FUNGI FROM THE SEDIMENTS OF THE HARBOUR OF LIVORNO AS POTENTIAL BIOREMEDIATION AGENTS

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Abstract – Due to anthropogenic activities, the harbour's sediments are among the biotopes most affected by pollutants (both organics and inorganics). Therefore, the polluted sediments are subjected to regular dredging that require remediation treatments. However, the growing ecological issue of sediment contamination leads to the need for economic and eco-friendly treatments. Bioremediation could be an efficient solution and fungi are among the most promising as bioremediation agents, thanks to their ability to produce extracellular enzymes such as lignin-modifying enzymes. The main objectives of this work were: i) to perform a preliminary screening on 74 fungi previously isolated in the polluted sediments of the harbour of Livorno; ii) to identify those endowed with oxidative capabilities, using MS-GP agar media supplemented with guaicol, syringaldazine and ABTS with or without seasalts; iii) to assess the production of enzymes such as laccases and/or peroxidases. The results have shown that 26 (35.1 %) out of 74 fungi produced positive oxidation signal on at least one media and 4 taxa displayed a positive oxidation of all the three indicators used, both in salt and saltless contitions, indicating their potentiality also in environments with high salt concentrations, such as the marine sediments. However, further studies are needed to fully identify the enzymes and their degradative capabilities.

Introduction

The Mediterranean Sea is strongly influenced by human activities with consequent pollution of the coastal marine environment. The seabed of harbours is continuously exposed to the effects of this type of pollution. In fact, these sediments store a wide range of pollutants (e.g. polycyclic aromatic hydrocarbons, PAHs, or heavy metals), derived from several activities [1]. To maintain the harbour depth suitable for navigation, these sites are subjected to regular dredging. However, the dredged polluted sediments require remediation treatment to be recovered. Generally, physico-chemical remediation techniques are used but they can be very costly and have several environmental drawbacks. Therefore, it is necessary to find more economic and environmentally-friendly remediation solutions. The biological remediation techniques could guarantee these prerogatives and the components of this biodegradation process are mainly bacteria and fungi [2].

Although most of the literature concerning organic pollutants biodegradation is focused on bacteria [3–5], fungi have become of great interest for bioremediation purposes, both in soil and marine habitats [6]. Indeed they are able to transform and/or degrade many hazardous

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and polluting chemicals thanks to their wide enzymatic production [7], to explore (contaminated) sediments thanks to the apical growth of their hyphal network [8] and adsorb hydrocarbons in low nutrient and pH environments [9]. Several genera of marine fungi have shown the ability to degrade recalcitrant compounds like aliphatic and aromatic hydrocarbons [10], while others (mainly earthborne basidiomycetous white rot fungi-WRF) have already been used for decontaminating polluted sites [11]. Indeed fungi isolated from the sea can be effective in the degradation of petroleum hydrocarbons [12], and, although poorly represented in marine environment, Basidiomycota might have a great biotechnological potential [13]. Hydrocarbon degradation, in aerobic conditions, involves a wide array of enzymes, the most studied of whitch are P450 monooxygenases and alkane-oxygenases. However the extracellular enzymes are the most promising for biodegradation purposes, particularly the lignin-modifying enzymes (LMEs) of the WRF [14]. The main LMEs enzymes are lignin-peroxidase (LiP), manganese-peroxidase (MnP), as regards peroxidase and laccase, divided into low potendial (LP) and high potential (HP) laccase according to their oxidative potential. As reported by Panno et al.[15], marine fungi can produce them in high-salinity conditions, such as marine sediments.

To assess the production of LMEs, it is common to use indicators capable of showing the oxidative abilities of fungi following colour shift of their growth media. Examples of indicators are 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), guaiacol (GCL), syringaldazine (SGZ) and, industrial dyes (e.g. Remazon Brilliant Blue R, Malachite green) [16]. Dyes are both indicators for these screenings, and pollutants. They have been known and used for long time, and in the last century many new dyes have been produced by chemical synthesis. Therefore, whether the final purpose is the remediation of marine sediments or the decolouration of industrial dyes, fungi have proved to be interesting organisms endowed with metabolic and enzymatic abilities adaptable to these purposes. In particular for port sediments, researchers share the idea that new fungi potentially exploitable for bioremediation applications, should be sought in the polluted sediments (or sites) themselves, as they should be adapted to the stresses and contaminants present [17,18].

The purpose of this work is: i) to perform a preliminary screening on fungi previously isolated from the sediments of a polluted port area, in order to identify fungal strains endowed with oxidative abilities; and ii) to evaluate the producers of metabolites or enzymes of interest, for applications in future environmental bioremediation.

Materials and methods

Sediment sampling and characterization

Marine sediment of the Yacht Club in the harbour of Livorno (43°33'00.1" N 10°17'51.8" E) were sampled by the "Interuniversity Consortium of Marine Biology G. Bacci, CIBM" of Livorno. Fungi were isolated and identified, according to Bovio et al. [19], at the *Mycotheca Universitatis Taurinensis* (MUT, University of Torino) and 74 strains (Table 1) were screened for LMEs production. The chemical analysis of the sediments was performed by CIBM (Table 2). Polycyclic aromatic hydrocarbons (PAHs as total content and 16 EPA congeners), and polychlorinated biphenyls (PCBs as total content) were extracted according to Salem et al. [20]. The extracts were then processed according to the method EPA 8270E [21].

The LOD was 0.5 µg/kg. The recovery rate was always over 89 %. Low molecular weight hydrocarbons (C<10) were determined following the method EPA5021A [22] and EPA 8015C [23]. High molecular weight hydrocarbons (C>10) were extracted and measured according to the method ISO 16703 [24] for mineral oils. C<10 and C>10 analyses were performed with a GC Trace 1300 (Thermo Scientific) equipped with a TG-5SilMS column (Thermo Scientific). The GC was coupled to a TriPlus RSH autosampler and flame ionization detector (FID). In all cases highly pure helium was used as carrier gas. For C<10 the LOD was 0.5 µg/kg, for C>10 the LOD was 1.6 mg/kg. The recovery rate was always over 90 %.

Table 1 – Fungal strains tested for LMEs production.

Acremonium pilosum	*Dichotomopilus funicola*	*Penicillium* sp.
Acrostalagmus luteoalbus	*Discula destructiva*	*Phaeosphaeriaceae* sp.
Alternaria alternata	*Emericella pluriseminata*	*Pholiota gummosa*
Amesia nigricolor	*Emericellopsis minima*	*Preussia* sp.
Annulohypoxylon multiforme	*Eupenicillium crustaceum*	*Pseudeurotium bakeri*
Ascomycota sp. 1	*Exophiala xenobiotica*	*Pseudeurotium ovale*
Ascomycota sp. 2	*Gaeumannomyces graminis*	*Pyrenochaetopsis tabarestanensis*
Ascomycota sp. 3	*Massarina* sp.	*Sporothrix inflata*
Ascomycota sp. 4	*Microascus paisii*	*Stachybotrys chlorohalonata*
Ascomycota sp. 5	*Microascus* sp.	*Stachylidium bicolor*
Aspergillus aureolatus	*Neocosmospora solani*	*Talaromyces flavus*
Aspergillus flavipes	*Parasarocladium debruynii*	*Talaromyces minioluteus*
Aspergillus fumigatus	*Parasarocladium radiatum*	*Talaromyces minioluteus*
Aspergillus heyangensis	*Parasarocladium wereldwijsianum*	*Talaromyces versatilis*
Aspergillus pseudodeflectus	*Penicillium antarcticum*	*Talaromyces wortmannii*
Aspergillus pseudoglaucus	*Penicillium crustosum*	*Talaromyces wortmannii*
Aspergillus tabacinus	*Penicillium fellutanum*	*Thelebolus* sp.
Aspergillus templicola	*Penicillium glabrum*	*Tilachlidium brachiatum*
Aspergillus terrreus	*Penicillium janczewskii*	*Trematosphaeria grisea*
Aspergillus ustus	*Penicillium javanicum*	*Trematosphaeria grisea*
Aspergillus versicolor	*Penicillium menonorum*	*Trichoderma harzianum*
Auxarthron thaxteri	*Penicillium paneum*	*Trichoderma longibrachiatum*
Beauveria felina	*Penicillium parvulum*	*Wardomycopsis humicola*
Cladosporium asperulatum	*Penicillium restrictum*	*Westerdykella dispersa*
Cladosporium cladosporioides	*Penicillium simplicissimum*	

Table 2 – Organic chemicals in sediments (LOD, limit of determination, for PAHs and PCBs 0.5 µg/kg, C >10 5 mg/kg, C<10 0.5 µg/kg ; U.O.M = unit of measurement).

Parameter	U.O.M. d.w.	Harbour
Σ PAHs	µg/kg	577.2
Acenaphtene	µg/kg	< LOQ
Acenaphthylene	µg/kg	4.1
Anthracene	µg/kg	3.9
Benzo[*a*]anthracene	µg/kg	40.4
Benzo[*a*]pyrene	µg/kg	51.2
Benzo[*b*]fluoranthene	µg/kg	120.4
Benzo[*ghi*]perylene	µg/kg	53.2
Benzo[*k*]fluoranthene	µg/kg	43.4
Chrysene	µg/kg	45.8
Dibenz[*a,h*]anthracene	µg/kg	34.5
Fluoranthene	µg/kg	55.9
Fluorene	µg/kg	< LOQ
Indeno[1,2,3-*c,d*]pyrene	µg/kg	81.2
Naphthalene	µg/kg	< LOQ
Phenanthrene	µg/kg	4.6
Pyrene	µg/kg	38.4
Σ PCBs	µg/kg	19.1
Hydrocarbons > 10C	mg/kg	116.5
Hydrocarbons < 10C	µg/kg	12.6

LMEs production screening

The screening was conducted using MS-GP agarised solid media (5.0 g/L glucose, 5.0 g/L peptone, 1.0 g/L KH_2PO_4, 1.0 g/L ammonium acetate, 0.01 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, 0.001 g/L $MnSO_4$, 0.001 g/L $FeSO_4 \bullet 7H_2O$, 0.0005 g/L $CuSO_4$, 3 % agar, at pH 6.0), in salted (40 g/L Sea Salts) and saltless lines, supplemented with different indicators: 1 mM SGZ, 1 mM ABTS or 1 mM GCL (redox potential 0.39 V, 0.48 V and 0.8 V, respectively). The LMEs production activity was defined as low (+, barely detectable), medium (++, clear and measurable halo) and high (+++, fully extended and intense halo) depending on the colour shift due to the enzyme activities. Fungi were inoculated by agar plugs of about 3 mm diameter into 35 mm Ø 6 wells plates and incubated at 25 °C. Plates were checked at days 1, 3, 6, 8 and 14; oxidation halos were measured where present. All the chemicals were purchased from Sigma-Aldrich (Merck Group KGaA, Darmstadt, Germany). All further analyses and graphics were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). All fungal strain that displayed positive result is currently preserved at MUT (http://www.tucc.unito.it/it/content/collezione-mycotheca-universitatis-taurinensis) for further investigations.

Results and discussion

Seventy-four fungal strains were screened for their LMEs production and 26 of them (35.1 %) gave positive oxidation signal on at least one of the media supplemented with the three different indicators, revealing LME production both in salted (SS+) and saltless (SS-) media (Table 3).

Eleven fungi (42.3 %) were considered LP laccases producers following the oxidation of SGZ in the SS- line, and 6 of them were able to produce these enzymes also in the SS+ line. In particular, *Westerdykella dispersa* and *Alternaria alternata* showed similar production in SS- and SS+ lines, indicating that these fungi, regarding the LP laccases production, were unaffected by the osmotic stress in the SS+ lines. Twenty-three fungi (88.5 %) were considered HP laccases producers following the oxidation of ABTS in the SS- line, and 16 were able to produce these enzymes also in the SS+ line. Interestingly, almost all the fungi investigated produced this class of enzymes, and only three (*Ascomycota* sp.2, *Penicillium antarcticum* and *Trichoderma harzianum*) produced peroxidases, which have higher potential than the HP laccases. Seven strains (*Penicillium fellutanum*, *Pyrenochaetopsis tabarestanensis*, *Acrostalagmus luteoalbus*, *Emericella pluriseminata*, *Microascus* sp., *Ascomycota* sp.4 and *Alternaria alternata)* showed similar performances in the SS- line and SS+ line, indicating their activity, in producing HP laccases, despite the osmotic stress. Finally, 15 fungi (57.7 %) were considered peroxidases producers following the oxidation of GCL in the SS- line, and 8 were able to produce these enzymes also in the SS+ line. Among the latter, 4 fungi (*Trichoderma harzianum*, *Ascomycota* sp.2, *Microascus* sp. and *Acrostalagmus luteoalbum*) performed similarly in the SS- than SS+ lines. Extremely interesting is *Alternaria alternata*, which displayed an higher peroxidase producion in the SS+ line in comparison to the SS- line, underlining its adaptation to the marine environment, as the high salt concentration has promoted its enzymatic production.

Among the LMEs producing strains, eight (*Westerdykella dispersa*, *Acrostalagmus luteoalbus*, *Emericella pluriseminata*, *Microascus* sp., *Stachybotrys chlorohalonata*, *Pholiota gummosa*, *Ascomycota* sp. 4, *Alternaria alternata*) displayed a positive oxidation of all the three indicators used, thus indicating the production of LP laccases, HP laccases and peroxidases in saltless conditions. Of these, four (*Westerdykella dispersa*, *Acrostalagmus luteoalbus*, *Microascus sp*., *Alternaria alternata*) produced the three classes of enzymes also in the SS+ lines, indicating the ability to produce LMEs also in environments characterised by high saline concentration (Table 3). Ascomycota are commonly associated with decaying mangroves leaves and seagrasses in marine environment and some of them are well-known producers of LMEs [25,26]. In line with this, among the best performing fungi here reported, seven trains out of eight are Ascomycota. Many recent works highlight LMEs production and potential applications of their enzymes. Toker *et al*. [27], assessed the dye decolourisation performances of six fungal strains (*Phoma* sp.1, *Phoma* sp.2, *Alternaria* sp.1, *Alternaria* sp.2, *Cadophora* sp. and *Cadophora luteo-olivacea*) isolated from surface water, sediment, algae and woody root samples collected in a lagoon. Interestingly, fungi belonging to the same genera (or specie) have been sampled by this work too, and exactly as Toker's research, one *Alternaria* is among the most promising strains, suggesting a high LMEs production by marine fungi belonging to this genus. No literature assessed *E. pluriseminata* LMEs production, although two works [28] confirmed high LiP, laccase and lignin degrading activity in soilborne *E. nidulans*, a close relative. Hence, the genus *Emericella* could be worth

Table 3 – Fungal taxa positive for LMEs production) in SS± and intensity of their activities (reported as low +, medium ++, or high +++). (LP lac = LP laccase, HP lac = HP laccase, Peroxi = peroxidase.

TAXA	SS- ENZYMES	SS+ ENZYMES
Acrostalagmus luteoalbus	LP lac +++ HP lac +++ Peroxi +	LP lac ++ HP lac +++ Peroxi +
Alternaria alternata	LP lac +++ HP lac +++ Peroxi +	LP lacc +++ HP lac +++ Peroxi +++
Amesia nigricolor	HP lac +	-
Ascomycota sp. 2	Peroxi +++	Peroxi +++
Ascomycota sp. 4	LP lac + HP lac+++ Peroxi+++	HP lac +++ Peroxi+
Ascomycota sp.5	LP lac + HP lac +++	HP lac +
Aspergillus pseudodeflectus	HP lac+++	HP lac +
Cladosporium asperulatum	HP lac +++	HP lac +
Cladosporium cladosporioides	LP lac ++ HP lac +++	LP lac + HP lac +
Discula destructiva	HP lac +++ Peroxi +++	HP lac + Peroxi +
Emericella pluriseminata	LP lac ++ HP lac +++ Peroxi +	HP lac +++
Emericellopsis minima	HP lac +++	HP lac +++
Gaeumannomyces graminis	HP lac + Peroxi +++	-
Massarina sp.	LP lac +++ HP lac+++	LP lac ++
Microascus sp.	LP lac ++ HP lac +++ Peroxi ++	LP lac + HP lac +++ Peroxi++
Neocosmospora solani	HP lac +++	-
Parasarocladium radiatum	HP lac + Peroxi +	-
Penicillium antarcticum	Peroxi ++	-
Penicillium fellutanum	HP lac +	HP lac +
Pholiota gummosa	LP lac +++ HP lac +++ Peroxi +++	-
Pyrenochaetopsis tabarestanensis	HP lac +++	HP lac +++
Stachybotrys chlorohalonata	LP lac + HP lacc +++ Peroxi+++	-
Talaromyces flavus	HP lac +++	HP lac +
Trichoderma harzianum	Peroxi+	Peroxi +
Wardomycopsis humicola	HP lac +++ Peroxi+	HP lac +
Westerdykella dispersa	LP lac +++ HP lac +++ Peroxi ++	LP lac ++ HP lac ++ Peroxi +

of deeper investigation. *W. dispersa* is a known source of interesting new secondary metabolites [29] (e.g. alkaloids), but low or none LMEs activity has been reported by Da Silva *et al*. [30] against PAHs.

This result was confirmed by de la Cruz-Izquierdo *et al*. [31] that reported only low LiP production by a soilborn *Westerdykella* sp. isolate. As stated for *S. chlorohalonata*, probably the origin of *W. dispersa* has been a determinant factor on its LMEs production. As *W. dispersa*, the genus *Microascus* has been studied for its metabolites and LMEs production [32]. Raybarman *et al*. [33] detected LMEs activity of a strain of *Microascus* sp. on coir fibres, with production of laccases, Mn and LiP. Although this report did not manage to identify the strain at species level, the genus *Microascus* remains consistent with the cited work and should be studied more carefully for its biodegradative performances. Although marine *A. luteoalbus* LMEs production is not yet reported in literature, many papers deal with its production of unusual metabolites [34]. This paper instead outlines the production of many lignin-modifying enzymes (peroxydases, LP and HP laccases) both with and without Sea Salts, indicating its biodegradative potential. Finally, *Pholiota gummosa* is the only Basidiomycota of the eight most performing fungal strains assessed. The genus *Pholiota* is known for producing LMEs [35]; the ability of this particular species to produce the three classes of enzymes investigated, has been reported here for the first time.

Conclusion

The fungal community of the Livorno's harbour sediments has shown strong oxidative abilities on model chemicals, indicating an adaptation to the polluting conditions present in the port area. Indeed, this screening shows that 26 out of 74 tested fungi can produce enzymes that modify lignin and that could degrade organopollutants (PAHs and PCB). Eighteen strains produced LME in high salinity conditions, meaning that i) they were fully adapted to the marine environment, and ii) they have the enzymatic potential to degradate most of the aromatic pollutants which characterise the harbour sediments. These LME producing strains potentially represent the starting point to create microbial consortia suitable for bioremediation approaches. Moreover, these fungi are a source of new extremozymes that can find application in future research and in several industrial fields.

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