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Selective factors involved in oil flotation isolation of black yeasts from the environment

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Abstract: The oil flotation isolation technique has been successfully applied to recover chaetothyrialean black yeasts and relatives from the environment. The selective mechanisms playing a role in isolation are unknown. The fungi concerned are supposed to occupy specialized microniches in nature, taking advantage of (1) oligotrophism. Mineral oil as a main selective agent may be based on (2) hydrophobicity or on (3) assimilation. All three hypotheses are tested in this paper. Results show that cell wall hydrophobicity is unlikely to be a selective factor. Incubation under poor nutrient conditions provides competitive advantage for black yeasts, especially for *Exophiala* strains, which are subsequently enriched by mineral oil which enhances growth in this group of fungi. Incubation under mineral media and mineral oil can be used as selective factor.

Key words: Black yeasts, oil flotation, polluted soil, selective isolation.

INTRODUCTION

Black yeasts belonging to the *Chaetothyriales* are infrequently isolated from the environment. Recent studies have shown, however, that if selective methods are applied, these fungi may be encountered in a wide diversity of environments (Badali *et al.,* 2008). Special attention has been paid to environments rich in hydrocarbons, because there are indications that these compounds can be used as substrates by black yeast and filamentous relatives (Prenafeta-Boldú *et al*. 2006); the fungi may have a significant potential for bioremediation. Particularly difficult is isolation of black yeasts from natural sources, classical techniques usually revealing only a limited number of strains (Iwatsu *et al.* 1981, Marques *et al.* 2006, Vicente *et al.* 2001). This is supposed to be due to the abundance of rapidly growing saprobes in the same samples. This is one of the reasons that knowledge on the distribution of this group of fungi is still incomplete (Marques *et al*. 2006).

The oil flotation technique has been reported as an effective method for isolation of chaetothyrialean black yeasts (Dixon *et al.* 1980, Gezuele *et al*. 1972, Iwatsu *et al.* 1981, Richard-Yegres *et al.* 1987, Vicente *et al.* 2001, Marques *et al.* 2006). This technique applies mineral oil in the procedure. The black yeast and relatives are able to assimilate monoaromatic hydrocarbons and are promoted in environments rich in these compounds (Prenafeta-Boldú *et al.* 2002, Sterflinger & Prillinger 2001, Woertz *et al.* 2001), so mineral oil – a complex mixture of petrol hydrocarbons – could act as an enrichment factor favoring their isolation. Another hypothesis is that the cells of black yeast and relatives are hydrophobic and remained on the interphase solution-oil which inoculum is subsequently plated. The other hypothesis, not related to the mineral oil, is that black yeasts could continue grow in poor nutrient media, like the solution of this technique, due to their oligotrophic metabolism. So the aim of this article is to verify which selective factor is determinant for the isolation of black yeast and relatives in this technique. Such approach is of great relevance because it raises useful information for the improvement of black yeast isolation methods and consequently the better understanding of the ecology of this group.

MATERIAL AND METHODS

Sampling area

The soil samples were collected in a landfarming area of Paulínia Oil Refinery (REPLAN) in São Paulo state, Brazil (-22.726213 latitude, -47.135259 longitude). This site receives large amounts of waste petrol hydrocarbons. Samples were collected from different regions of the landfarming cell at 0–10 cm depth using sterile lab tools, they were placed in plastic bags and maintained at 4 ºC until use. In the laboratory the samples were homogenised and processed within a period of 2–15 d.

Fungal isolation

The oil flotation technique was based in previous studies (Iwatsu *et al.* 1981, Marques *et al.* 2006, Vicente *et al.* 2001). Twenty g soil sample was added to a sterile Erlenmeyer flask (250 mL) with 100 mL saline containing 200 U/mL penicillin; 200 µg/mL chloramphenicol; 200 µg/mL streptomycin and 500 µg/mL cycloheximide. The solution was homogenised and incubated for 30 min at 20–22 ºC. Subsequently 20 mL mineral oil was added, followed by vigorous vortexing for 5 min. Flasks were allowed to settle for 20 min. Aliquots from the oil/saline interphase were plated

Table 1. Composition of the media in different culture conditions of the assimilation test (volumes in µL).

	Culture Condition				
Component	**A**	**B**	**C**	**D**	**E**
Cell suspension (1.0 × 10^6 cells/mL)	40	40	40	40	40
Basal growth media	360	280	280	---	---
Mineral oil	---	80	---	360	---
n-Hexadecane	---	---	80	---	360
Total	400	400	400	400	400

on Mycosel agar and incubated at 28 °C until dark, slow-growing colonies appeared (about 4 wk). Colonies were purified by plating and transferred to slants with 2 % malt extract agar (MEA) incubated at 28 °C for 2 wk and then, they were maintained at 4 °C.

Morphology

Macroscopic morphology was observed by growing isolates on MEA at 28 ºC. Slide cultures were prepared with strains grown on MEA at 28 ºC and mounted in lactophenol cotton blue.

Molecular identification

About 1 cm^2 mycelium of 20 to 30-d-old cultures was transferred to a 2 mL Eppendorf tube containing 400 µL TEx buffer (pH 9.0) and about 80 mg of glass beads (Sigma G9143). Samples were homogenised for 1 min in MoBio vortex and subsequently 120 µL SDS 10 % and 10 µL Proteinase K (Merck 124568) were added, the mixture was vortex and incubated in water bath for 30 min at 55 °C. The samples were vortex again for 3 min on MoBio vortex. Then, 120 µL 5 M NaCl and 0.1 vol CTAB (hexadecyltrimethylammoniumbromide, Sigma H-5882) 10 % was added and the tubes were incubated in a water bath for 1 h at 55 ºC. Subsequently, samples were submitted to agitation in MoBio vortex for 3 min. One vol of SEVAG was added and it was carefully mixed by hand, inverting the flasks 50 times. Then, the solution was centrifuged at 20 400 g, 4 ºC for 5 min and the supernatant was transferred to a new tube. 225 µL 5 M NH_4-acetate was added and mixed carefully by inverting. After 30 min incubation on ice water samples were centrifuged for 5 min at 4 ºC at 20 400 g. Supernatant was transferred to a new Eppendorf tube and 0.55 vol isopropanol was added and mixed carefully. Samples were incubated at –20 ºC for 1 h and centrifuged for 5 min at 20 400 g. The supernatant was decanted and the pellet was washed with cold 70 % EtOH. After drying at room temperature it was resuspended in 100 µL TE-buffer and incubated for 5 min at 37 °C prior to storage at –20 °C. rDNA Internal Transcribed Spacer (ITS) was amplified using primers V9G and LS266 and sequenced with ITS1 and ITS4. Amplicons were cleaned with GFX PCR DNA and gel band purification kit (GE Healthcare, U.K.). Sequencing was performed on an ABI 3730XL automatic sequencer. Sequences were edited using the SEQMAN package (DNAStar Inc., Madison, United States of America) and aligned using BIONUMERICS v. 4.61 (Applied Maths, Kortrijk, Belgium).

Preparation of cell suspensions

Stock cultures were transferred to 5 MEA slants and incubated for 10–15 d at 28 °C. Five millilitres physiological salt solution was added to the grown culture and vortexed for 1 min. Aliquots were filtered and cell densities were measured using a Neubauer's counting chamber. Cell concentration was adjusted by adding physiological salt solution, mineral medium or inoculum.

Hydrophobicity

Six isolates and three reference strains from CBS collection were tested. The test was an adaptation of methodology of Göttlich *et al*. (1995). One millilitres of n-hexadecane or mineral oil was added to a 20 mL glass flask with rubber cap containing 5 mL of cell suspension and submitted to vigorous vortexing for 30 s. The flask was allowed to settle for 2 min for the complete separation of the phases. A small volume of the aqueous phase was removed with a 1 mL syringe and the cell density in the aqueous phase was established by visual counting using a Neubauer's counter chamber. The procedure was repeated at least five times for each culture condition and the results were expressed as the percentage of cells remained in the aqueous phase. Values above 50 % were considered to indicate hydrophily, whereas with values below 50 % the strain was considered to be hydrophobic.

Assimilation

Mineral oil and n-hexadecane assimilation tests were performed with a spectophotometer Bioscreen C (Labsystems, Helsinki, Finland). Yeast nitrogen base (Difco) was used as basal growth medium. Mineral oil and n-hexadecane were filter-sterilised. Five different culture conditions were established: Test A: only mineral medium (Yeast Base Nitrogen 0.65 % YNB); B: YNB + 20 % mineral oil; C: YNB +20 % n-hexadecane; D: YNB + 80 % mineral oil; E: YNB + 80 % n-hexadecane. The volumes of the reagents are summarised in Table 1. Each culture condition was done with five replications. Initial densities of cell suspensions were set to 1.0 x 10^6 cells/mL. Control tests without inocula were done to measure the blank (absorbance of the media) in order to compare the readings of the different culture conditions. The Bioscreen was set to maintain a temperature of 28 °C for 7 d with continuous shaking and absorbance reading at 540 nm every 2 h. Data were registered automatically. Growth curves were done with values resulted of the absorbance readings discounted of the blank, so the values shown in the graphics refer only to the growth of the strain in different culture conditions.

Oligotrophism

The same procedure of the assimilation test was used, but basal growth media was replaced by physiological salt solution.

Table 2. Molecular identification of melanised isolates from oil-polluted soil.

Accession no.	Identification based on ITS
dH 18460	*Cladophialophora minourae*
dH 18466 / CBS 122275	*Cladophialophora minourae*
dH 18463	*Cladophialophora immunda*
dH 18465 / CBS 122257	*Cladophialophora immunda*
dH 18468 / CBS 122253	*Cladophialophora immunda*
dH 18469	*Cladophialophora immunda*
dH 18471 / CBS 122255	*Cladophialophora immunda*
dH 18473 / CBS 122636	*Cladophialophora immunda*
dH 18474	*Cladophialophora immunda*
dH 18476	*Cladophialophora immunda*
dH 18477	*Cladophialophora immunda*
dH 18478	*Cladophialophora immunda*
dH 18462	*Cladosporium halotolerans*
dH 18458 / CBS 122258	*Exophiala xenobiotica*
dH 18459	*Exophiala xenobiotica*
dH 18461	*Exophiala xenobiotica*
dH 18464	*Exophiala xenobiotica*
dH 18467	*Exophiala xenobiotica*
dH 18470	*Exophiala xenobiotica*
dH 18472	*Exophiala xenobiotica*
dH 18475	*Exophiala xenobiotica*

RESULTS

Isolation

A total of 107 strains suspected to belong to chaetothyrialean black yeast and relatives were isolated from three landfarming soil samples. 20 of them were identified based on molecular techniques and one appeared to be a *Cladosporium* species (*Capnodiales*). The remaining strains belonged to at least three different species (Table 2). *Exophiala xenobiotica* was the preponderant species, while also *Cladophialophora minourae* was isolated. Eleven *Cladophialophora* strains did not match with any known species, neither in GenBank nor in a research database containing about 7,000 black yeast sequences maintained at CBS. Their nearest, undescribed neighbours in the latter database all originated from environments rich in hydrocarbons or had been isolated using alkylbenzene enrichment method.

Tested chaetothyrialean black yeast-like fungi (*Cladophialophora* and *Exophiala*) differentially responded to our hydrophobicity test (Table 3). The n-hexadecane test, applied previously to fungi by Göttlich *et al*. (1995) was validated using the same control strains, viz. hydrophilic strain *Rhodotorula graminis* (CBS 2826) and hydrophobic strain *Penicillium chrysogenum* (CBS 776.95). The strains responded as expected, viz. with very few *versus* many cells remaining in the oil phase. Isolated black yeast-like strains responded differentially to culture condition: *Cladophialophora*-type strains were hydrophobic, whereas *Exophiala*-type strains (strains of *Exophiala xenobiotica* CBS 122258 and CBS 118157) proved to be hydrophilic.

In the strains from genus *Cladophialophora*, high hydrophobicity values were observed and they were higher with n-hexadecane except strain CBS 122255.

Table 3. Identification, CBS numbers and cell density results of hydrophobicity tests.

			Cell density		
			Initial		
Species	**Accession no.**	**Culture Condition**	**(1.00×10^7)**	**Aqueous phase**	**Oil phase**
Penicillium chrysogenum	CBS 776.95	Mineral oil	100 %	2.40 %	97.60 %
		n-Hexadecane	100 %	2.81 %	94.75 %
Rhodotorula graminis	CBS 2826	Mineral oil	100 %	79.91 %	20.81 %
		n-Hexadecane	100 %	85.98 %	14.01 %
Cladophialophora minourae	CBS 122275 = dH 18466	Mineral oil	100 %	5.25 %	94.75 %
		n-Hexadecane	100 %	3.30 %	96.70 %
Cladophialophora immunda	CBS 122253 = dH 18468	Mineral oil	100 %	31.91 %	68.08 %
		n-Hexadecane	100 %	17.17 %	82.82 %
	CBS 122257 = dH 18465	Mineral oil	100 %	16.93 %	83.07 %
		n-Hexadecane	100 %	10.22 %	89.78 %
	CBS 122255 = dH 18471	Mineral oil	100 %	25.80 %	74.20 %
		n-Hexadecane	100 %	36.92 %	63.08 %
	CBS 122636 = dH 18473	Mineral oil	100 %	47.27 %	52.73 %
		n-Hexadecane	100 %	32.40 %	67.60 %
Exophiala xenobiotica	CBS 118157	Mineral oil	100 %	55.20 %	44.80 %
		n-Hexadecane	100 %	56.70 %	43.30 %
	CBS 122258 = dH 18458	Mineral oil	100 %	67.70 %	32.30 %
		n-Hexadecane	100 %	83.75 %	16.25 %

Assimilation of mineral oil and n-hexadecane

The readings of 80 % hydrocarbon concentration were not considered because at this percentage an interference on the absorbance occurred. Growth curves of the strains in culture conditions A, B and C (values with blank discounted) are shown (Figs 1–3).

Strains not belonging to the black yeast group: *Rhodotorula graminis* (CBS 2826) and *Penicillium chrysogenum* (CBS 776.95) showed minimum growth in culture conditions A, B and C (Figs 1–3). Similar growth was observed for the black yeast strain CBS 122275. Ability to use hydrocarbons as sole source of carbon and energy seemed to be absent for these strains.

Cladophialophora sp. CBS 122255 showed growth in all culture conditions, however its growth was higher in culture condition A, without hydrocarbon (Fig. 1). Better growth with hydrocarbon as sole source of carbon and energy was observed in strains

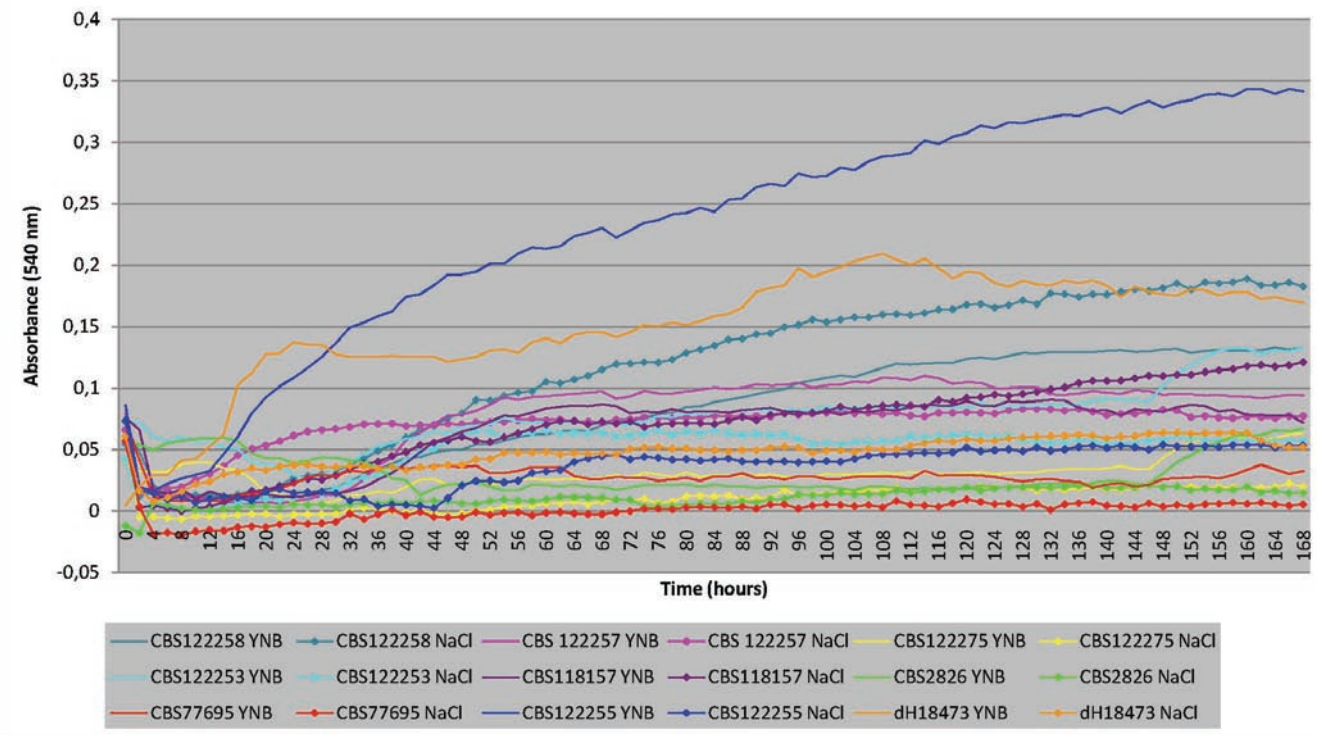

Fig. 1. Culture condition A: growth curves of studied strains in mineral medium, YNB (continuous line), or in physiological salt solution, NaCl (line and dots).

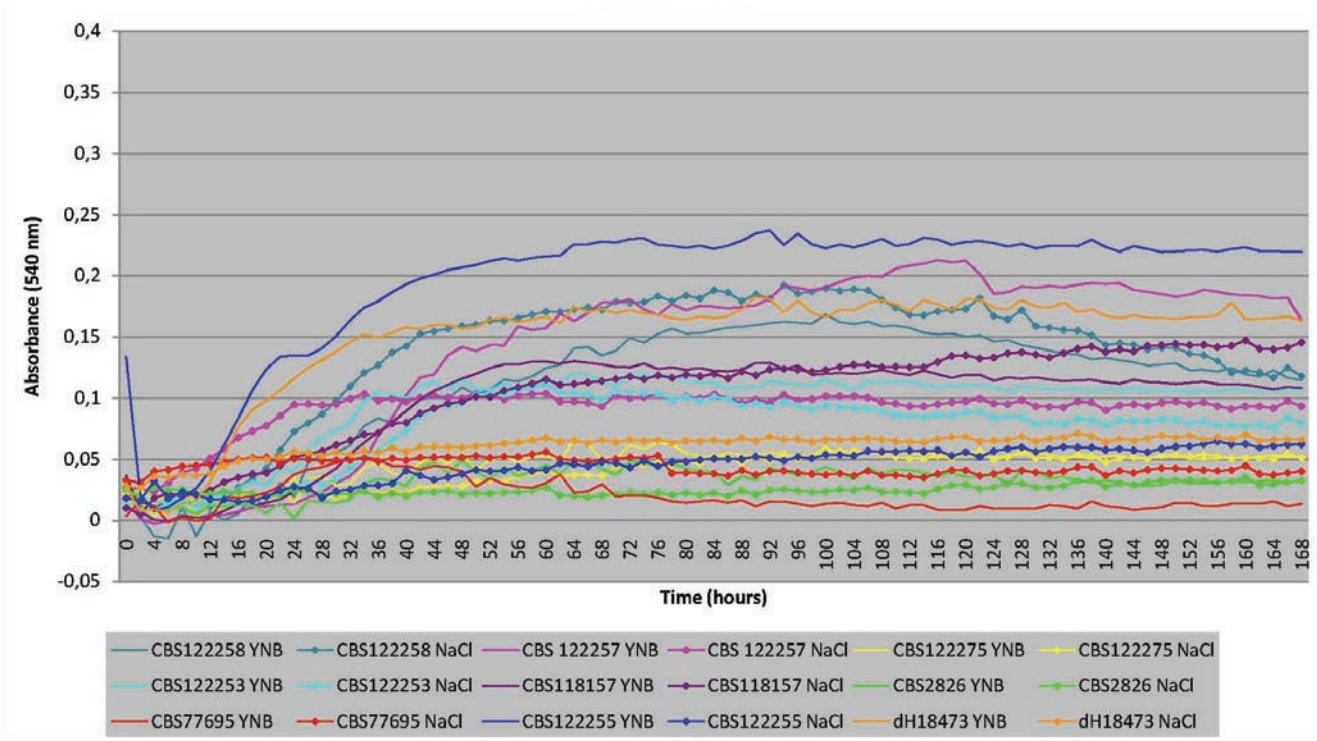

Fig. 2. Culture condition B: growth curves of studied strains in mineral medium (YNB) + 20 % mineral oil (continuous line), and in physiological salt solution (NaCl) + 20 % mineral oil (line and dots).

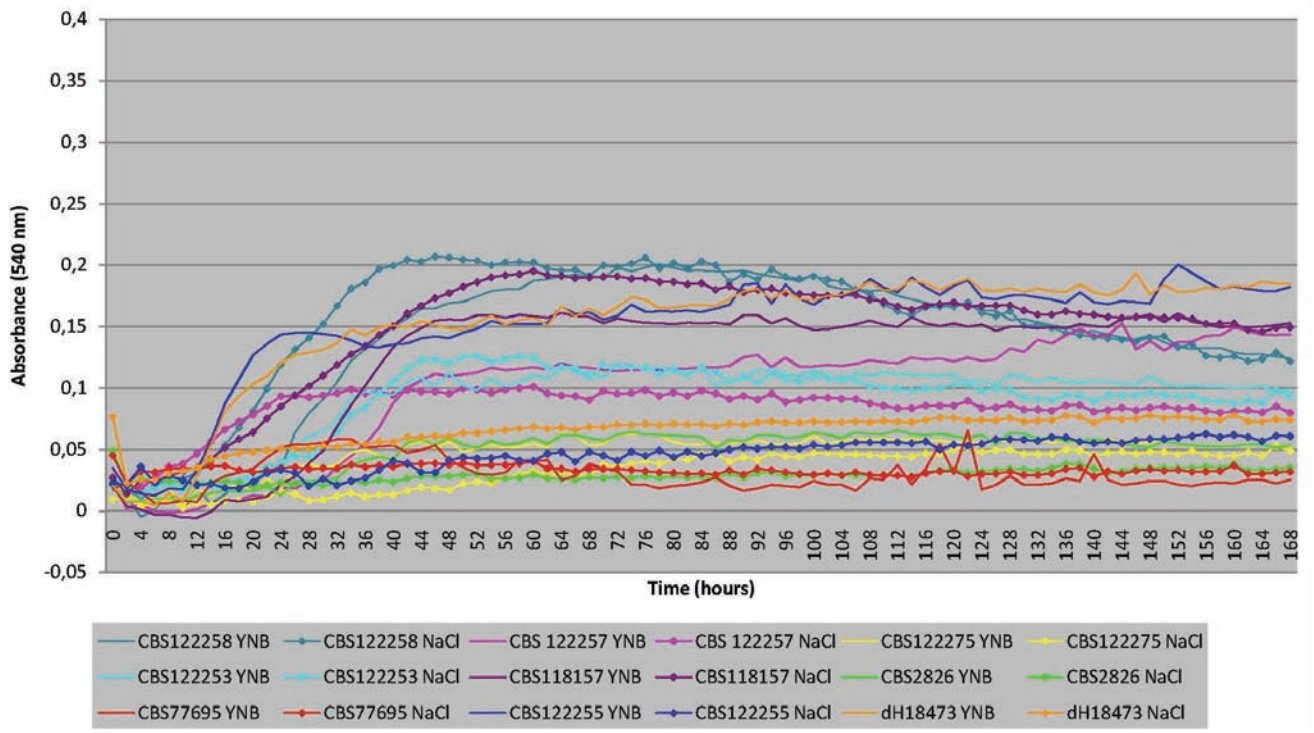

Fig. 3. Culture condition C: growth curves of studied strains in mineral medium (YNB) + 20 % n-hexadecane (continuous line), and in physiological salt solution (NaCl) + 20 % n-hexadecane (line and dots).

CBS 122253, CBS 122257, CBS 122258 and CBS 118157. The difference between the growth rates in culture conditions B and C were small: strain CBS 122253 and CBS 122258 showed the same rates for both culture conditions; higher growth rates with mineral oil was observed for strain CBS 122257; and strain CBS 118157 seemed to be stimulated by adding n-hexadecane (Figs 2–3). Strain dH 18473 had similar growth in culture conditions A, B and C (Figs 1–3).

Oligotrophism

When growth was tested in physiological salt solution, several black yeasts and relatives (CBS 122258; CBS 122257; CBS 122253; CBS 118157) were able to grow for few ds. However, growth rates were lower than in assimilation tests as presumed due to the poor concentration of nutrients. Stationary phase was mostly reached after 36−68 h. *Cladophialophora minourae* (CBS 122275) and *Cladophialophora* sp. (dH 18473) showed the same pattern as reference strains (*Rhodotorula graminis* and *Penicillium chrysogenum*): they all did not have significant growth in NaCl (Figs 1–3). Surprisingly some growth curves in mineral oil and n-hexadecane 20 % from oligotrophism tests were higher than observed in assimilation tests. Strain CBS 122258 and CBS 118157 (both *Exophiala xenobiotica* strains) continued growth at a minimal level. Addition of 20 % mineral oil slightly stimulated growth of black yeast-like fungi in that stationary phase was reached somewhat earlier (Fig. 2). Addition of 20 % n-hexadecane stimulated growth in *Exophiala* strains that performed best in previous tests; clear exponential and stationary phases were distinguishable (Fig. 3). Strains CBS 122255 and dH 18473 also did not show significant growth in NaCl. Strains CBS 122257 and CBS 122253 showed ability to grow in media poor in nutrients; growth levels were not so high as for *Exophiala* strains but were higher than in *Cladophialophora* sp.

DISCUSSION

Previous studies on black yeasts isolation using the oil flotation technique reported that soil was poor in black yeast (Iwatsu *et al.,* 1981; Marques *et al*., 2006; Vicente *et al.,* 2001), but in the present article using polluted soil, a large number of isolates was obtained. In these researches few black yeast strains per sample were recovered: Iwatsu *et al.* (1981) isolated 83 dematiaceous strains from 177 samples; Vicente (2000) obtained 81 strains from 540 samples and Marques *et al.* (2006) 9 isolates from 68 samples. In this study 107 strains were isolated from 3 samples. Hence it may be concluded that hydrocarbons present in the soil might favor the growth of the black yeasts and function as an enrichment factor, as supposed earlier (Prenafeta-Boldú *et al.* 2002, 2006, Sterflinger & Prillinger 2001) or inhibited growth of competing species. Judging from the number of strains recovered, and the number of strains for which no match was obtained with any known species, land farming soil was considered an interesting substrate for recovering this group of fungi. Studies on the ecology, phylogeny and bioremediation seem to be promising. Previous studies in this area obtained strains able to degrade aromatic hydrocarbons such as fungi from genera *Fusarium*, *Penicillium*, *Trichoderma*, *Aspergillus* (Kataoka 2001, Satow 2005) and some fungi with black colonies, however, molecular identification was not done to confirm if they belonged to black yeast-like fungi (Conceição *et al.* 2005).

The recovery of *Exophiala xenobiotica* from land farming soil confirms that particularly those members of *Chaetothyriales* showing a preference for habitats rich in monoaromatic hydrocarbons and alkanes are recovered (de Hoog *et al.* 2006, Sterflinger & Prillinger 2001, Woertz *et al.* 2001). Given their potential as opportunists or human pathogens (*e.g.*, Prenafeta-Boldú *et al*. 2006, Zeng *et al.* 2007) the results of this study may contribute for the biosafety rules established for the employees of the refinery. On the other hand, black yeasts and relatives could be also developed as agents for bioremediation for industrial purposes, such as treatment of volatile

pollutants in bioreactors (Woertz *et al.* 2001) and other types of bioremediation in soil and water.

Cladophialophora strains were also isolated from land farming soil; its natural niche is not well known yet, but Prenafeta-Boldú *et al.* (2002, 2004, 2005) observed the ability of strains from this genus to degrade aromatic compounds and to survive in habitats with high concentrations of volatile hydrocarbons.

In the present study, besides the fact that only one fifth of isolated strains was identified by molecular techniques, it was shown that they belonged to only three genera. Prevalence of these fungi seems to be due to the niche, rich in hydrocarbons which could select the strains able to survive under toxic conditions, and also the absence of plant matter that could inhibit the growth of fungi associated to this substrate. *Fonsecaea*, *Phialophora*, *Rhinocladiella* and *Veronaea* species were observed in previous isolations (Iwatsu *et al.*1981, Marques *et al.* 2006, Vicente 2000) from diverse sources from nature, using the same technique, indicating that the method can recover different strains from the black yeast group and the diversity depends on the conditions of the habitat.

The application of the method on landfarming soil samples revealed that cell hydrophobicity is not the main selective factor of the method, because *Exophiala xenobiotica* (hydrophilic) as well as *Cladophialophora* species (hydrophobic) were repeatedly recovered from soil samples. Considering the aim of this technique, this result supports its efficiency because it succeeds in recovering both types of black yeast-like strains observed in the hydrophobicity test. However, the method seems not to be adequate for quantitative studies due to the different rates of hydrophobicity shown by black yeast strains. Strains with higher cell hydrophobicty tend to be more prevalent in the interphase than the hydrophilic ones.

Melanised fungi of the order *Chaetothyriales* are highly polymorphic. Closely related members may be morphologically very different, and even a single strain may exhibit various types of morphology. For that reason a practical, ecological classification of anamorphs has been proposed: *Cladophialophora* for catenate anamorphs which are mostly hydrophobic, and *Exophiala* for annellidic anamorphs producing slimy, mostly hydrophilic conidia and budding cells. In this paper we have proven that *Cladophialophora* cells are indeed strongly hydrophobic, and *Exophiala* cells are hydrophilic, underlining the differential ecological roles of these morphotypes. The presence of yeast cells does not *a priori* indicate hydrophilic character, as yeast cells of *Horteae werneckii* have 94–98 % hydrophobicity suggesting distribution by rain splash (Göttlich *et al.* 1995). These authors also showed that *Exophiala* species are differentially hydrophilic: *Exophiala dermatitidis* has a hydrophobicity of 63 % and *Exophiala jeanselmei* of 37 %.

Assimilation test revealed that most black yeast strains were able to grow in media with mineral oil or n-hexadecane as sole source of carbon and energy, so incubation in medium with hydrocarbon may improve the selection of this group of fungi, as enrichment factor or inhibiting other fungal species with faster growth rate. Growth differences between the culture conditions B and C are probably due to the composition of the reagents: n-hexadecane has a defined chemical composition ($C_{16}H_{34}$) and a short, simple molecular chain; mineral oil is a complex mixture of long chain hydrocarbons, where degradation is more difficult.

As mentioned above strain CBS 122275 has low ability to use hydrocarbons as sole carbon source, but high cell hydrophobicity what may indicate that the selective factor, in this case, could be the hydrophobicity instead of hydrocarbon assimilation or oligotrophism.

Mineral oil and n-hexadecane serve to be an enrichment factor for members of both genera, *Exophiala* and *Cladophialophora*, enhancing growth of many black yeast-like but not of competing saprobes. The addition of a hydrocarbon source for a short period (2–3 d) in media lacking adequate nutrients might just be sufficient to be used as a selective factor for this group of fungi. Extended periods of incubation under these conditions seem to further promote the isolation of members of *Chaetothyriales*, as several tend to continue growth at a very low level (Fig. 1). Since they are able to show some growth with or without these compounds and with or without additional N-source, they can be regarded as being truly oligotrophic. In this study, the primary advantage of the isolation of *Cladophialophora* hydrophobic strains above the *Exophiala* hydrophilic ones could be compensated by a short incubation under oligotrophic conditions and mineral oil enrichment. *Exophiala* strains showed faster growth than most *Cladophialophora* strains under these conditions, so after some ds of incubation, they could have more chance to be recovered.

Incubation of the sample for 2 d in mineral medium and 20 % mineral oil (culture condition B) seems to enrich the growth of most of black yeast strains and could be used to increase the chance to isolate them. In the present study it was not possible to clearly identify the main selective factor of mineral oil in black yeast isolation. The strains of this fungal group are very diverse concerning their ecology, physiology and phylogenetic aspects so it is presumed that their cell surface hydrophobic characteristics, hydrocarbon assimilation and oligotrophism should be distinct, as demonstrated with the tests performed.

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