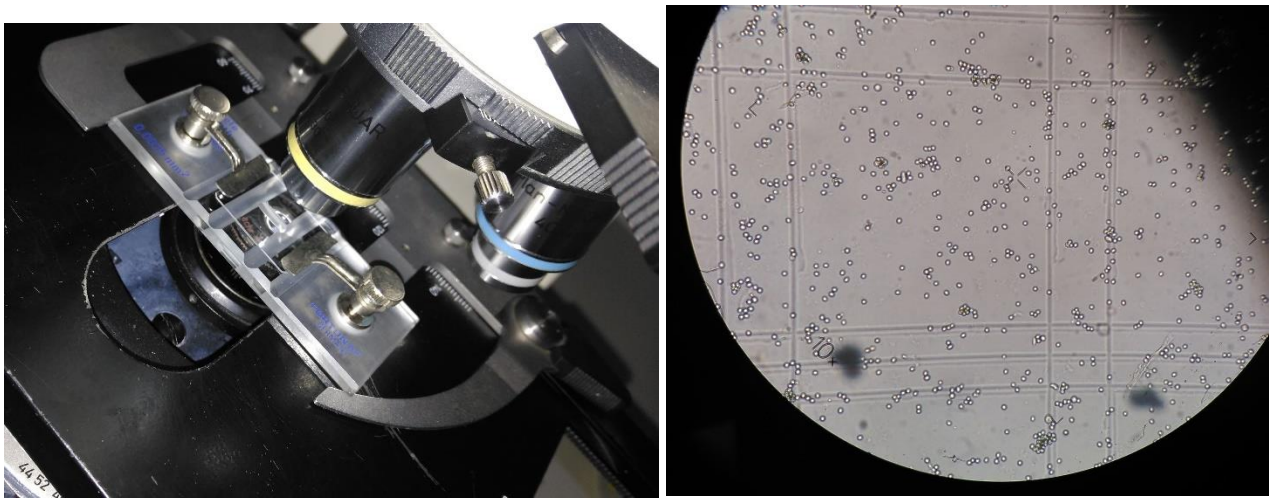


Figure 19 Burker Chamber.

These suspensions were employed in the bioreactor to promote the formation of mycofilm on the fibers of the module. The deposition of conidia and hyphae fragments on the membrane surface is the starting point of biofilm development (Simões et al. 2015).

4.2.5. Fungal growth on single fiber

The tests to verify the usability of the *Accurel PP S6/02* membrane and to observe the early stages of mycofilm formation were carried out on a single fiber. In this way it was possible to easily take samples for analysis relating to the various tests described in this paragraph.

Several tests changing the Agar concentration in the MEA were carried out in order to select the best gel composition to coat the membranes. The MEA contains 2% of agar, malt, glucose and peptone. It is solid at temperatures below 45 °C and is suitable for microbiology plates and tubes. For the experimentation, the percentage in agar was fixed to 0.5%, since it remains liquid already at 30 °C. The choice of the percentage was made following immersion and weight gain tests of membrane segments, modifying the temperature and the percentage of agar. The procedure involves few steps: the membranes were perforated on one side and sealed on the other by heat to allow better immersion and drying. They were left in a dry environment for 24 hours to remove moisture and then immersed in the MEA at a controlled temperature for 10 seconds. After the immersion the membranes were dried in an oven for 24 hours at 50 °C. The segments were weighed before the immersion and then after the drying process. The experimental setup for the immersion of several samples at the same time is shown in Figure 20. With this technique, multiple segments were tested simultaneously in exactly the same environmental conditions and it was possible to calculate the mean weight increase for each test.

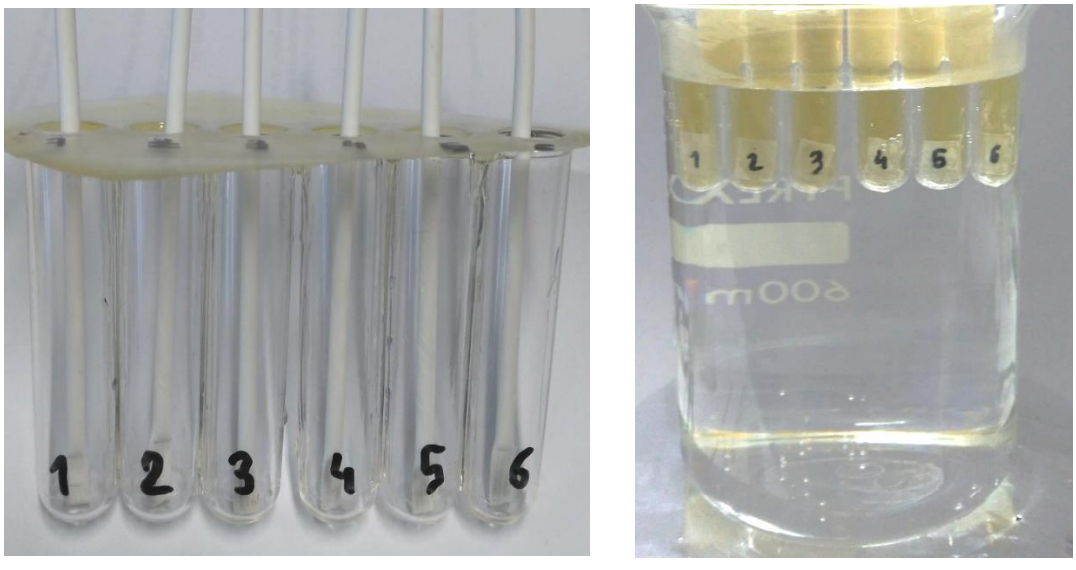


Figure 20 Experimental setup for immersion of membranes at a controlled temperature.

After selecting the most optimal percentage of agar for gel formation around the fibers, the segments were immersed in a conidia solution for 10 sec and then left in sterile water for 1 week. This allowed to highlight the first stages of biofilm development around the segments, analyzed by taking a sample every 48 hours. The mycelium growth on the membrane surface was observed by means of a FE-SEM analysis as reported in (Villena et al. 2010) for the growth of mycofilm on other media.

4.2.6. Fungal growth on the MABR

The growth of *P. expansum* was studied directly on the hollow fiber membrane module in a laboratory-scale MABR bioreactor, in not sterile conditions. In the MABR the module was housed in a cylindrical transparent polypropylene vessel in which oxygen was supplied inside the fiber lumen and the liquid phase was recirculated from a 1L tank (Cerqueira et al. 2013).

The module was first dipped in MEA 0.5% agar and then fixed in the bioreactor in batch mode. In the liquid phase, the inoculum was dissolved in not sterile water and left in contact with the module for a few days. After 4-5 days it was possible to observe a biofilm on the membranes surface: the liquid media was replaced with a model wastewater without inoculum. A balanced fungal medium for growth contain a ratio 10:1 of carbon to nitrogen (Sankaran et al. 2010). The medium used as a model wastewater was formed by glucose and peptone solution. Then water samples were taken a few days later for the analysis of the COD, acronym for Chemical Oxygen Demand. COD is a chemical parameter that quantifies the concentration of chemically oxidizable substance in an aqueous matrix sample. During the experiment at laboratory-scale, the COD value was sampled to study the consumption curve of the solution: its behavior was observed to decrease in time (Bashaar 2004). The COD measurement method involves the oxidation of organic and inorganic substances present in a water sample by means of a potassium dichromate solution in the presence of concentrated sulfuric acid and silver sulphate as a catalyst. The excess of dichromate is titrated with a solution of ammonium sulphate and iron (IRSA-CNR 2003).

In particular, in this study the MERCK cuvette tests at different concentration scales were used for the measurement of COD and the result was automatically acquired with the MERCK spectrophotometer (model Spectroquant ® Pharo 300).

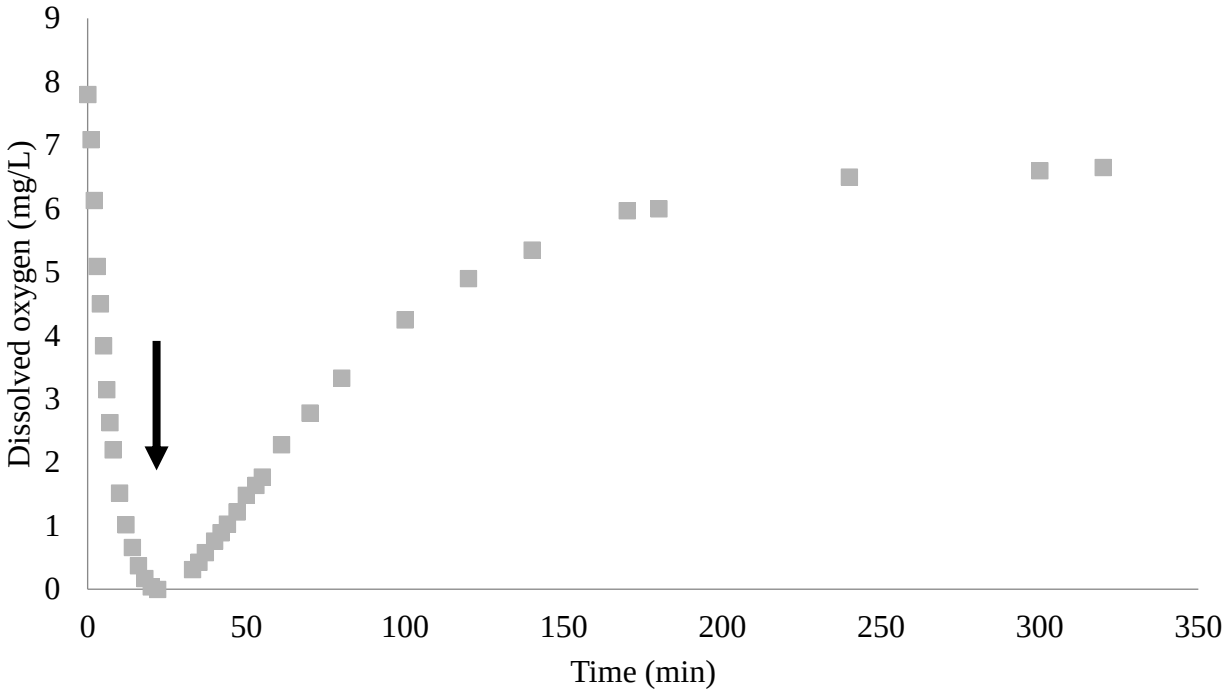
This chemical parameter was used in the present study as an index of the consumption of organic substances by the biomass formed in the reactor. Normally was used the Biological Oxygen demand (BOD) to have this data: from the difference in pressure of a sample left in the dark for a determined time, the quantity of oxygen that the microorganisms contained within it have consumed is obtained.

We decided to do not measure BOD, considering the fact that in this study the organic substances used as nutrients are biodegradable and detectable in the same way with BOD or COD.

5.3. Result and discussion

5.3.1. Oxygen transfer rate

Graph 2 shows an example of the trend over time of the oxygen concentration for one of the modules tested. After the start of aeration, an induction time may occur in which the oxygen concentration does not increase until the complete consumption of the sulphite. The data for the evaluation of the oxygen transfer rate are recorded from the moment when the oxygen concentration starts to increase. The transfer speed of the module corresponds to the slope of the curve at each point. The transfer rate will reach the maximum value at the inflection point of the almost linear growth zone, then it will decrease while the plateau is reached.

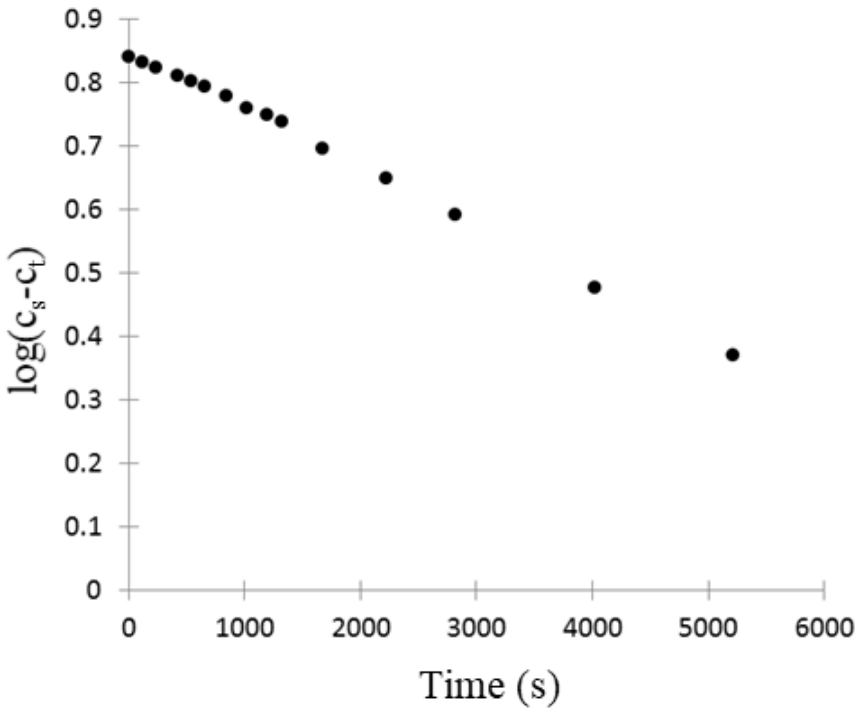


Graph 2 Oxygen concentration profile.

From the analysis of the data of the aeration tests carried out with different modules it was possible to observe the linear relation between time and *log* (c_s-c_t). In Graph 3 an example of the aeration tests of one of the module, where the abscissa is time and the ordinate is *log* (c_s-c_t): K_La can be extrapolated as the intercept of the equation that describes the linear trend (*Graph 3).* The details of one of the modules used for the aeration tests are shown below.

Number of fiber	*fiber length (mm)*	*Surface(mm^2)*	*K_La (s^{-1})*	*V (m/s)*
5	250	10205	1.61E-04	1.80

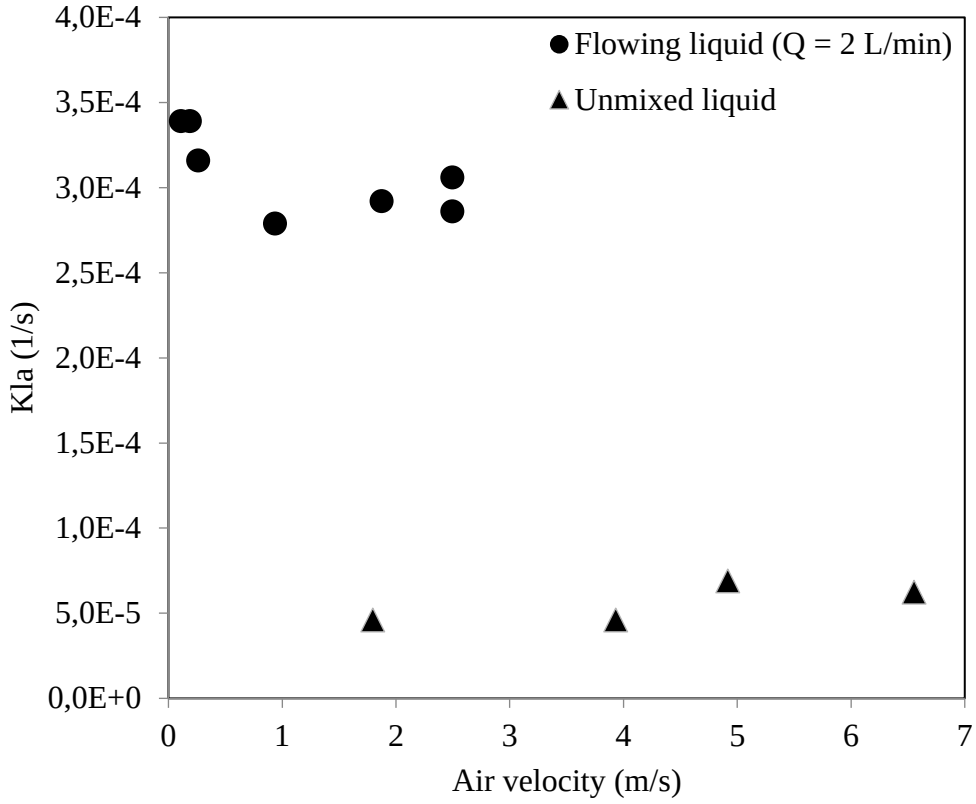
Table 15 Characteristics of one of the tested modules.



Graph 3 Trend line obtained from the oxygenation tests of one of the modules.

From the curve, considering the Lewis double film law, it is possible to derive the parameter K_La that allows to compare the transfer performance of the modules in different conditions.

Graph *4* shows the value of different mass transfer coefficients measured in batch system (unmixed liquid) or recirculated liquid flow (Q = 2 L/m). The oxygen transfer performance of the membrane module without biofilm was improved by increasing both the air and liquid velocity.



Graph 4 Oxygen transfer rate coefficients.

Considering the tests carried out in the batch systems (▲ in

Graph 4), the mass transfer coefficient is slightly improved by increasing the air velocity inside the membrane lumen. By doing so, the partial oxygen concentration inside the membranes was increased and the local driving force governing the process was enhanced.

The tests performed by flowing the liquid phase (● in

Graph 4) highlighted higher K_La values. In these conditions, the turbulence reduced the thickness of the liquid boundary layer and lowered the mass transfer resistance in the liquid section. Moreover, the effect of the gas velocity was less evident, since in these conditions the mass transfer resistance in the gas layer was less influent than that of the liquid section.

5.3.2. Fungal growth on single fiber

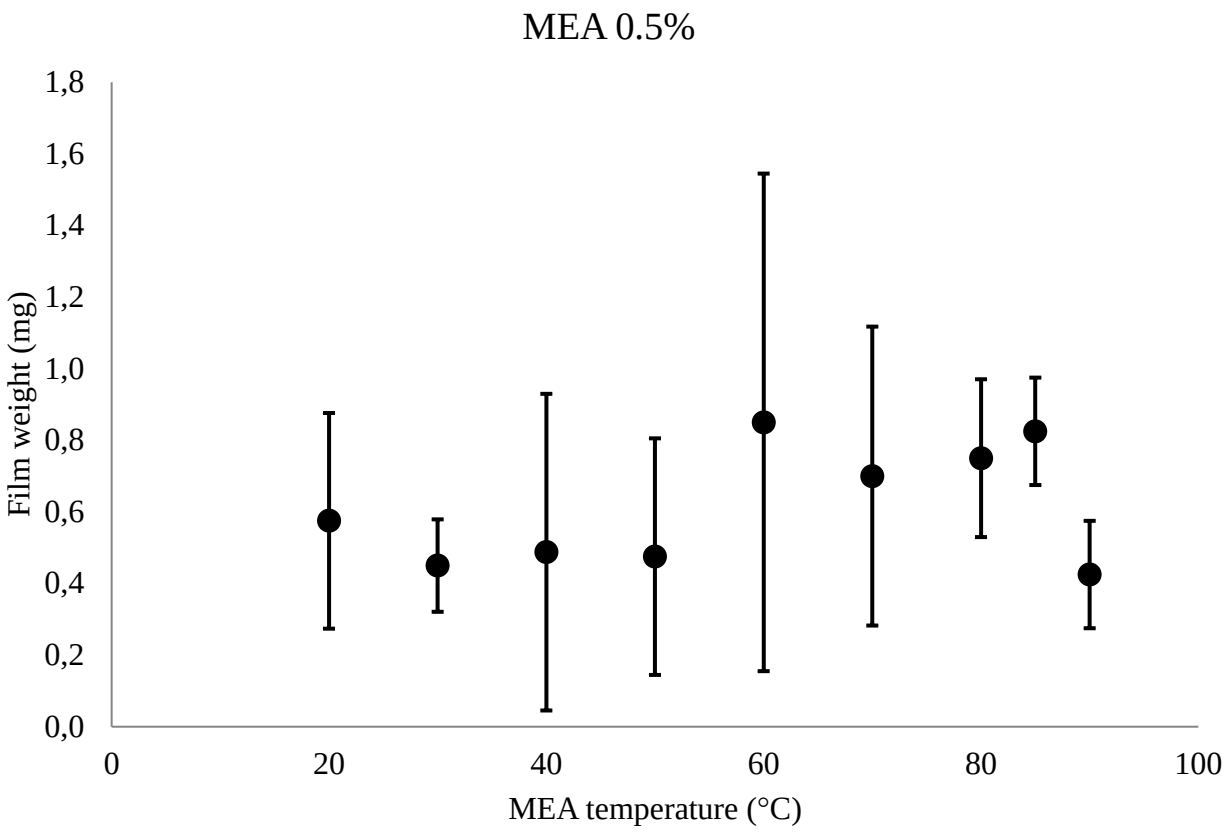
In order to define the best conditions to create a culture medium film on the membrane surface, different compositions and temperatures were tested, as reported in Table 16.

Agar concentration range	*MEA temperature range*	*Immersion time range*
0,5 - 2,0%	65 - 90°C	30 - 10 sec
0,5%	20 - 90°C	10 sec

Table 16 Preliminary conditions tested.

For each test, several membrane samples with similar lengths were tested following the procedure reported in material and method of the chapter five. The preliminary tests were performed using a MEA concentration similar to the one commonly used for fungal growth in petri dishes. However, when the agar concentration was higher than 2%, it was found that the medium viscosity was too high to perform the membrane immersion at temperatures below 70 °C. At such high temperatures the air trapped inside the membrane pores expanded creating bubbles on the surface and the partial detachment of portions of the films.

It was then decided to use a lower agar concentration (0,5%) in the MEA. The amount of culture medium deposed on the membrane at different immersion temperatures was measured. The results are reported in Graph 5: all tests shows that part of the broth adheres to the outer surface of the membrane. Moreover, it seems that the additional weight due to the MEA adhesion is not dependent from the temperature.



Graph 5 Culture medium film weight obtained at different temperatures.

The first tests were performed to confirm the formation of the biofilm in a sterile environment: a single fiber and a microbiological tube. In order to have a sterile condition, the membranes were placed under the UV rays in a biological hood and the water was autoclaved at 121 °C. It is necessary to be sure that this strain is able to grow in biofilm within an aqueous environment on the membranes in the absence of competitors. In sterile condition the *P. expansum* biofilm develops on the membrane surface without invading the pores of membranes (

Figure 21), the mycelium adheres tightly to the membranes and the simple washing or touching the fibers do not cause damage to the mycofilm (Mcdonald et al. 2012). The P. *expansum* biofilm takes 7 days to develop uniformly on the polypropylene membranes (Siqueira & Lima 2012). During this period of time, the film it creates expands in one dimension, creating a thin film in contact with the hydrophobic membrane on one side and with the aqueous environment on the other. The hyphae form a texture with smaller pores than those of which the *Accurel PP/S6* membrane is composed as can be seen by comparing the A) and D) of the

Figure 21. There are no conidiophora but only filaments of sterile mycelium.

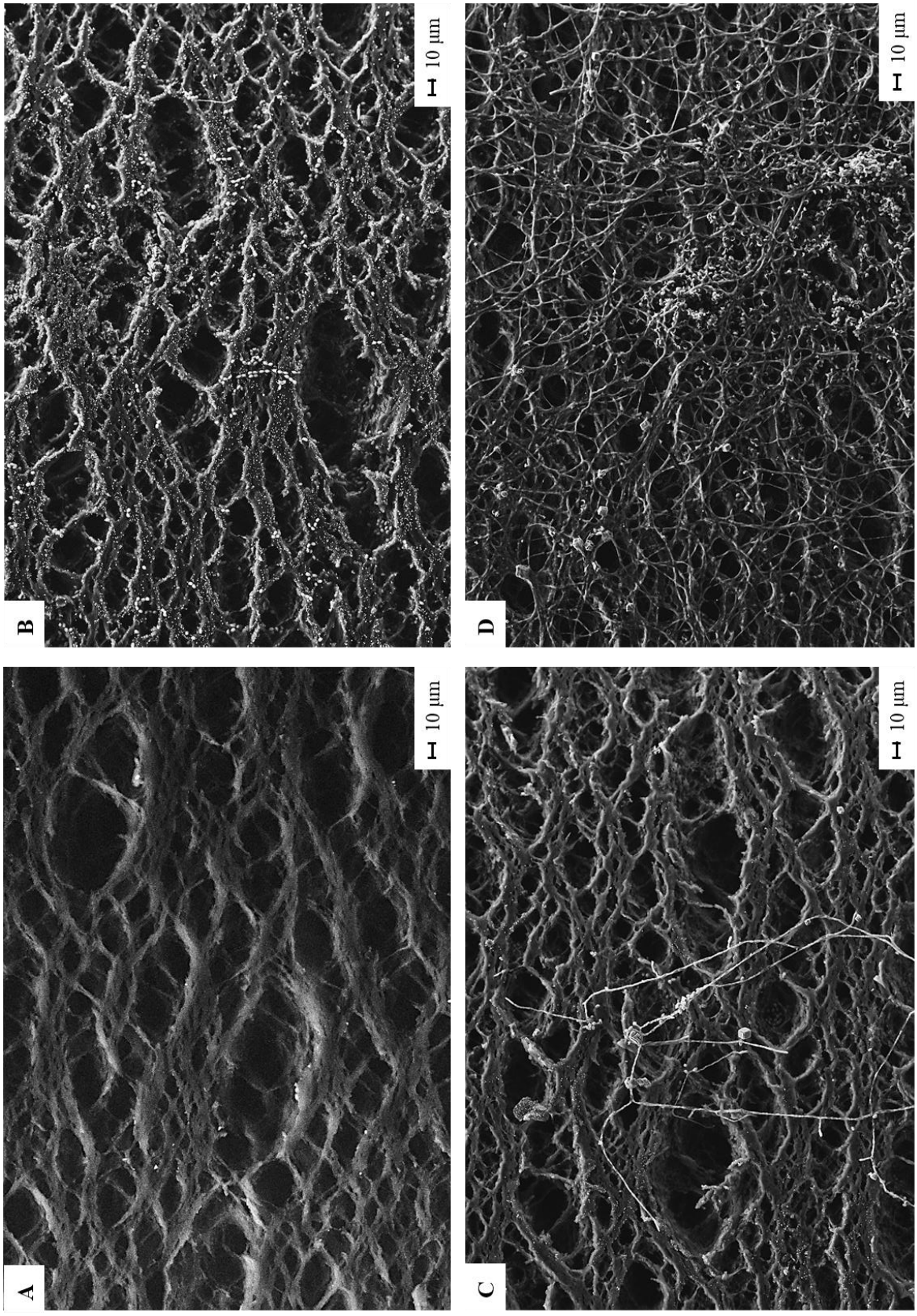


Figure 21 P. expansum mycofilm development in one week: A) Membrane surface clean, B) Membrane surface after 1 day, C) Membrane surface after 3 days and D) Membrane surface after 7 days.

5.3.3. Fungal growth on the MABR

Subsequent tests were carried out in a MABR: a not-sterile environment where was verify if the concentration of inoculated conidia was sufficient to win the competition with other microorganisms possibly present in the water, in the air or on the membranes used. In not sterile conditions, the P. expansum was able to form a biofilm on the hollow fiber outer surface. Despite the use of non-sterile water in the bioreactor and the possibility of contaminations on the module, the cultivation sample performed on MEA substrate demonstrated the absence of other microorganisms in the biofilm after 3 weeks. Figure 22 shows the biofilm accumulation on the membrane surface during a three weeks time interval. The biofilm of P. expansum developed homogeneously on all the bioreactor membrane surface and after two weeks of growth, it appeared uniform and with a color similar to the P. expansum mature colony cultivated on a plate.

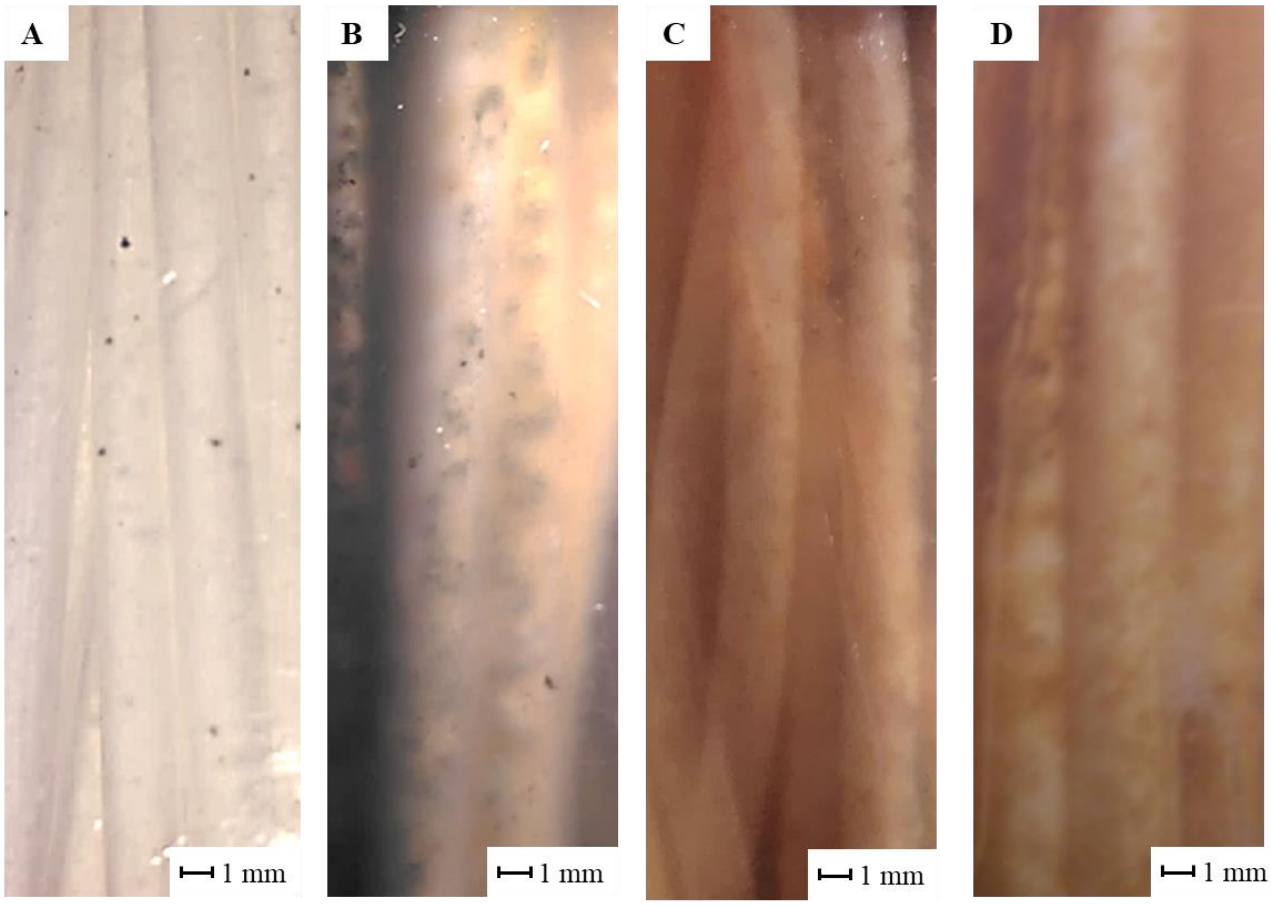
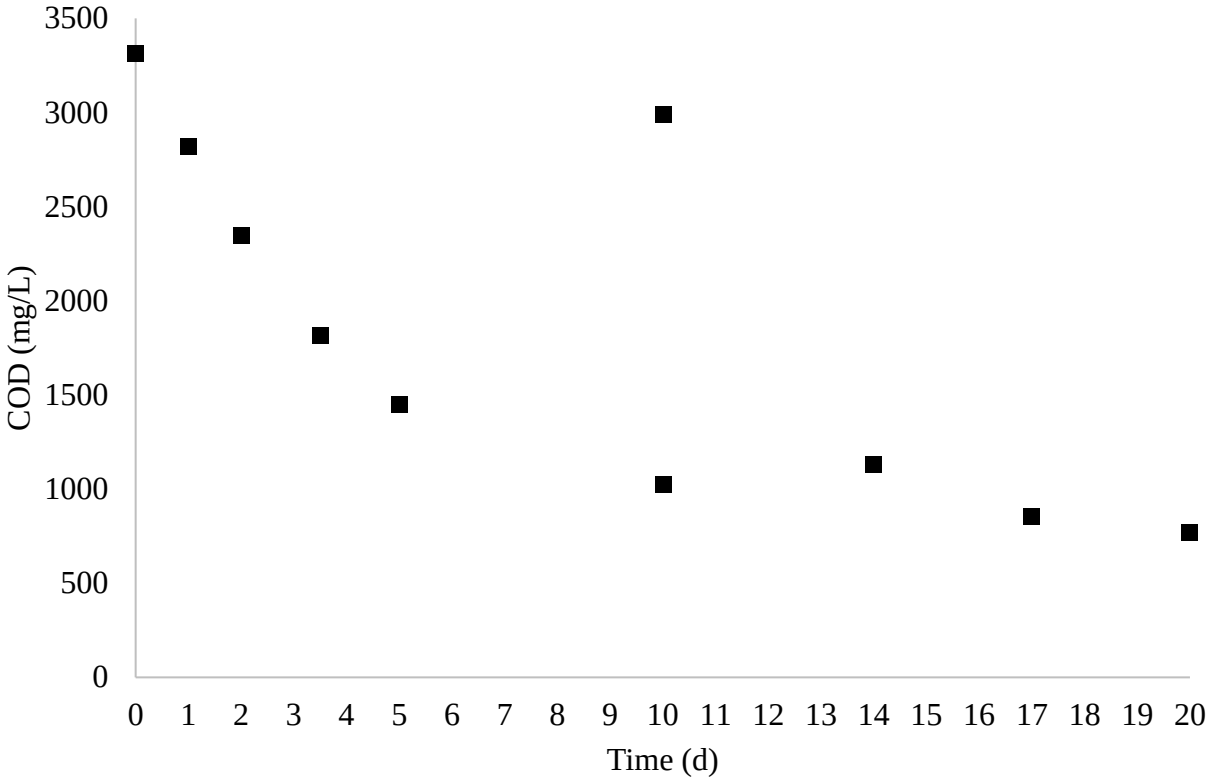
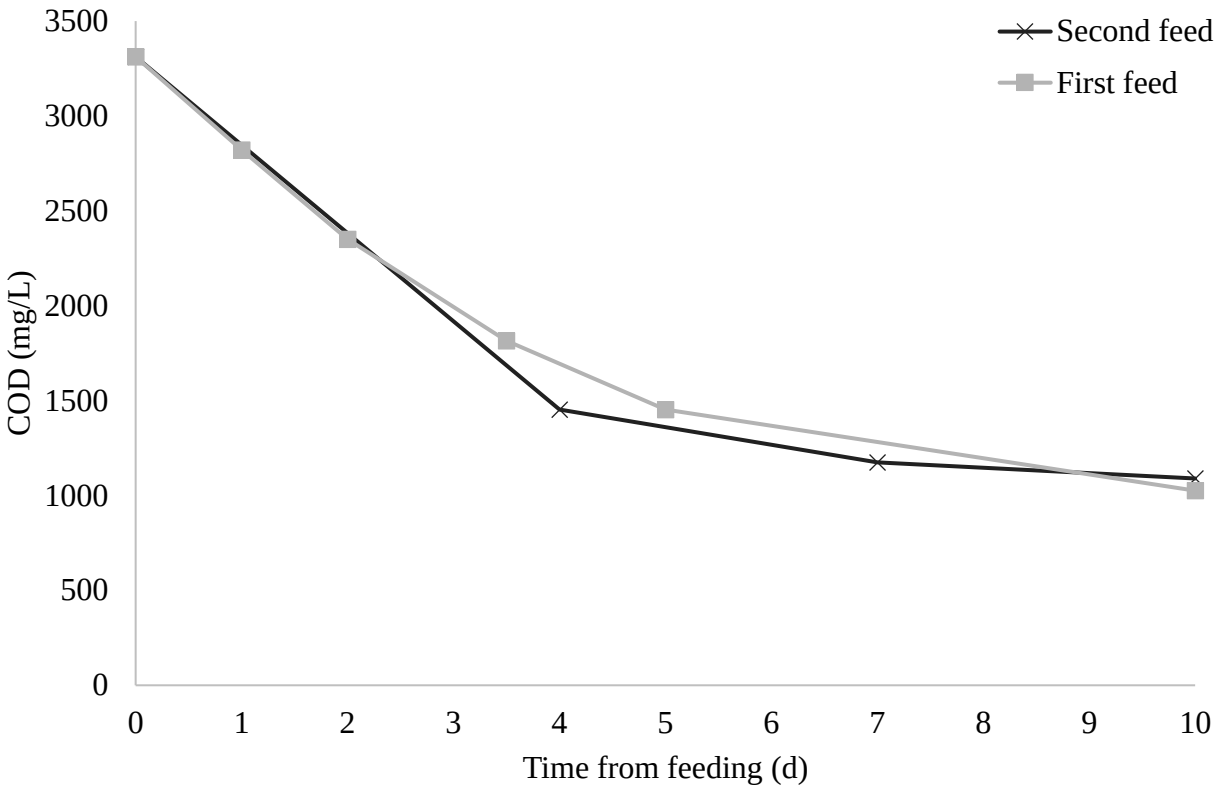


Figure 22 P. expansum mycofilm development in MABR test: A) Membrane surface after 2 days, B) Membrane surface after 7 days, C) Membrane surface after 14 days and D) Membrane surface after 21 days.

The depuration ability of the bioreactor was estimated by measuring the COD value of a model solution at fixed time intervals. To this purpose, a solution of glucose and peptone was added twice during the tests. The consumption of organic substance was measured by the chemical analysis of COD as shown in Graph 6. The Graph 7 shows that the trend in nutrient consumption is comparable in the two weeks probably because the growth of the biofilm has not undergone great differences compared to the first feed time. The COD decrease indicates that there is a vital community in the bioreactor that needs nutrients to be supplied. At the time of the analysis, the refueling was done manually but in a scale-up of the project an automatic feeding system will be provided from a tank where the solution is contained, and subsequently the wastewater to treat.



Graph 6 COD trend during the test.



Graph 7 COD trend based on feed supplied.

5.4. Conclusion

The use of membranous supports can improve the management of fungal biomass in the aqueous environment. Moreover, the MABR system provides a more efficient oxygenation of the biofilm. The ability to form resistant biofilms on porous surface is useful to support different stresses: liquid flow, air pressure, touch, etc.

Currently, membranes designed for MABR do not exist and the commercial *Accurel PP S6/02* membranes, built for MBR filtration systems, have proved to be a good type of fibers both for the ability to transfer oxygen to the biofilm and for the porous surface that favors adhesion. The oxygen transfer rate was influenced by both the air and the liquid flow velocity and the main transfer oxygen resistance appeared to be located in the liquid phase.

P. expansum mycofilm can grow on the surface of hollow fiber membranes, in both sterile and not sterile conditions and became stable in few weeks. The *P. expansum* is able to grow in not sterile conditions, using water and a glucose and peptone solution as growth substrates. The mycofilm spread uniformly on membrane surface in a not sterile MABR rector. The filamentous structure of the mycelium in the MABRs becomes an advantage because it results in more strength and resistance to the structure of the biofilm.

In future it will be interesting to test different filamentous fungi with the same type of membranes to verify if there are differences in the development of the biofilm, such as the timing and speed of consumption of the substrate. It will also be interesting to continue the studies with *P. expansum* with different substrates, using simple and complex pollutants at different concentrations.

5.5. References

Bashaar YA. 2004. Nutrients requirements in biological industrial wastewater treatment. African J Biotechnol. 3(4):236–238.

Bienati B, Bottino A, Capannelli G, Comite A. 2008. Characterization and performance of different types of hollow fibre membranes in a laboratory-scale MBR for the treatment of industrial wastewater. Desalination. 231(1–3):133–140.

Biomedical P. 1986. The uptake of metals from aqueous solution by bacteria , yeast , filamentous fungi , and algae has received a great deal of attention over the past decade . Heavy radionuclides , especially Ra , Th , and U , have been singled out (Galun etaL , 1983a , b . 33(1987):359–371.

Boonchan S, Britz ML, Stanley GA. 2000. Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl Environ Microbiol. 66(3):1007–1019.

Casey E, Glennon B, Hamer G. 1999. Oxygen mass transfer characteristics in a membrane-aerated biofilm reactor. Biotechnol Bioeng. 62(2):183–192.

Cerqueira AC, Nobrega R, Sant'Anna GL, Dezotti M. 2013. Oxygen air enrichment through composite membrane: Application to an aerated biofilm reactor. Brazilian J Chem Eng. 30(4):771–779.

Cooke WB, Pipes WO. 1970. The occurrence of fungi in activated sludge. Mycopathol Mycol Appl. 40(3–4):249–270.

Dantigny P, Bensoussan M, Vasseur V, Lebrihi A, Buchet C, Ismaili-Alaoui M, Devlieghere F, Roussos S. 2006. Standardisation of methods for assessing mould germination: A workshop report. Int J Food Microbiol. 108(2):286–291.

Diener UL, Morgan-Jones G, Hagler WM, Davis ND. 1976. Mycoflora of activated sewage sludge. Mycopathologia. 58(2):115–116.

Downing LS, Nerenberg R. 2008. Effect of oxygen gradients on the activity and microbial community structure of a nitrifying, membrane-aerated biofilm. Biotechnol Bioeng. 101(6):1193–1204.

Fan T, Liu Y, Feng B, Zeng G, Yang C, Zhou M, Zhou H, Tan Z, Wang X. 2008. Biosorption of cadmium (II), zinc (II) and lead (II) by Penicillium simplicissimum: Isotherms, kinetics and thermodynamics. J Hazard Mater. 160(2–3):655–661.

Fleury S. 2007. Method for treatment of sewage plant sludges by a fungal process.

Garon D, Krivobok S, Wouessidjewe D, Seigle-Murandi F. 2002. Influence of surfactants on solubilization and fungal degradation of fluorene. Chemosphere. 47(3):303–309.

Garon D, Sage L. 2004. Effects of fungal bioaugmentation and cyclodextrin amendment on fluorene degradation in soil slurry. Biodegradation. 15(1):1–8.

Ghaly AE, Kok R. 1988. The effect of sodium sulfite and cobalt chloride on the oxygen transfer coefficient. Appl Biochem Biotechnol. 19(3):259–270.

Gusakov A V, Sinitsyn AP. 2012. Cellulases from Penicillium species for producing fuels from biomass. Biofuels. 3(4):463–477.

Hofrichter M, Bublitz F, Fritsche W. 1994. Unspecific degradation of halogenated phenols by the soil fungus Penicillium frequentans Bi 7/2. J Basic Microbiol. 34(3):163–172.

Holan Z nR, Volesky B n. 1995. Accumulation of cadmium, lead, and nickel by fungal and wood biosorbents. Appl Biochem Biotechnol. 53(2):133–146.

Ianis M, Tsekova K, Vasileva S. 2006. Copper biosorption by Penicillium cyclopium: equilibrium and modelling study. Biotechnol Biotechnol Equip. 20(1):195–201.

IRSA-CNR A. 2003. Metodi analitici per le acque. APAT Manuali e Linee Guid. 29:2003.

Jimenez AM, Borja R, Martin A, Raposo F. 2005. Mathematical modelling of aerobic degradation of vinasses with Penicillium decumbens. Process Biochem. 40(8):2805–2811.

Jiménez AM, Borja R, Martín A, Raposo F. 2006. Kinetic analysis of the anaerobic digestion of untreated vinasses and vinasses previously treated with Penicillium decumbens. J Environ Manage. 80(4):303–310.

Kacprzak M, Neczaj E, Okoniewska E. 2005. The comparative mycological analysis of wastewater and sewage sludges from selected wastewater treatment plants. Desalination. 185(1–3):363–370.

Kondepati VR, Heise HM. 2009. ChemInform Abstract: The Potential of Mid- and Near-infrared Spectroscopy for Reliable Monitoring of Bioprocesses. ChemInform. 40(21):117–132.

Launen L, Pinto L, Wiebe C, Kiehlmann E, Moore M. 1995. The oxidation of pyrene and benzo [a]

pyrene by nonbasidiomycete soil fungi. Can J Microbiol. 41(6):477–488.

Leitão AL. 2009. Potential of penicillium species in the bioremediation field. Int J Environ Res Public Health. 6(4):1393–1417.

Leitão AL, Duarte MP, Oliveira JS. 2007. Degradation of phenol by a halotolerant strain of Penicillium chrysogenum. Int Biodeterior Biodegradation. 59(3):220–225.

Lewis WK, Whitman WG. 1924. Principles of Gas Absorption. Ind Eng Chem. 16(12):1215–1220.

Marr J, Kremer S, Sterner O, Anke H. 1996. Transformation and mineralization of halophenols by Penicillium simplicissimum SK9117. Biodegradation. 7(2):165–171.

Martin KJ, Nerenberg R. 2012. The membrane biofilm reactor (MBfR) for water and wastewater treatment: Principles, applications, and recent developments. Bioresour Technol [Internet]. 122:83–94. http://dx.doi.org/10.1016/j.biortech.2012.02.110

Mcdonald JE, Houghton JNI, Rooks DJ, Allison HE, Mccarthy AJ. 2012. The microbial ecology of anaerobic cellulose degradation in municipal waste landfill sites: Evidence of a role for fibrobacters. Environ Microbiol. 14(4):1077–1087.

Mendil D, Tuzen M, Soylak M. 2008. A biosorption system for metal ions on Penicillium italicum–loaded on Sepabeads SP 70 prior to flame atomic absorption spectrometric determinations. J Hazard Mater. 152(3):1171–1178.

Mishra P, Srivastava P, Kundu S. 2005. A comparative evaluation of oxygen mass transfer and broth viscosity using Cephalosporin-C production as a case strategy. World J Microbiol Biotechnol. 21(4):525–530.

More TT, Yan S, Tyagi RD, Surampalli RY. 2010. Potential use of filamentous fungi for wastewater sludge treatment. Bioresour Technol [Internet]. 101(20):7691–7700. http://dx.doi.org/10.1016/j.biortech.2010.05.033

Nerenberg R. 2016. The membrane-biofilm reactor (MBfR) as a counter-diffusional biofilm process. Curr Opin Biotechnol [Internet]. 38:131–136. http://dx.doi.org/10.1016/j.copbio.2016.01.015

Ni BJ, Yu HQ. 2012. Microbial products of activated sludge in biological wastewater treatment systems: A critical review. Crit Rev Environ Sci Technol. 42(2):187–223.

Niu H, Xu XS, Wang JH, Volesky B. 1993. Removal of lead from aqueous solutions by Penicillium biomass. Biotechnol Bioeng. 42(6):785–787.

Noman E, Al-Gheethi A, Mohamed RMSR, Talip BA. 2019. Myco-Remediation of Xenobiotic Organic Compounds for a Sustainable Environment: A Critical Review. [place unknown].

Onken J, Berger RG. 1999. Biotransformation of citronellol by the basidiomycete Cystoderma carcharias in an aerated-membrane bioreactor. Appl Microbiol Biotechnol. 51(2):158–163.

Prasad R. 2018. Mycoremediation and environmental sustainability. [place unknown]: Springer.

Ravelet C, Krivobok S, Sage L, Steiman R. 2000. Biodegradation of pyrene by sediment fungi. Chemosphere. 40(5):557–563.

Robles A, Lucas R, de Cienfuegos GA, Gálvez A. 2000. Biomass production and detoxification of wastewaters from the olive oil industry by strains of Penicillium isolated from wastewater disposal ponds. Bioresour Technol. 74(3):217–221.

Roussos S, Hannibal L, Aquiahuatl MA, Trejo Hernandez M del R, Marakis S. 1994. Caffeine degradation by Penecillium verrucosum in solid state fermentation of coffee pulp: critical effect of additional inorganic and organic nitrogen sources. J Food Sci Technol. 31(4):316–319.

Roussos S, De los Angeles Aquiahuatl M, del Refugio Trejo-Hernández M, Perraud IG, Favela E, Ramakrishna M, Raimbault M, Viniegra-González G. 1995. Biotechnological management of coffee pulp—isolation, screening, characterization, selection of caffeine-degrading fungi and natural microflora present in coffee pulp and husk. Appl Microbiol Biotechnol. 42(5):756–762.

Sankaran S, Khanal SK, Jasti N, Jin B, Pometto AL, Van Leeuwen JH. 2010. Use of filamentous fungi for wastewater treatment and production of high value fungal byproducts: A review. Crit Rev Environ Sci Technol. 40(5):400–449.

Saraswathy A, Hallberg R. 2002. Degradation of pyrene by indigenous fungi from a former gasworks site. FEMS Microbiol Lett. 210(2):227–232.

Saraswathy A, Hallberg R. 2005. Mycelial pellet formation by Penicillium ochrochloron species due to exposure to pyrene. Microbiol Res. 160(4):375–383.

Say R, Yilmaz N, Denizli A. 2003. Removal of heavy metal ions using the fungus Penicillium canescens. Adsorpt Sci Technol. 21(7):643–650.

Schwimmer S, Kurtzman RH. 1972. Fungal decaffeination of roast coffee infusions. J Food Sci. 37:921–924.

Simões LC, Simões M, Lima N. 2015. Kinetics of biofilm formation by drinking water isolated

Penicillium expansum. Biofouling [Internet]. 31(4):349–362. http://dx.doi.org/10.1080/08927014.2015.1042873

Siqueira V, Lima N. 2012. Surface Hydrophobicity of Culture and Water Biofilm of Penicillium spp . :93–99.

Skowroński T, Pirszel J, Skowrońska BP. 2001. Heavy metal removal by the waste biomass of Penicillium chrysogenum. Water Qual Res J. 36(4):793–803.

Subramanian SB, Yan S, Tyagi RD, Surampalli RY. 2008. A New, Pellet-Forming Fungal Strain: Its Isolation, Molecular Identification, and Performance for Simultaneous Sludge-Solids Reduction, Flocculation, and Dewatering. Water Environ Res. 80(9):840–852.

Townsley CC, Ross IS. 1985. Copper uptake by Penicillium spinulosum. Microbios. 44(178):125–134.

Vesilind P. 2003. Wastewater treatment plant design. [place unknown]: IWA publishing.

Villena GK, Fujikawa T, Tsuyumu S, Gutiérrez-Correa M. 2010. Structural analysis of biofilms and pellets of Aspergillus niger by confocal laser scanning microscopy and cryo scanning electron microscopy. Bioresour Technol [Internet]. 101(6):1920–1926. http://dx.doi.org/10.1016/j.biortech.2009.10.036

Wanner J. 1994. Activated sludge: bulking and foaming control. [place unknown]: CRC Press.

Conclusion

This thesis work presented several aspects of fungi research applicable to bioremediation procedures. It is possible to isolate fungi from matrices of the most extreme environments and to tests them to evaluate their potential in bioremediation protocols. In this specific case, fungi were isolated from landfill leachate and from a composting plant. The isolated, cultivated, identified and cryopreserved fungi have contributed to increase the collection ColD by over fifty units. The collection of the laboratory is part of the MIRRI project: in this way, the isolated strains will be part of a network of exchanges between Italian and European researchers for studies on biodiversity, genetics, environmental biotechnologies or pharmaceuticals. Viability tests on the strains stored in vivo and cryopreserved in the collection were carried out after several months up to a year and showed the effectiveness of this type of conservation.

Fungi isolated from environmental matrices can be vital and therefore resist to extreme environments: for this reason, toxicity tests are performed as the starting point for selecting the strains suitable for mycoremediation. In the first case study reported in this thesis, fungi already used in mycoremediation were isolated from the leachate but did not show an optimal yield in the leachate itself. They could still be good candidates for treating specific substances such as metals and other compounds present in large concentrations in the landfill. Further studies on the resistance to toxicity of individual substances could be the next steps: this is possible because all the strains have been cryopreserved in the collection of the mycology laboratory. The second case study involved the isolation and identification of fungal strains from another extreme environment: a composting plant. More than forty fungi were isolated, among them a new species of *Penicillium* has been identified: the strain kept also in the laboratory where the thesis was carried out is the *typus*.

The second part of the thesis concerns the development of a bioreactor for wastewater treatment. Few studies have been published concerning the development of MABR bioreactors using filamentous micro-fungi, and even fewer studies have been done on a large scale. For this reason, the study reported in this thesis started with preliminary tests regarding the verification of biofilm adhesion and formation, the choice of suitable membranes and fungi. These studies were carried out on individual fibers and on different environmental and sterility conditions. The results allowed us to move on to the next phase of construction: a laboratory-scale MABR was entirely designed, engineered and built for this thesis in the industrial chemistry laboratories. Hydraulic sealing and oxygen transfer tests were the second step. In this study the plant was tested with a model strain stored in the laboratory and a model wastewater consisting of glucose and peptone. In this way, the growth on the plant and the consumption of organic matter were controlled. The attachment of the inoculum on the fibers requires few days, after which it is possible to introduce a nutrient solution recirculated in the system.

The construction of MABR with filamentous micro-fungi requires again other tests and investigations but this study showed that this solution has a great potential because the mycelium of film-like fungi creates a dense network of hyphae that are not washed away even in the case of high water or air flow. The use of fungi for wastewater treatment is a new field yet to be explored, but we hope that this and other studies will give visibility to fungi as biodegraders.

“Research is formalized curiosity.
It is poking and prying with a purpose.”

Zora Neale Hurston

CANDIDATA **Ester Rosa**