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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

Ability of marine-derived fungi isolated from polluted saline environment for enzymatic hydrocarbon remediation

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Abstract

Marine-derived fungi have attracted much attention due to their ability to present a new biosynthetic diversity. About 50 fungal isolates were obtained from Tunisian Mediterranean seawater and then screened for the presence of lignin-peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase (Lac) activities. The results obtained from both qualitative and quantitative assays showed that four of marine fungi isolates had a high potential to produce lignin-degrading enzymes. They were characterized taxonomically by a molecular method, based on international spacer (ITS) rDNA sequence analysis, as *Chaetomium jodhpurense* (MH667651.1), *Chaetomium maderasense* (MH665977.1), *Paraconiothyrium variabile* (MH667653.1), and *Phoma betae* (MH667655.1) which have been reported as producers of ligninolytic enzyme in the literature. The enzymatic activities and culture conditions were optimized using a Fractional Factorial design (2^{7-4}). Then, fungal strains were incubated with the addition of 1% of crude oil in 50% of seawater for 25 days to evaluate their abilities to simultaneously degrade hydrocarbon compounds and to produce ligninolytic enzymes. The strain *P. variabile* exhibited the highest crude oil degradation rate (48.3%). Significant production of ligninolytic enzymes was recorded during the degradation process, which reached 2730 U/L for the MnP, 410 U/L for LiP, and 168.5 U/L for Lac. The FTIR and GC–MS analysis confirmed that the isolates rapidly biodegrade crude oil under ecological and economic conditions.

Keywords Marine-derived fungi · Hydrocarbons biodegradation · Optimization · Ligninolytic enzyme · GC–MS analysis

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed as environmental contaminants. In addition to being ubiquitous, recalcitrant, bioaccumulative, and toxic, they have carcinogenic, teratogenic, mutagenic, and other toxic properties [1]. PAHs were investigated on the surface of Mediterranean seawater with a total concentration resulted of 43 ng/L [2]. However, The PAHs distribution in the Mediterranean sediment samples varied from 26.9 to 364.4 ng/g in Sicily Channel and 14.7 to 618.1 ng/g in the Gulf of Tunis. The proportion of PAH 2–3 and PAH 4–5 rings concentration showed that the main origins are characteristic of petroleum sources [3]. Hydrocarbon contamination is a serious threat to ecosystems and bioremediation has proved to be the most promising method, which is known to be practical and eco-friendly. Thereby, micro-organisms can produce several metabolites involved in the degradation of hydrocarbons and may generate less toxic products [4]. As a syntrophic bacterial association, biofilm is crucial to the breakdown of PAHs. It has been highlighted that the biofilm matrix's three-dimensional structure promotes the quick and effective degradation of PAH, the effectiveness depends on several physicochemical biofilm characteristics [5].

The marine ecosystem represents a largely unexplored niche. Marine microflora found to be effective to address some current environmental contamination. Hence, bacteria isolated from marine sources bear great attention in bioremediation of a wide spectrum of hydrocarbon contaminants such as synthetic plastics [6].

Marine-derived fungi capable of degrading pollutants such as PAHs are largely uncharacterized and still poorly studied [7]. The use of these fungi for the bioremediation

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of polluted saline environments is facilitated by their tolerance to saline and oligotrophic conditions [8]. Therefore, the introduction of this type of microorganisms adapted to the marine environment to improve the biodegradation rate of PAHs may be an important approach to decrease these compound concentrations in a contaminated site.

Microbial enzymes have attracted a considerable attention due to their significant biotechnological and environmental applications. In recent years, besides lignin degradation, ligninolytic enzymes have been implicated in the degradation of recalcitrant environmental pollutants such as xenobiotic compounds and various industrial dyes or effluents that can cause both serious environmental and health hazards because of their harmful effects [9].

The extracellular ligninolytic enzyme system, consisting essentially of manganese-dependent peroxidase (MnP) (E.C:1.11.1.13), lignin peroxidase (LiP) (E.C:1.11.1.14), and laccase (Lac) (E.C:1.10.3.2), is sufficiently non-specific and non-stereoselective to promote degradation abilities of several persistent aromatic pollutants. Their catalytic action generates more polar and soluble metabolites such as quinones, phthalate, or diphenic acid, increasing their bioavailability and enhancing their further degradation and mineralization by indigenous microorganisms in contaminated sites [10].

White-rot fungi, belonging to the basidiomycetes, are considered as the most efficient producers of ligninolytic enzymes in nature namely *Phanerochaete chrysosporium*, and *Trametes versicolor* [11]. However, there has been a growing interest in screening research for new and alternative fungal strains for producing efficient ligninolytic enzymes. Marine fungi have been proposed as an alternative organism that can produce biologically active secondary metabolites such as ligninolytic enzymes because of their broad capacities to adapt to the marine harsh environment [8].

Considering that petroleum compounds are pollutants commonly found in the marine environment and that little is known about their degradation by marine microorganisms, this study aims to isolate local marine fungi producing lignin-degrading enzymes and assess their ability to degrade crude oil in saline conditions. The isolated strains were screened and identified based on molecular methods. Crude oil was used to evaluate strains' degradation abilities and metabolic products were investigated by GC–MS analysis.

Material and methods

Chemicals and substrates

2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), Guaiacol, were purchased from Sigma–Aldrich Pvt Ltd. (USA). All the chemicals used were procured of analytical grade.

Sample collection and isolation

Water and sediment were collected from different saline point from the north of Tunisia (Fig. 1). The salinity in site (A) ranged from 20 g/L in summer to 38.5 g g/L in winter [12]. In site B the salinity is more than 40 g/L [13] and site (C) is characterized by extreme salinity which varies between 32 and 43 g/L [14]. The collected samples were brought to the laboratory in clean plastic bags in a cooler.

Fig. 1 Satellite imagery of water and sediment samples location: **A** the Lake of Bizerte, **B** Sebkha Sijoumi, and **C** Lake of Tunis

Fungi were isolated in modified Malt Extract Agar (MEA) containing an antibiotic (Chloramphenicol) to inhibit bacterial growth. A standard dilution method was adopted to isolate fungi from water and sediment suspension. 100 µl of each dilution was plated on MEA medium. Plates were incubated at 28 °C for 7 days. For purification, a minimal amount of mycelia was picked up from young colonies and subcultured on MEA plates. These assays led to collect 50 pure and morphologically different fungal isolates.

The modified MEA was prepared with artificial seawater which is composed of 30 g/L NaCl, 0.73 g/L KCl, 10.7 g/L $MgCl_2{\cdot}6H_2O$, 5.4 g/L $MgSO_4{\cdot}7H_2O$, g/L $CaCl_2{\cdot}2H_2O$.

Screening of fungi for ligninolytic enzyme activity

Preliminary screening of the fungal isolates for the presence of lignin-degrading enzymes (Laccase and Peroxidases) was qualitatively performed by growing them on modified MEA supplemented with 4 mM of guaiacol as an aromatic model compound [15].

Guaiacol-supplemented MEA plates were inoculated with a 7 mm plug of each fungus and then, were incubated at 28 °C for 7 days. A strain was described as a producer of extracellular ligninolytic enzymes if a reddish-brown halo appeared under and around the fungal colonies in the first week of incubation.

Identification of fungal isolates

The selected pure fungal strains were identified based on internal transcribed spacer (ITS) rDNA sequence analysis. The total genomic DNA of the isolates was extracted as described by [16]. DNA extracts were assessed using a Nano drop ND-1000 Spectrophotometer.

The ITS1-5.8S-ITS4 regions were amplified using two universal fungal primers: internal transcribed spacer 1 (ITS1:5' TCCGTAGGTGAACCTGCCG-3') and internal transcribed spacer (ITS4 5' TCCTCCGCTTATTGATATGC-3').

PCRs were performed in final reaction mixtures (25 µL) containing 2 µl genomic DNA, 2 µl of each primer (20µmoles/µl), 1 µl dNTPs (20 mM), 1.5 µL $MgCl_2$ (25 mM), 0.25 µl Taq polymerase (5U/L) (Promega) and 5 µl of reaction buffer (Promega). Thermocycling conditions consisted of an initial denaturation for 2 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 53 °C and 1 min at 72 °C, and a final extension for 10 min at 72 °C and cooling to 4 °C. The amplicons obtained were sequenced and analyzed. The DNA sequences were compared with those available on the database using the BLAST program at the National Center for Biotechnology Information (NCBI). The phylogenetic tree was constructed using neighbour-joining method [17]. Distances were estimated by the Tamura-Nei method [18]. The sequences of 18S rRNA gene identified were deposited in GenBank under accession number.

Optimization of culture conditions

Fractional Factorial design (2^{7-4}) was adopted to determine the optimum conditions that affect significantly the production of ligninolytic enzymes. Seven variables were applied (inoculum size, glucose, $(NH_4)_2SO_4$, Veratryl alcohol, and sodium chloride concentration, agitation speed, and the initial pH) and three responses (Lac, LiP, and MnP activities) were considered for the design analysis. The factor values and their range were chosen based on the results of a preliminary analysis of the isolated strains (data not shown) and on previous studies. Veratryl alcohol, an aromatic compound, was used in this study to induce ligninolytic enzyme activities [19]. pH is an important factor for microbial growth and secondary metabolite secretion. It can alter enzyme stability by changing its surface properties [20]. The rate of agitation is a crucial factor because it facilitates nutrient access and impacts oxygenation [21]. The carbon and Nitrogen sources are essential nutrients for fungal growth [22]. The salinity was investigated in this work because fungal strains were obtained from saline biotopes.

To obtain the parameters that affect the ligninolytic activity of the four selected strains, each independent variable was investigated at two levels, high and low, which were respectively denoted by (+) and (−). The coded values in Fractional Factorial design matrix were transformed into real values; therefore, eight runs were applied for each strain (Table 1).

After seven days of incubation, the content of each flask was centrifuged at 5000 × *g* for 20 min, the supernatant was used for enzymatic analyses (Lac, LiP, and MnP).

The experimental design was analyzed using the statistical software "Statistica". These parameters were screened according to previous studies that were shown to be critical in enhancing enzyme production [23, 24].

Fungal degradation of crude oil

Preparation of degradation medium

To determine the ability of selected fungi to degrade hydrocarbon, a specially prepared media with 1% of crude oil was used (according to the best condition found for each fungus after optimization). Fungal spores were inoculated in the broth. The media was incubated at 28 °C for 25 days in a shaker incubator rotating at 120 rpm. The control flasks were inoculated without fungi.

Quantitative Analysis for ligninolytic enzymes

The enzymatic quantification was performed in 250-mL Erlenmeyer flasks containing 50 mL Kirk's modified

Table 1 Matrix for the $2^{(7-4)}$ fractional factorial experimental design

Factors runs	Inoculum concentration (spore/mL)	Inducer veratryl alcohol (mM)	Salinity (g/L)	Agitation (rpm)	Glucose concentration (g/L)	$(NH_4)_2SO_4$ Concentration (g/L)	PH
Low level	10^3	0	15	0	5	1	4
High level	10^6	1.5	30	150	10	3	6
1	−1	+1	+1	−1	−1	+1	−1
2	−1	−1	−1	+1	+1	+1	−1
3	+1	−1	−1	−1	−1	+1	+1
4	−1	−1	+1	+1	−1	−1	+1
5	+1	+1	−1	+1	−1	−1	−1
6	−1	+1	−1	−1	+1	−1	+1
7	+1	+1	+1	+1	+1	+1	+1
8	+1	−1	+1	−1	+1	−1	−-1

medium [25]. This medium contained per liter: glucose, 10 g; peptone, 5 g; yeast extract, 1 g; ammonium tartrate, 2 g; KH_2PO_4, 1 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; KCl, 0.5 g; trace elements solution, 1 ml. The composition of trace elements solution per liter was: $B4O_7Na_2{\cdot}10H_2O$, 0.1 g; $CuSO_4{\cdot}5H_2O$, 0.01 g; FeSO4·$7H_2O$, 0.05 g; MnSO4·$7H_2O$; 0.01; $ZnSO_4{\cdot}7H_2O$, 0.07 g; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.01 g. The pH of the solution was adjusted to 5. Cultures were incubated at 28 °C under agitation at 150 rpm. All enzyme activities were followed on a UV–visible spectrophotometer. Cultures were harvested by filtration and centrifuged at 10,000 × *g* for 20 min, and the supernatants were used as an enzyme sources.

Laccase activity was estimated by oxidation of 2.2-azino-bisethylbenthiazolina (ABTS) according to [26]. A 50 µl of culture supernatant was added to a reaction buffer of 0.1 M sodium acetate (pH 4.5). The reaction was initiated after the addition of 5 mM ABTS to the reaction mixture and the oxidation was determined spectrophotometrically at 420 nm.

Lignin peroxidase (LiP) activity was determined by measuring the rate of oxidation of veratryl alcohol to veratraldehyde as described by [27]. The mixture reaction contained 2 mM veratryl alcohol and 0.4 mMH_2O_2 in 50 mM sodium tartrate buffer, pH 3, and 500µL enzyme extract. The reaction was started by adding hydrogen peroxide and the appearance of veratraldehyde was determined at 310 nm.

MnP activity was determined spectrophotometrically at 270 nm through the oxidation of 0.5 mM MnSO4 in 50 mM sodium malonate buffer (pH 4.5) in the presence of 0.5 mM H_2O_2. The absorbance was measured in 1 min intervals after the addition of hydrogen peroxide [28]. One enzyme unit was defined as a 1 µmol product formed per minute under the assay conditions.

Biomass accumulation by fungal strains under crude oil degradation

Biomass was investigated during the process of crude oil degradation by gravimetric method. The culture broth was filtered through Whatman No. 1 filter paper. The harvested biomass samples were washed twice with distilled water and dried in an oven at 85 °C for 72 h.

Analytic methods

Crude oil extraction and gravimetric analysis

The liquid culture was transferred into a 100-ml separatory funnel and the metabolites of crude oil degradation were extracted using ethyl acetate (1:1 v/v) by shaking vigorously for 2 min with periodic venting. The organic layer was recuperated and the aqueous layer was re-extracted twice. The combined extract was dried with anhydrous sodium sulfate columns (3 g). The dried extracts were concentrated with a rotary evaporator using ethyl acetate as an organic solvent [29]. The residual crude oil was accurately weighed and quantified gravimetrically. The percentage of degradation was then calculated using the followed formula:

$$\%\ \text{of degradation} = \frac{(a-b)}{a} \times 100$$

where *a* is the weight of crude oil in the control, *b* is the weight of residual crude oil after fungal treatment.

FTIR analysis

FTIR spectroscopy was used in the present study to detect structural changes in the functional group of crude oil

degradation metabolites. Perkin Elmer, Thermo Scientific IR 200 FT-IR, was used for the analysis of crude oil extracts before and after fungal treatment. The spectrum was recorded in the mid-IR region of $400-4000\ cm-^{1}$ at a rate of 16 nm/s.

GC–MS analysis

To detect and identify crude oil metabolites after fungal degradation, extract samples were analyzed by gas chromatography coupled with mass spectrometry according to Luan et al. [30] with minor modifications. The column was held at 100 °C for 3 min and raised to 300 at a rate of 5 °C/min, and finally kept for 10 min. The injector temperature was held isothermally at 280 °C with a split-less mode for 3 min. The solvent cut time was set to 4 min. Helium was used as the carrier gas with a flow rate of 1 ml/min. The MS was operated under electron impact (EI) with electron energy of 70 eV, and scanned ranging from 50 to 500 amu (atom to mass unit) to collect appropriate masses for selected ion monitoring. The identification of metabolites was based on matching their retention times of standards.

Statistical analysis

An analysis of variance (a one-way ANOVA) was conducted by employing performed (SPSS) version 20.0 software. SAS 9.0 software was used for all statistical analysis with multiple comparison tests effects were considered significant when the P value was < 0.05.

Results and discussion

Isolation and screening of ligninolytic enzyme producers

In this study, a collection of 50 fungal isolates was obtained from contaminated marine biotopes in North of Tunisia. Out of 50 fungal strains, 29 were isolated from Sebkha Sijoumi (site A), 19 from Bizerte lagoon (site B), and 2 fungal strains were isolated from Lake of Tunis (site C) (Fig. 1).

Based on the qualitative screening on the MEA supplemented with guaiacol, four fungal isolates (G1, SO2, M4, and E12) were considered as the most promising for lignin-degrading enzymes (Fig. 2). The ability of the fungus to oxidize and decolorize guaiacol confirms that they are capable of producing phenol oxidases, peroxidases, and hydrogen peroxide–producing oxidases [31].

Similar results were found by Chen et al. [32], who isolated about 20 fungi from sea mud, seagrass, and mangrove samples. Among these fungi, one isolate showed a positive reaction when cultured in a guaiacol medium. In another screening from the Çakalburnu Lagoon, Izmir Bay, Aegean Sea in Turkey, marine origin fungi were obtained from the sea and lagoon environment [33]. In a study conducted in Tunisia, among 20 fungi isolated from marine environments and screened [34], 5 fungi showed positive oxidative activity on both 2,6-dimethoxyphenol and ABTS added as substrates. The isolation of fungi producing ligninolytic activity from marine environments is less explored than their terrestrial homologs. However, due to their great ability to adapt to extreme conditions (high pressure, low temperature, oligotrophic nutrients, salinity), fungi of marine origin can provide a new biosynthetic diversity [35, 36].

Taxonomic identification of selected fungi

The four fungal isolates were characterized taxonomically based on morphology and molecular analyses using internal transcribed spacer (ITS) regions. The phylogenetic tree is shown in Fig. 3. Results indicated that all isolates were representative of the Ascomycota phylum SO2 and G4 was 100% identical to that of *Chaetomium maderasense* and *Paraconiothyrium variabile* (Accession No. MH665977.1 and MH667653.1 respectively), while the partial 18S rDNA sequence of isolates M1 and E12 were 99% identical

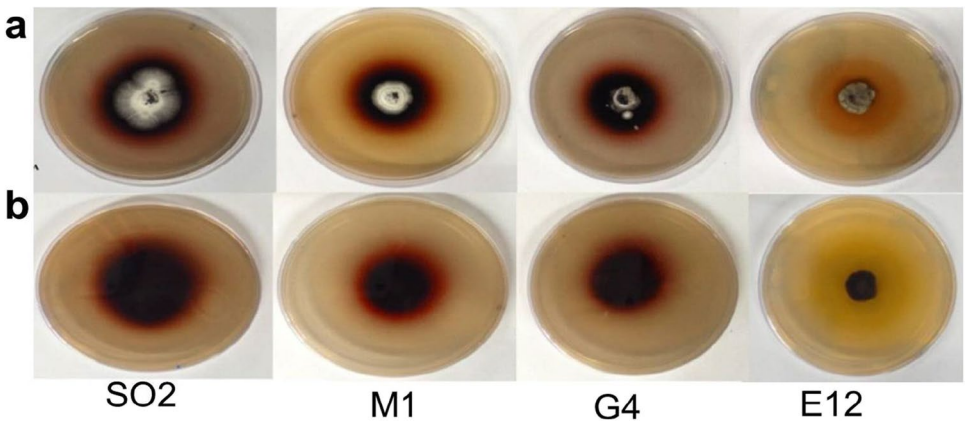

Fig. 2 Ligninolytic enzyme production in solid media by positive guaiacol oxidation after 7 days of incubation of four marine fungal isolates: **a** front view, **b** back view

to *Chaetomium jodhpurense* and *Phoma betae* respectively (Accession No MH667651 and MH667655).

Prasannarai and Sridhar [37] and Dhanasekaran et al. [38] discussed the prevalence of ascomycetes in aquatic habitats, which can be explained by the adaptation of their spores to the aquatic ecosystem. Nevertheless, basidiomycetes fungi are rarely isolated from marine samples [39].

The genus *Chaetomium* is ubiquitous occurring at the ground, air, and marine environments [40]. Studies have shown significant production of a wide range of secondary metabolites by different species belonging to this genus [41–43]. In 2022, Tian and Li [44] reviewed the structural diversities and biological activities and properties of 122 secondary metabolites isolated from marine-derived *Chaetomium* species between 2001 and 2021 with a very broad spectrum of biological activity including cytotoxicity, enzyme inhibitory activity, radical-scavenging activity, antiparasitic, antibacterial, and antifungal activity. Indeed, the genus *Chaetomium* has a large range of applications in bioremediation [45, 46].

Paraconiothyrium variabile is the homotypic synonym of *Didymosphaeria variabile.* In 2004, [47] proposed *Paraconiothyrium* as a new genus, and then it was found to be a commonly occurring fungal genus ranging from marine to soil. Species belonging to this genus have shown great interest in producing antimicrobials [48, 49], anti-inflammatory [50], and cytotoxic metabolites against human cancer cell lines [51, 52]. The production of extracellular enzymes and their application in bioremediation have been reported in numerous investigations. An extracellular laccase purified and characterized from *P. variabile* [53] has been shown highly efficient for decolorization of synthetic dyes [54], as well as removing effectively chlorophenolic derivatives [55]. The isolation of *P. variabile* and some other *Paraconiothyrium* species from the Mediterranean Sea was reported for the first time by Garzoli et al. [56]. In 2020, Gonçalves et al. [57] isolated a new species named "*Paraconiothyrium salinum*" from saline water in the estuary of the Ria de Aveiro (Portugal).

The genus *Phoma* is very important because it is known for its excellent production of bioactive secondary metabolites. There are two major groups of *Phoma* species: terrestrial and marine. Marine *Phoma* represents an important source of many secondary, which have demonstrated antiviral, antifungal, antibacterial, antiprotozoal, and weedicide activities [58]. Isolation of strains from the aquatic environment belonging to *Phoma* genus has been previously reported by [33, 59 and 60]. Species belonging to *Phoma* genus were highlighted as good producers of laccase. Isolation of *Phoma sp.* with great efficiencies for synthetic azo and anthraquinone dyes decolorization had been studied by Junghanns et al. [59]. Debnath et al. [61] reported the production of thermophilic and alkali-stable laccase by *Phoma herbarum* with a promising result for industrial dye-decolorization. Immobilized and free purified laccase from *Phoma beate* also showed great efficiencies for synthetic dyes degradation [62].

The exploration of fungi living in saline water has increased considerably in recent years due to their great ability to produce secondary metabolites [63]. In this work, we isolated four fungal strains from saline environments belonging to *Ascomycota*, which have been documented as good producers of ligninolytic enzymes and efficient candidates for bioremediation process.

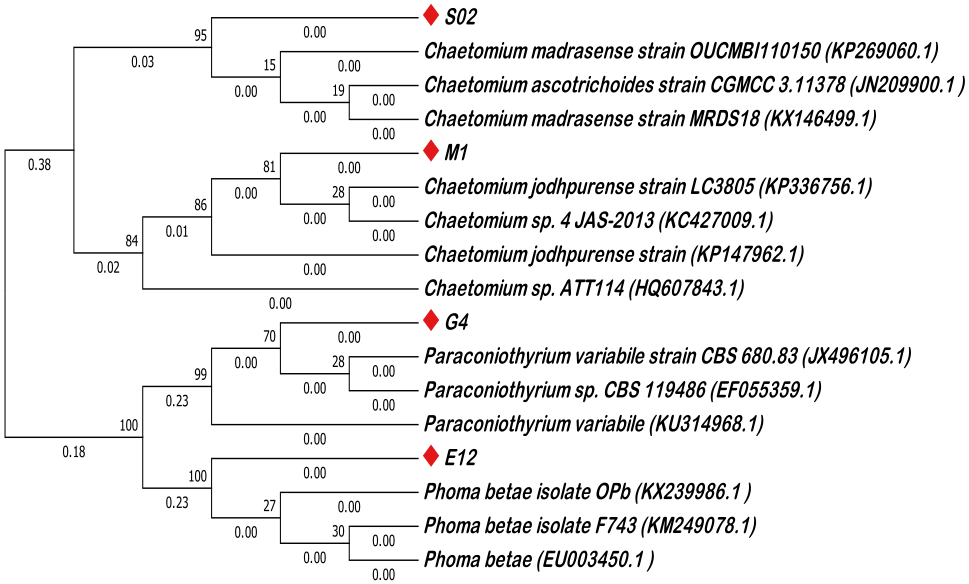

Fig. 3 Phylogenetic tree of fungal strains based on ITS rDNA sequence. Numbers following the names of the strains are accession numbers of published sequences. The tree was constructed by neighbor-joining algorithm using maximum composite likelihood model. Bootstrap percentages from 1000 replicates are shown

Table 2 Optimization of enzymatic activities (U/L) of laccase (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP) using a 2^{7-4} fractional factorial design

	C. jodhpurenses			*C. madrasense*			*D. variabile*			*P. beate*		
Run	Lac	LiP	MnP	Lac	LiP	MnP	Lac	LiP	MnP	Lac	LiP	MnP
1	2.224	**94.39***	112.3	**4.449***	81.54	6.048	**4.635***	75.82	93.6	0	81.97	31.14
2	3.152	79.272	**334.4***	3.337	75.92	95.76	0.743	**80.03***	**347.4***	2.874	81.11	31.14
3	**4.450***	80.14	319.7	2.039	**84.89***	127.2	0.556	78.95	249.7	**120.5***	**84.56***	314.5
4	0.927	91.15	268.2	2.781	80.14	107.5	2.039	72.58	290.7	0	80.78	286.9
5	0.556	91.15	88.56	1.298	80.35	3.672	3.152	72.36	57.42	2.132	81	49.14
6	0.742	82.30	209.3	3.708	78.62	3.744	4.264	73.33	58.32	3.708	76.36	88.2
7	1.298	71.82	108	2.781	81.43	4.464	2.781	77.76	86.76	1.205	79.38	130.9
8	2.225	76.57	273.1	1.483	83.81	**174.7***	3.893	75.82	311.8	2.966	78.41	**318.8***

*The highest value of enzymatic activity

Optimization of culture conditions for enzyme production

The study of nutritional and environmental conditions of fungal cultures is very crucial to increase enzymatic activities. The maximum MnP activities were achieved in culture without veratrylic alcohol and containing 15 g/L of NaCl, 10 g/L of glucose, 3 g/L of $(NH_4)_2SO_4$, and 10^6 spores/mL with 150 rpm speed agitation at initial pH 4 (Table 2).

The high LiP production was found in a static medium added by 15 g of NaCl, 5 g/L glucose, 10^6 spore/mL, 1 g/L $(NH_4)_2SO_4$ at initial pH 6, and with the addition of 1.5 mM of veratryl alcohol as an inducer. The most suitable medium for Lac production contained 30 g/L NaCl, 5 g/L glucose, 10^6 spore/mL, and 3 g/L of $(NH_4)_2SO_4$ at initial pH 4 and without agitation and veratryl alcohol (Table 2).

Pareto charts represented the factor's effect on enzyme production (Fig. 4). The vertical line chart represents a reference line, any factor that extends past this line is of significant effect at $p = 0.05$ (significance level). In this matrix, a screening step was performed when very low coefficient effects have been discarded.

Pareto charts show clearly the significance of glucose concentration on LiP production only with the strain *C. maderasense* requesting a high concentration of glucose. The other tested fungi can produce maximum LiP activity in a low glucose concentration. Nitrogen-rich condition is suitable for LiP production with strains *P. betae* and *P. variabile*. Veratryl alcohol, known as an inducer of LiP enzyme production [64, 65], enhanced LiP activity only in *C. judhpurense* culture. However, it negatively affects MnP activity with all tested fungi. Schneider et al. [19] reported also that veratryl alcohol induced LiP activity and decreased MnP activity in the strain *Marasmiellus palmivorus* VE111. In this context, Arora and Gill [66] highlighted that some white rot fungi can produce veratryl alcohol and the exogenous addition could make it toxic against enzyme production.

Production of laccase in this experimental design was limited compared to LiP and MnP production. Static conditions and high nitrogen concentration have the most significant effect on Lac activity. This result suggests that the production of significant levels of Lac may require different conditions than those tested in this work.

This study demonstrated that the selected marine-derived fungi could economically produce extracellular enzymes. High activities were found in a static condition, without inducer, with a low concentration of NaCl, and glucose, and in limited Nitrogen conditions. These are promising results in further application for industrial technologies. Tested fungi can be considered as slightly halophilic because they can grow and produce extracellular enzymes in the presence of low (1.5%) or high levels (3%) of NaCl. This was supported by Gonçalves et al. [57], who highlighted the ability of two marine-derived fungi "*Paraconiothyrium salinum* and *Neoascochyta fuci*" to survive similarly in the presence or not of 3% of sea salt.

Crude oil evolution in optimal synthetic media

Enzyme activity

The fungal crude oil degradation was carried out in optimum conditions found with the experimental design after the addition of 1% of crude oil. The production of laccase, MnP, and LiP enzymes was recorded in most of the cultures of the four tested fungi in treatment and control cultures (Fig. 5). The results showed that the enzymatic activities had increased considerably as compared to the controls.

In the presence of crude oil, the enzymatic activity recorded by the strains shows a dominance of peroxidase enzymes. The strain *C.ascotrichoides* showed 2500 U/L of MnP, 410.8 U/L of LiP, and 13.29 U/L of lac. For *C. judhuprensis*, MnP, LiP, and laccase activities were 2232.2 U/L, 278.864, and 49.27 respectively. The strain *P. variabile*

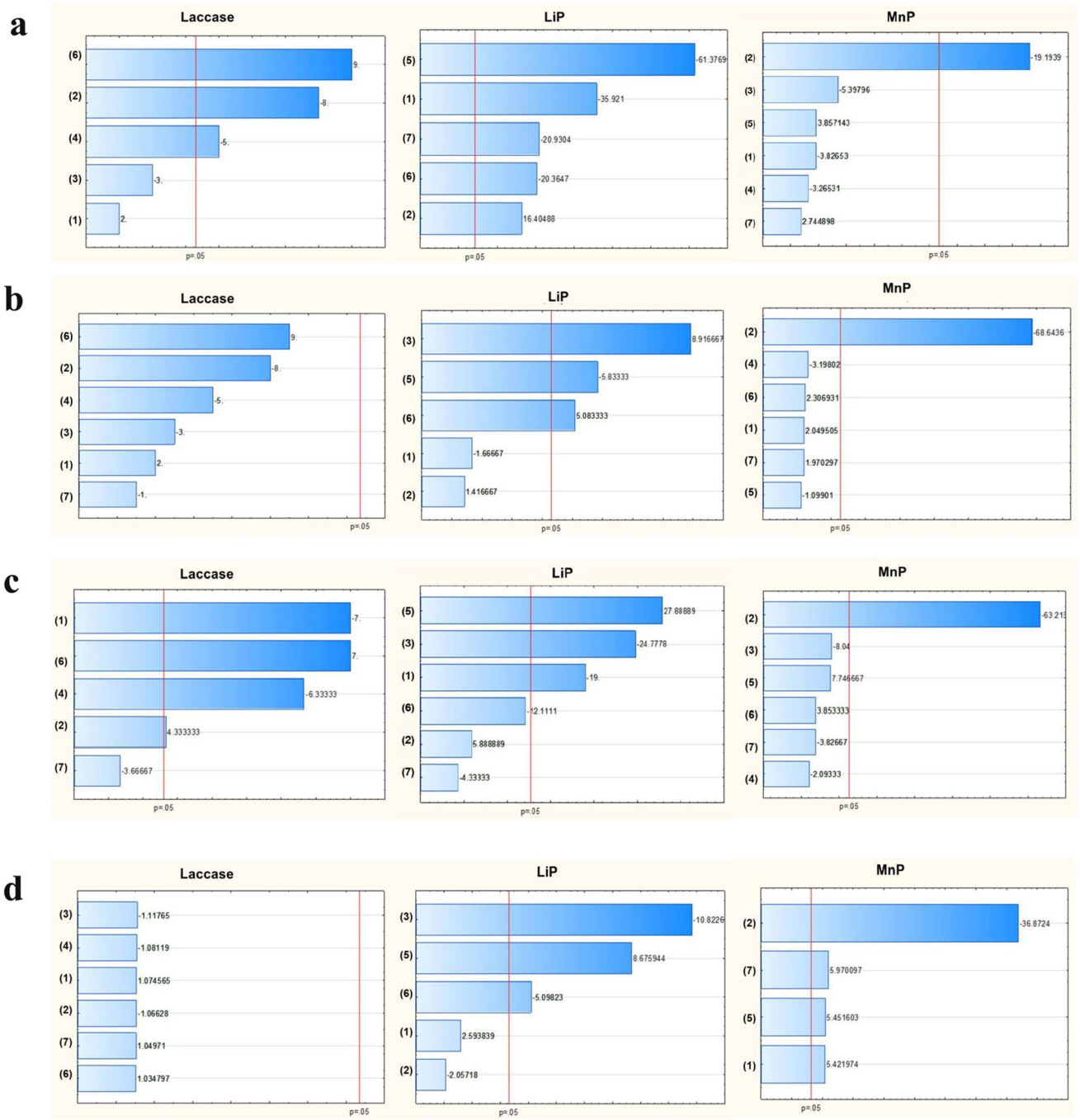

Fig. 4 Pareto charts of standardized effects of Lac, LiP, and MnP production by **a** *C. jodhpurense*; **b** *C. madrasense*; **c** *P. variable*; **d** *P. betae* (The red vertical line chart represents significance level $p = 0.05$). (1): Inoculum concentration, (2): Inducer, (3): salinity, (4): agitation, (5): glucose concentration, (6): $(NH_4)_2SO_4$, and (7): pH

produced 2730.24 U/L of MnP, 369.36U/L of LiP, and 168.52U/L of Lac. *P. betae* presented 1318 U/L of MnP, 223.2 U/L of LiP, and 128 U/L of Lac.

In control cultures, the highest level of MnP production is recorded with *Chaetomium* genus at more than 700 U/L followed by *P. betae* and *P. variabile* with 636 U/L and 617 U/L respectively. *P. betae* produced the highest LiP activity with 175U/I and the lowest level showed in *P. variabile* culture (163U/L). However, the laccase activity was ineffective and unstable.

Our results showed that the marine isolated ascomycete could produce extracellular lignin peroxidase (LiP)

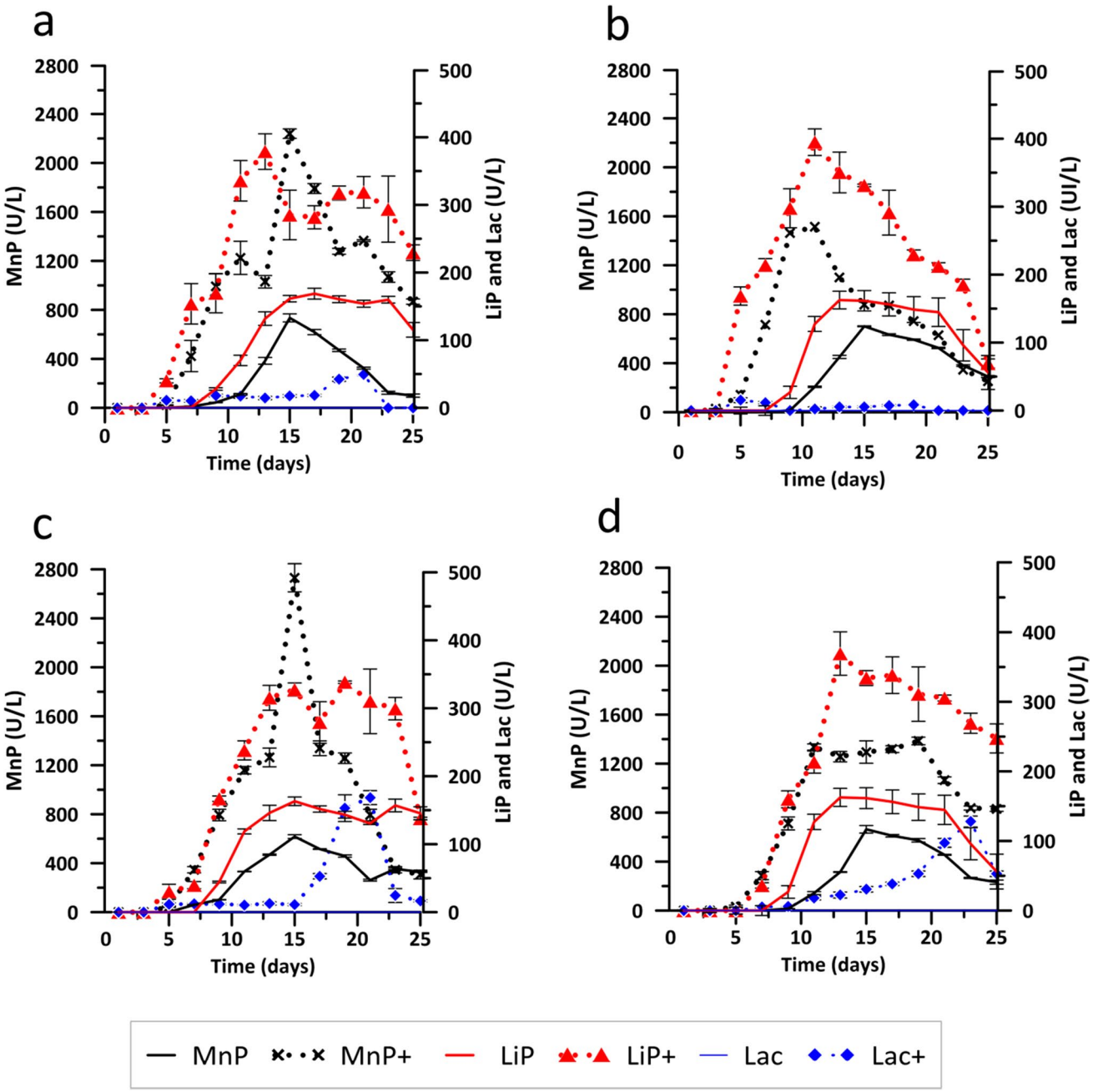

Fig. 5 Ligninolytic enzyme activities by fungal isolates **a** *C. jodhuprense*, **b** *C. maderasense*, **c** *P. variabile*, and **d** *P. betae* in free crude oil media (controls) and in treatments. + indicated the treatment culture

and manganese peroxidase (MnP), Furthermore; the enzyme activity was largely stimulated by the presence of hydrocarbon compounds. In this study, Lac production was enhanced by the presence of crude oil particularly with two strains *P. variabile* and *P.betae* that can produce noticeable levels, which rushed 168 U/L and 128 U/L respectively. Similarly, the induction of Lac activity of *P. ostreatus* by the PAHs (i.e., naphthalene, anthracene, phenanthrene, pyrene, and benzo[a]pyrene) was noticed by Pozdnyakova et al. [67]. The higher level of the three sought enzymes in crude oil-supplemented media than in control cultures can be explained by the great demand for enzymes for hydrocarbon breakdown reactions [68]. The improved activity of these enzymes was recorded by Agrawal and Shahi [69] in *Coriolopsis byrsina* in presence of pyrene. Ameen et al. [68] demonstrated that the culture of marine-derived fungi in the presence of diesel is also associated with the production of ligninolytic enzymes.

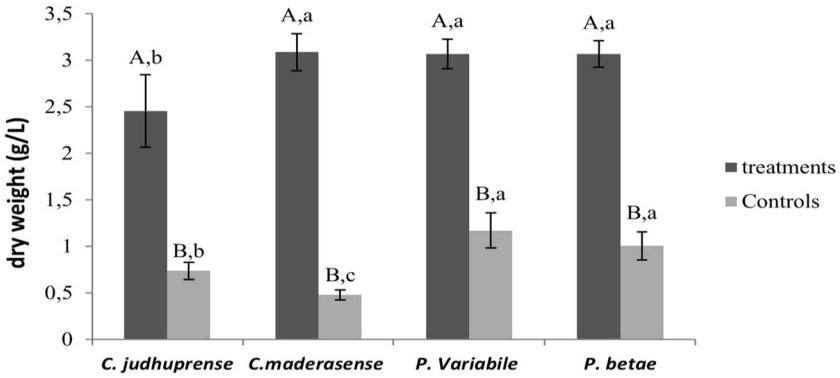

Fig. 6 Dry weight accumulation by fungi under treatment with crude oil. Error bars represent standard deviations of means ($n=3$). Different uppercase letters designate significant differences ($p \leq 0.01$) among crude oil treatments and controls within the same isolate. Different lowercase letters show significant differences ($p \leq 0.05$) between isolates within the same culture medium

It was suggested that the rate of PAH bioremediation directly correlates with the rate of ligninolytic enzyme production [69].

Dry weight accumulation by fungal isolates

The culture of the various strains was carried out on an optimal synthetic medium containing 1% of the crude oil for 25 days. The growth of fungi was determined by the measuring of the mycelium weight at the end of the culture (Fig. 6). Biomass accumulation is more important in presence of crude oil than in control culture. The *C. maderasense* strain had the highest weight gain with five times more, followed by both the *C. jodhpurense* and *P. betae* strains (more than two times) and the *P. variabile* was only one and a half times heavier. The means were significatively different for the biomass accumulation in crude oil-supplemented media and control ($p < 0.01$) but were not significatively different among different species ($p < 0.05$) except between *P. variabile* and *P. beta*, which exhibited the lowest biomass accumulation.

The accumulation of biomass associated with this type of treatment has been extensively studied. The biomass gain recorded by the strains in the presence of crude oil in this work has reached more than 5 times the control medium. This biomass gain can be considered to be due to the fungus biodegradation. Several other studies, which demonstrated the biodegradation potential of different fungi, also reported the accumulation of biomass under similar conditions. Hasan [70] recorded a significant biomass gain of *Aspergillus niger* and *Rhizopus stolonifer* strains in 10% kerosene broth, accumulating 0.530 g and 0.522 g dry weight respectively. Ameen et al. [68] reported a maximum weight gain of 43.4% in the presence of diesel fuel. All fungal strains studied were able to grow optimally in environments polluted with 1% crude oil.

Crude oil removal

After 25 days of incubation, the percentage of crude oil degradation was determined by the gravimetric method (Fig. 7). The *P. variabile* strain showed the highest rate of 48%, followed by *P. betae* (32%), *C. jodhpurense* (27%), and *C. maderasense* (20%). A significant difference ($p < 0.05$) was observed between the fungal strains except between *C. jodhupense* and *P. betae*. In term of crude oil removal, the highest rate was observed with strains producing high laccase activity.

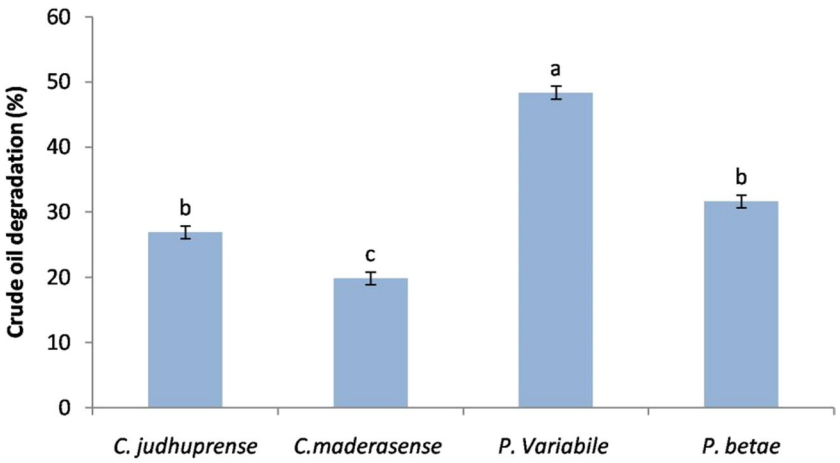

Fig. 7 Crude oil degradation rates by fungal strains after 25 days of incubation. Error bars represent standard deviations of means ($n=3$). Different letters designate significant differences ($p \leq 0.05$) between isolates

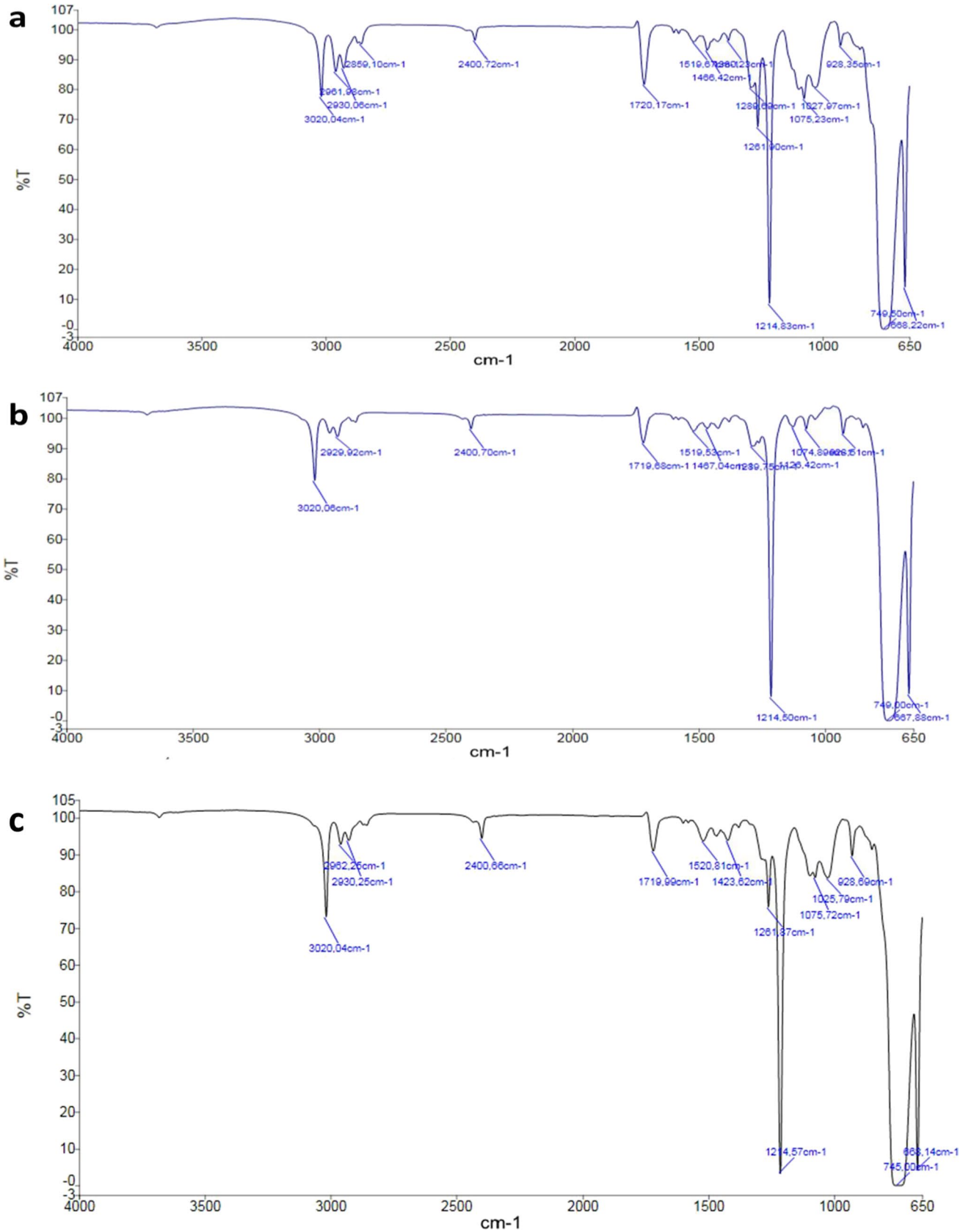

Fig. 8 FTIR spectra of untreated crude oil (**a**) and after fungal treatment with (**b**) *C. jodhuprense*, **c** *C. maderasense*, **d** *P.variabile*, and **e** *P. betae*

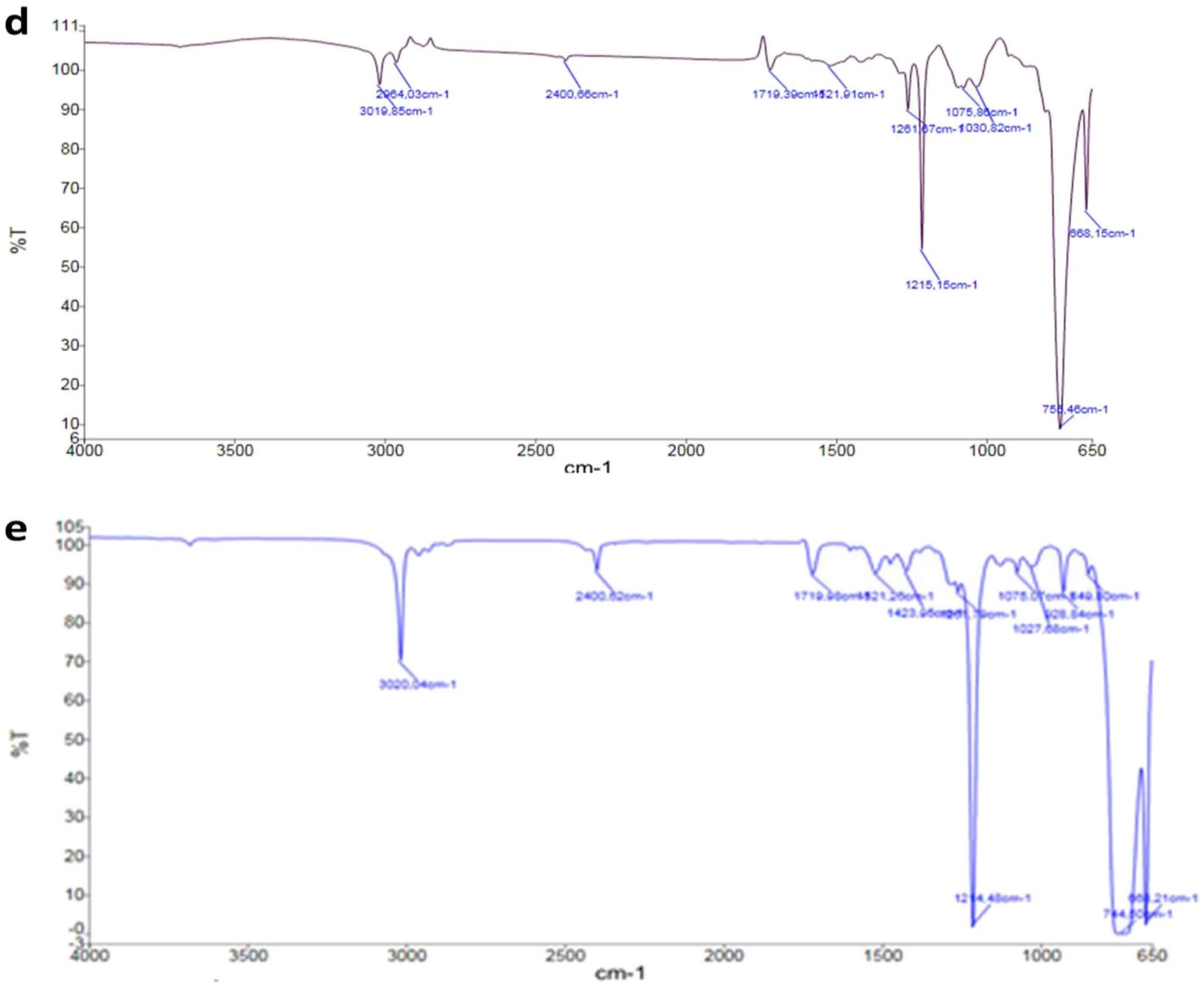

Fig. 8 (continued)

FTIR analysis

The FTIR spectrum of untreated crude oil revealed the presence of linear, chained, and cycloaliphatic hydrocarbons, and aromatic compounds (Fig. 8a). The band observed at 3020 cm^{-1} corresponds to aromatic C-H, and the bands ranging from 2960 and 2860 cm^{-1} represent stretching aliphatic bands (asymmetric and symmetric stretching). The absorption band at 1720 cm^{-1} indicates the presence of carbonyl groups. The presence of aromatic carbon (C = C) was assigned by the presence of distinct bonds ranging from 1520 to 1400 cm^{-1}. The absorbing bands around 1300–1100 cm^{-1} correspond to N- and S-related stretching and bending vibration. While C–C compounds ranged in 1025–1075 cm^{-1} and in 928 cm^{-1}. The same typical vibrational bands were detected in FTIR spectra of crude oil characterization [71, 72].

The FTIR spectrum of crude oil after fungal degradation showed an important increase in the transmittabce of the major peaks with the disappearance of some bands (Fig. 8b–e). In the IR spectrum of the strain *P. variabile*, the peaks at the range of 2950–2860 cm^{-1}, representing C-H aliphatic stretch, have totally disappeared and the transmittance at 3020 cm^{-1} (aromatic C-H) was lower than the control (Fig. 8d). The observed bands at 1720 cm^{-1} (C = O bond), 1520, and 1465 cm^{-1} (C = C) have disappeared. The transmittance of the peaks representing the C-O bond was about half as high as the control. The intensity of the band at 1025–1075 cm^{-1} has been decreased; while the peak at 928 cm^{-1} has disappeared.

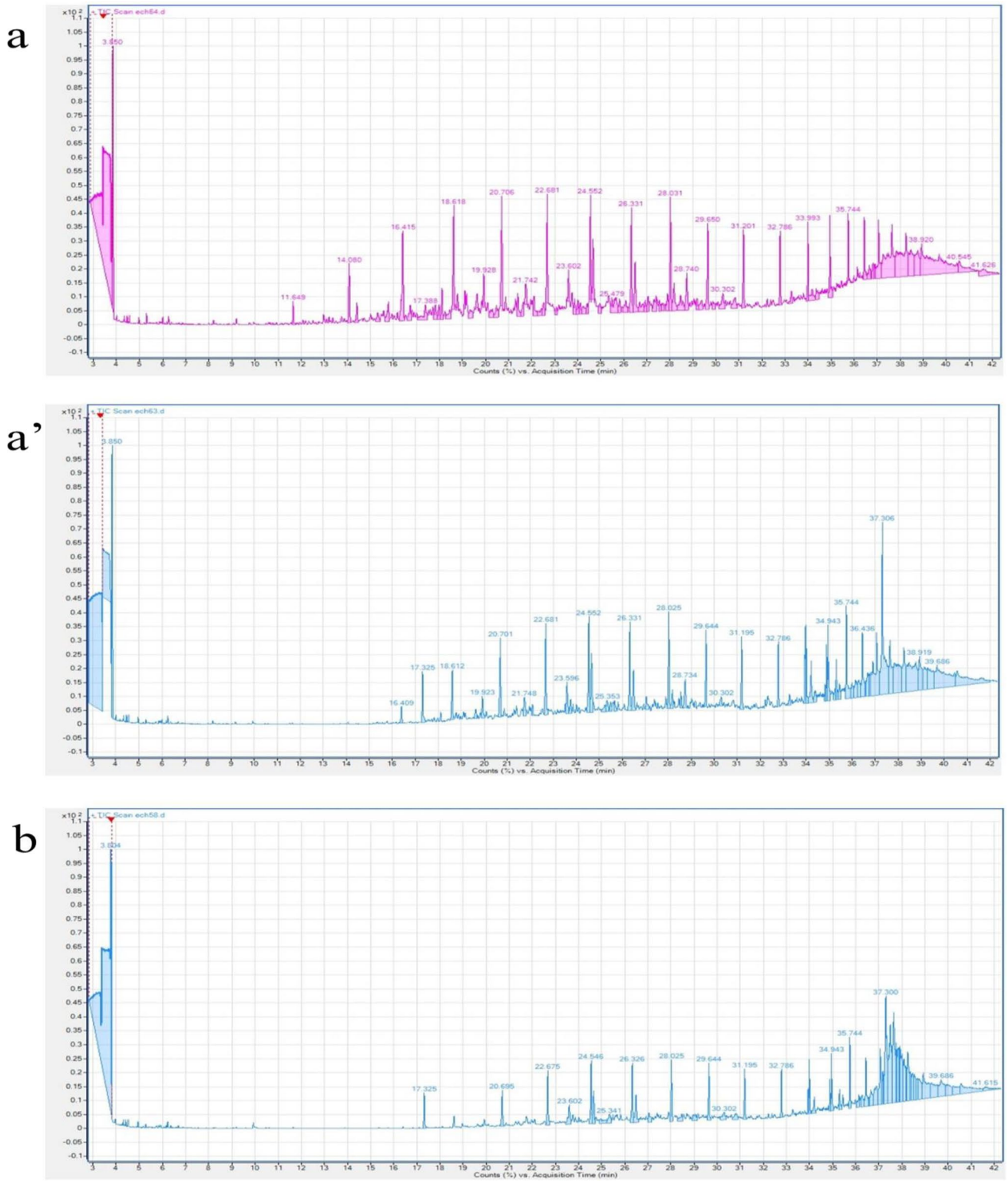

Fig. 9 Biodegradation of crude oil analyzed by GC–MS. Control culture at time 0 (**a**) and after 25-day incubation (**a'**) and treated cultures with **b** *C. jodhuprense*, **c** *C. maderasense*, **d** *P. variabile*, and **e** *P. betae*

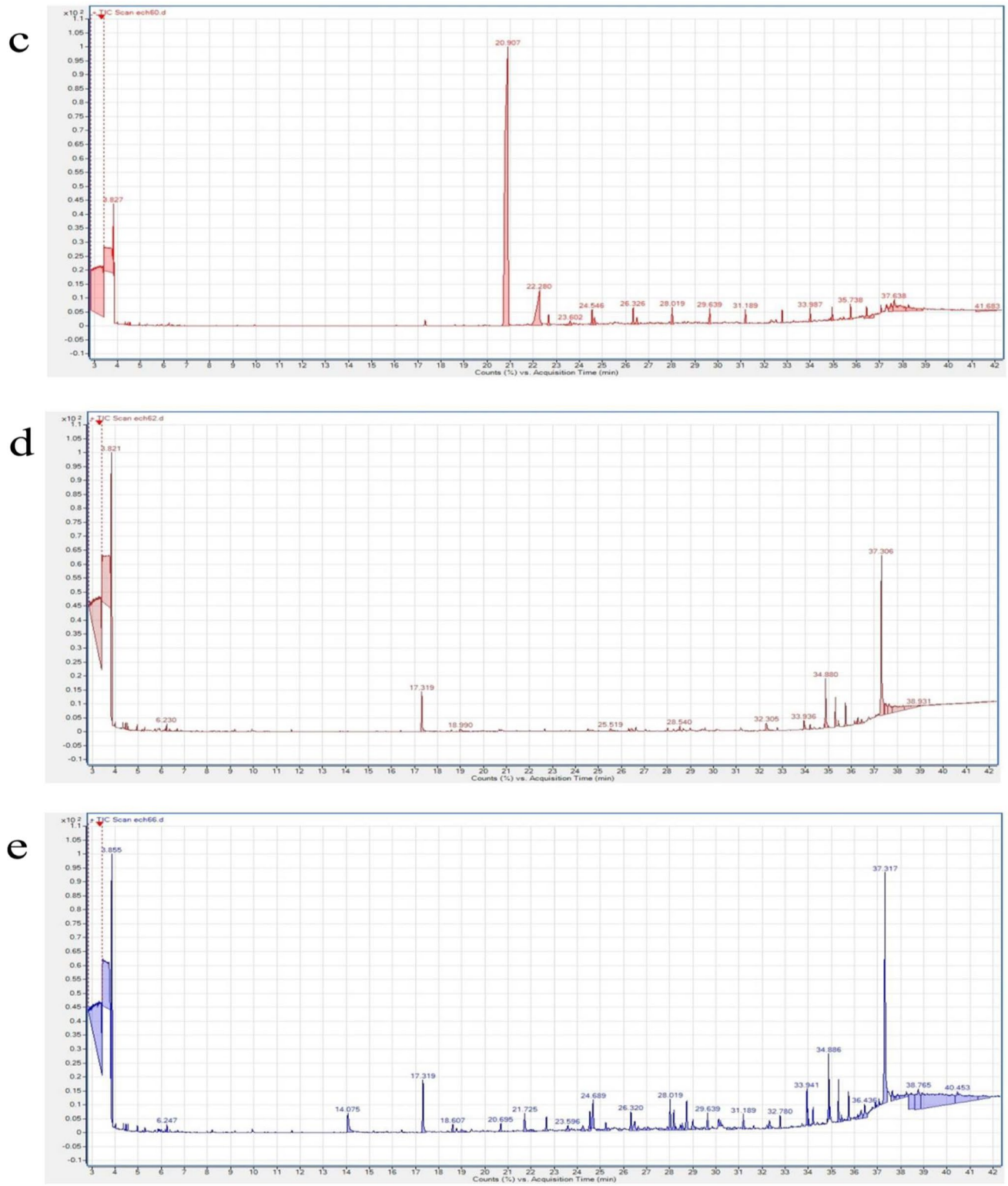

Fig. 9 (continued)

The spectrum for strain the *Phoma betae* showed also the disappearance of the bands at the range of (2930 and 2860 cm^{-1}) (Fig. 8e). The transmittance of the peaks at 1720 and 1075–1025 cm^{-1} was significantly increased.

After treatment with the strains *C. jodhpurense* and *C. maderasense*, it did not seem to be big change in band detected in the control, but a slight increase in the transmittance of some characteristic bands was observed. This result suggests a possible reduction of hydrocarbon compounds (Fig. 8b, c).

The post-treatment FTIR confirmed the efficiency of fungal crude oil degradation with the disappearance of some characteristic bands observed in the control, plus a noticeable reduction in the intensity of the preserving peaks.

GC–MS analysis of crude oil degradation metabolites

There are no significant changes in GC–MS spectrum in the control sample before and after ^{2}25 days incubation without fungi (Fig. 9a, a'). Only two peaks of undecane and dodecane were disappeared. This suggested that light chain alkenes could be lost by volatilization during the 25 days of incubation and that crude oil could resist abiotic degradation and physical interaction. This result is in concordance with those found by Olukunle and Oyegoke [73] after 16 days of incubation of crude-oil fungal degradation.

It was observed that all four fungal strains were able to degrade crude oil efficiently after 25 days of incubation (Fig. 9b–e). The disappearance of initial peaks and the appearance of new peaks confirmed the breakdown of crude oil products. *P. variabile* was the most efficient in crude oil degradation, as confirmed by the chromatogram with the disappearance of the major peaks (Fig. 9d). However, the main remaining aromatic peaks were benzaldehyde and benzenamine. The other peaks represented alkene and cycloalkene chains. *P. betae* is an efficient crude oil degrader. The persisting peaks in GC profile were essentially for Naphtalene and its derivatives as well as alkenes (Fig. 9e). The chromatographic patterns of crude oil degraded by *C. maderasense* showed a decrease in the area of some peaks (heptadecane, octadecane, nanodecane, Eicosane) (Fig. 9c). Aromatic compounds found in control such as Naphthalene, Fluorene, Anthracene, and Phenanthrene, and their derivatives were dissapeared. While a new peak of tetraphtalic acid at 20:907 min appeared which could be the result of the cleavage of the aromatic compound.

The strain *C. judheprens* GC–MS analysis of treated crude oil demonstrates the missing of some peaks of naphthalene and derivatives and the decrease in the area of the other peaks, which were present in the control (Fig. 9b).

These observations demonstrated the capacity of the tested strains to degrade the main hydrocarbon compounds present in crude oil. There were no toxic metabolites that were identified after fungal crude oil degradation.

Gnanasekaran et al. [74] also used GC data to analyze the biodegradation of crude oil by filamentous fungi and found that *Aspergillus* species strongly degrade hydrocarbons and convert toxic compounds into non-toxic after 35–40 days of incubation. The degradation of diesel fuel by fungi was studied by Ameen et al. [68] and the GC–MS analysis showed an effective degradation of this product. This degradation may be due to the high amounts of ligninolytic enzymes produced by the different strains. In addition, further studies have shown the critical role of these enzymes in the degradation of PAHs. Batista-García et al. [75] reported the involvement of ligninolytic enzymes (Laccase and peroxidase) in the degradation of PAHs and Phenols. Experiments have proved that high degradation of pyrene in a new fungal strain *Coriolopsis byrsina* is more efficient due to their ligninolytic enzyme activity [69]. Further studies have demonstrated the important role of these enzymes in hydrocarbon compound degradation [76, 77].

Overall, the highest crude oil removal was observed with *P. variabile* (48%) followed by *P. beta* (32%) and this result was confirmed with FTIR and GCMS spectra of these strains. This could be associated with the fact that only these two strains showed significant laccase activity in presence of crude oil which rushed 168 U/L and 127 U/L for *P. variabile* and *P. beta* respectively. Therefore, Li et al. [78] showed that laccase plays the main role in PAHs transformation.

Conclusion

Four fungal strains were isolated from marine Tunisian habits and identified as *Chaetomium maderasense*, *Chaetomium jodhpurense*, *Paraconiothyrium variabile*, and *Phoma betae*, which have also been reported in the literature as producers of ligninolytic enzyme. In the presence of crude oil, the selected fungi increased the ligninolytic enzyme production with a high prevalence of peroxidase. The results of FTIR and GCMS analyses demonstrated a great ability of the tested fungi to degrade crude oil efficiently in 25 days in the presence of 50% seawater. The selected strains can be used for further biotechnological processes, and even for the purpose of environmental bioremediation.

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Author contribution All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by Neila Hkiri, Frah Aounallah, and Khaoula Fouzai. The first draft of the manuscript was written by Neila Hkiri and Nedra Asses. The authors Neila Hkiri, Chedly Choucheni, and Nedra Asses

commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability The data used to support the finding of this study are available from the corresponding author upon request.

Declarations

Competing interests The authors declare no competing interests.

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