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ORIGINAL PAPER

Talaromyces sayulitensis, Acidiella bohemica and *Penicillium citrinum* in Brazilian oil shale by-products

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Abstract Fine shale particles and retorted shale are waste products generated during the oil shale retorting process. These by-products are small fragments of mined shale rock, are high in silicon and also contain organic matter, micronutrients, hydrocarbons and other elements. The aims of this study were to isolate and to evaluate fungal diversity present in fine shale particles and retorted shale samples collected at the Schist Industrialization Business Unit (Six)—Petrobras in São Mateus do Sul, State of Paraná, Brazil. Combining morphology and internal transcribed spacer (ITS) sequence, a total of seven fungal genera were identified, including *Acidiella*, *Aspergillus*, *Cladosporium*, *Ochroconis*, *Penicillium*, *Talaromyces* and *Trichoderma*. *Acidiella* was the most predominant genus found in the samples of fine shale particles, which are a highly acidic substrate (pH 2.4–3.6), while *Talaromyces* was the main genus in retorted shale (pH 5.20–6.20). *Talaromyces sayulitensis* was the species most frequently found in retorted shale, and *Acidiella bohemica* in fine shale particles. The presence of *T. sayulitensis, T. diversus* and *T. stolli* in oil shale is described herein for the first time. In conclusion, we have described for the first time a snapshot of the diversity of filamentous fungi colonizing solid oil shale by-products from the Irati Formation in Brazil.

Keywords Filamentous fungi · Fine shale particles · Shale · Oil shale · Retorted shale

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Introduction

The main requirement for successfully utilizing oil shale as a source of liquid and gaseous fuels is a technically and economically feasible retorting process to thermally decompose the kerogen into oil, gas and carbonaceous residue (Dinneen 1976). In 1972, Petrobras, the Brazilian state oil company, started oil shale processing activities by developing Petrosix® technology for extracting oil from the shale of the Irati Formation in São Mateus do Sul, Brazil. Petrosix® technology is an above-ground retorting process in which mined oil shale is reduced to particles of 10–75 mm and then conveyed to a retorter, where the shale is heated up to approximately 500 °C, breaking

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down the kerogen to yield oil vapour and gas (Cogo et al. 2009). Currently, the Petrobras Petrosix® plant generates a large volume of waste, mainly consisting of fine shale particles (FS) and retorted shale (RS). FS consists of small fragments of mined shale rock (particle size <6.35 mm) that are not usable in oil and gas processing, and RS is made up of retorting residues with high silicon content. RS also contains organic matter, micronutrients, hydrocarbons and other elements, such as phosphorus, calcium, magnesium and sulphur (Mangrich et al. 2001).

Polycyclic aromatic hydrocarbons are common environmental pollutants and were described by Nicolini et al. (2011) in Brazilian retorted oil shale samples taken from inside storage piles at the Schist Industrialization Business Unit (Six)—Petrobras, Brazil, the same place where our study was carried out.

Studies on mineral-microbe interactions have concentrated on bacteria and archaea (Dragutinović et al. 2012; Matlakowska and Sklodowska 2009), and, to some extent, fungi have been neglected (Burford et al. 2003). Few studies have reported on fungal species and diversity on the surface of oil-shale particles, such as Pfister et al. (1991) in the deposits of New Albany, US, and Jiang et al. (2016) in China by combining the clone libraries of ribosomal deoxyribonucleic acid internal transcribed spacer (rDNA ITS) with traditional pure culture. As reviewed by Watling (2015), studies on the role and contributions of microorganisms in mineral bioprocessing and bioleaching of mineral resources, including low- or high-organic content (shales or schists), emphasised mostly bacterial diversity.

For this reason, the aims of this study were to isolate and to evaluate fungal diversity present in FS and RS generated by oil shale industrialization in Brazil using Petrosix® Technology. The results will provide useful knowledge on fungal resources for the bio-utilization of oil shale and bioremediation of the by-products.

Materials and methods

Site and sampling description

Two samples of FS and two samples of RS were collected at the Schist Industrialization Business Unit (Six)—Petrobras in São Mateus do Sul, Paraná, Brazil (25°52′26″S, 50°22′58″W) (Fig. 1). The mine area is located at an altitude of 770 m above sea, according to Köppen's classification, the climate is type Cfb, temperate humid subtropical, oceanic without dry season, with temperate summer and severe frost and annual rainfall of 1550 mm. The average temperature of the hottest month is less than 22 °C, and that of the coldest month is below 18 °C (Alvares et al. 2014). The samples were collected with sterile tools at twelve discrete locations inside storage piles to obtain a composite sample. To avoid the collection of air-contaminated material, sampling was done after removal of a 30 cm surface layer. After combination into composite samples, two FS and two RS samples, each of approximately 1.0 kg, were placed in sealed and sterilized polyethylene bags, taken to laboratory, and split into two subsamples for chemical analyses and fungal isolation.

Chemical analyses

FS and RS samples were oven dried at 55 °C and sieved to <2 mm before chemical analysis using the methods described by Pavan et al. (1992). Briefly, the pH of the FS or RS was determined potentiometrically: $CaCl_2$ solution (0.01 mol L^{-1}) ratio of 1:2.5 (w/v) after shaking for 15 min and settling for 30 min. Calcium (Ca^{2+}), magnesium (Mg^{2+}) and aluminium (Al) were extracted with a non-buffered solution of KCl (1.0 mol L^{-1}) and measured by coupled plasma optical emission spectrometer (ICP-AS optima $_{830}$, Perkin-Elmer, USA). The total organic carbon concentration was evaluated by the Walkley–Black potassium dichromate–sulphuric acid oxidation procedure (Nelson and Sommers 1982). Phosphorus and potassium were extracted using the Mehlich-1 method, and their concentrations were determined colorimetrically using a UV–visible spectrophotometer and a flame photometer, respectively.

Isolation of fungi

Ten grams of each subsample were resuspended in 90 mL of sterile saline solution (NaCl 0.85%), shaken at 100 rpm for 1 h at 25 °C, and serially diluted (di Menna 1966). Aliquots of 100 µL were plated on two media: Sabouraud Agar (Silva et al. 2004) and Martin Medium Agar (Martin 1950). Two sets of Petri plates were prepared for incubation for 7 d at 28 and 37 °C to

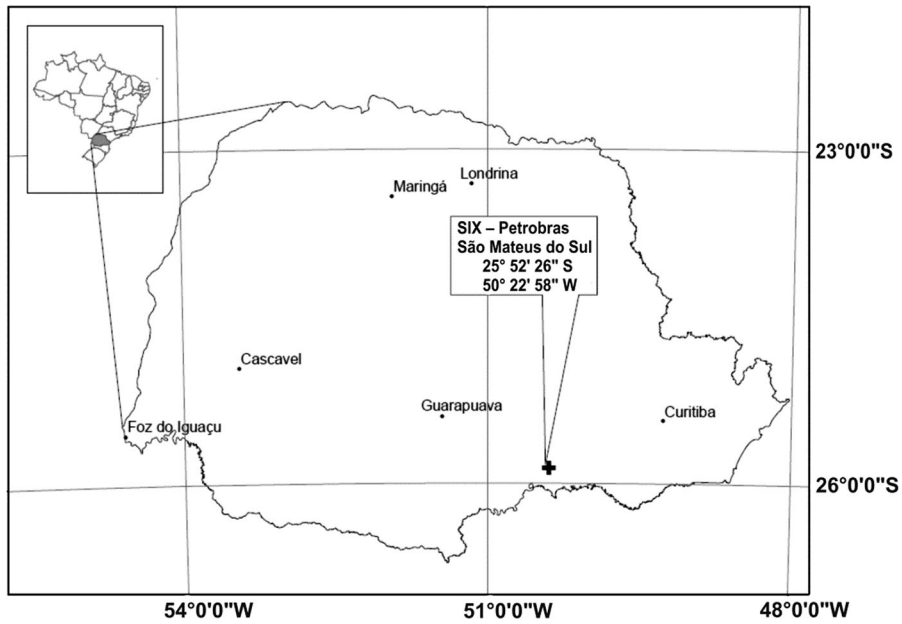

Fig. 1 Geographic location of the SIX-Petrobras mine in Paraná State, the sampling site for fine shale particles and retorted shale Source: IBGE, 2014 – adapted by João Henrique Caviglione

provide information on the growth range of some groups of fungi.

After the incubation period, the colonies were purified by streaking. Isolates are designated with the collection code Institute of Paraná (IPR6000 to IPR6057) and kept at the Collection of Microorganisms of the Laboratory of Soil Microbiology, Instituto Agronômico do Paraná (IAPAR), Brazil.

Morphological examination

The isolates were grown on standard media, Czapek Yeast Agar (CYA) and Malt Extract Agar (MEA). Macromorphological characteristics such as colony diameter, degree of sporulation, production of sclerotia, colour of mycelia and presence of exudate, were then checked. Microscopic features such as shape and size of vesicles and conidia were also observed in each culture medium.

The obtained isolates of the genus *Penicillium* were identified according to Pitt (1988) and Samson et al. (2007). Isolates of *Aspergillus* were identified according to Samson et al. (2007) and members of the genus *Cladosporium* were identified according to Bensch et al. (2012). Members of the genera *Acidiella*, *Talaromyces*, *Ochroconis* and *Trichoderma* were identified as described by Hujslová et al. (2013), Samson et al. (2011), Bissett (1991) and Samerpitak et al. (2014), respectively.

Molecular examination

For ITS nucleotide sequence analysis genomic DNA extraction was performed using approximately 100 mg of mycelia following a standard phenol: chloroform extraction protocol (Sambrook and Russell 2001). The ITS region was amplified using the primers ITS1 and ITS4 (White et al. 1990). The PCR products were cleaned up using ExoProStar® (GE Healthcare, UK) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Ten microliter of Hi-Di formamide was added to the sequencing products, which were then processed in an ABI3500XL Genetic Analyzer (Applied Biosystems, USA).

All ITS sequences were compared using the Basic Local Alignment Search Tool (BLASTn) against the NCBI database to recognize fungal species with similar DNA sequences. To avoid BLAST hits to misidentified sequences in the GenBank database, whenever possible we used the BLAST tool with the "sequences from type material" (RefSeq) option. If the identity values generated by BLAST were not sufficient to precisely identify the species,

phylogenetic trees were constructed using MEGA 6.0 software based on the Neighbor-joining (NJ) method and Tamura-Nei Model (Saitou and Nei 1987; Tamura et al. 2013). Nonparametric bootstrap analysis was performed with 1000 re-samplings. From the here obtained ITS sequences, a representative selection was deposited in GenBank under the accession numbers KX363445 to KX363463 (Table 1).

Statistical analysis

For chemical data, we used non-parametric independent Man-Whitney Rank Sum test ($\alpha = 0.05$) to determine if within each chemical element the mean values of two RS and FS are significantly different using the SIGMA Plot v 11.0.

Results

Chemical characteristics and fungi counting

The samples of FS (I and II) and RS (I and II) collected at SIX-Petrobras showed differences for some of the selected chemical characteristics (Table 2). There was a statistically significant difference between FS and RS for pH ($p < 0.01$) and aluminium concentration ($p < 0.05$). FS showed lower pH values with an average of 3.0 while RS samples showed a pH of 5.85. The average aluminium content was in FS samples 12.11 $cmol_c\ kg^{-1}$ and in RS samples only 0.05 $cmol_c\ kg^{-1}$. Calcium, phosphorus, magnesium, potassium and total carbon had similar concentrations in both by-products ($p = 0.100$).

Fungi were present in all samples and growth was observed only in Sabouraud Agar medium. The

Table 1 Fungal species isolated from samples of fine shale (FS) particles and retorted shale (RS) collected at Schist Industrialization Business Unit (Six)—Petrobras in São Mateus do Sul, Paraná—Brazil

Species	Isolates	
Aspergillus flavus	6001FS (KX363445)	
Aspergillus sydowii	6017FS (KX363451)	
Aspergillus niger/ welwitschiae		6029RS (KX363462)
Acidiella bohemica	6008FS (KX363448), 6014FS, 6015FS (KX363450), 6016FS, 6040FS (KX363456), 6043FS, 6044FS, 6045FS (KX363458), 6046FS	6030RS
Cladosporium cladosporioides complex		6051RS, 6053RS, 6057RS (KX363461)
Cladosporium sphaerospermum complex	6018FS (KX363452), 6047FS	6031RS, 6032RS, 6033RS
Ochroconis sp.	6042FS (KX363457)	
Penicillium citrinum	6000FS, 6002FS (KX363446), 6009FS, 6037FS (KX363454), 6041FS, 6010FS	6025RS, 6048RS, 6049RS
Penicillium parvum	6003FS (KX363447)	
Penicillium sp.	6038FS (KX363455)	
Talaromyces diversus		6035RS (KX363453)
Talaromyces sayulitensis	6004FS, 6005FS, 6006FS, 6007FS, 6011FS (KX363449), 6012FS, 6013FS, 6039FS	6019RS, 6020RS, 6021RS, 6022RS, 6023RS, 6024RS, 6026RS, 6027RS, 6028RS, 6050RS, 6052RS
Talaromyces stolli		6054RS (KX363459)
Trichoderma atroviride		6055RS (KX363460), 6056RS

The GenBank accession numbers are given in parentheses

Table 2 Chemical characteristics of fine shale particles (FS I and FS II) and of retorted shale (RS I and RS II) collected at Schist Industrialization Business Unit (Six)—Petrobras in São Mateus do Sul, Paraná, Brazil

Characteristics	FS I	FS II	Mean[e]	RS I	RS II	Mean[e]	*P*-values[f]
pH[a]	3.60	2.40	3.00	5.20	6.50	5.85	0.005
Ca ($cmol_c\ kg^{-1}$)[b]	50.47	49.15	49.81	35.57	21.95	28.76	0.100
Mg ($cmol_c\ kg^{-1}$)[b]	14.39	7.44	10.92	11.92	6.70	9.31	0.700
Al ($cmol_c\ kg^{-1}$)[b]	4.63	19.59	12.11	0.10	0.01	0.05	0.050
C ($g\ dm^{-3}$)[c]	29.22	27.23	28.22	31.79	31.63	31.71	0.100
P ($mg\ dm^{-3}$)[d]	145.30	134.00	139.65	150.20	154.10	152.15	0.100
K ($cmol_c\ kg^{-1}$)[d]	0.05	0.03	0.04	0.14	0.47	0.30	0.100

[a] pH in $CaCl_2$ 0.01 mol L^{-1}

[b] Ca (Calcium), Mg (Magnesium), and Al (Aluminum) by KCl 1 mol L^{-1}

[c] C (total Carbon) from Walkley–Black

[d] P (Phosphorus) and K (Potassium) in Mehlich-1

[e] Means of I and II by-product (FS and RS) samples

[f] *P* values from Mann–Whitney Rank Sum Test

numbers of fungal colony-forming units (CFU) per gram of by-product were 1.3×10^5 in RS I and 3.9×10^5 in RS II, while in FS I it was 3.8×10^4 and 4.1×10^4 in FS II, meaning that the retorted shale harboured higher fungal population density than the fine shale.

A slight decrease in the number of isolates was observed when the plates were incubated at 37 °C (eight FS isolates, nine RS isolates) in contrast to incubation at 28 °C (22 FS isolates, 17 RS isolates).

Fungal diversity

A total of seven fungal genera were found, including *Acidiella*, *Aspergillus*, *Cladosporium*, *Ochroconis*, *Penicillium*, *Talaromyces* and *Trichoderma*. By using morphological and physiological characteristics *Penicillium*, *Aspergillus*, *Cladosporium, Talaromyces* and *Trichoderma* were identified, but *Acidiella* and *Ochroconis* were not recognized. Morphological identification was confirmed by neighbor-joining analysis of the nrDNA internal transcribed spacer (ITS), the official DNA barcode for fungi that was determined for all fungi (30 from FS and 26 from RS—see Table 1).

The ten isolates recognized as *Acidiella* shared 99–100% sequence identity with *Acidiella bohemica* and *Fodinomyces uranophilus*.

The twenty *Talaromyces* ITS sequences were most similar to those belonging to *Talaromyces* section *Talaromyces*. An ITS-based neighbor-joining (NJ) tree using the obtained sequences and those for each type strain of *Talaromyces* section *Talaromyces* (Visagie et al. 2014), revealed that 19 isolates belonged to *Talaromyces sayulitensis* and the isolate 6054RS belongs to *Talaromyces stolli* (Fig. 2).

Isolate 6035RS showed 100% ITS sequence similarity to the type strain *Talaromyces diversus* of the section *Trachyspermi*.

The ten *Penicillium* ITS sequences were most similar to those from species belonging to *Penicillium* section *Citrina*. The identity values generated by BLAST were insufficient to precisely identify the species. An ITS-based neighbor-joining (NJ) tree using the obtained sequences and those for each type strain of *Penicillium* section *Citrina* (Visagie et al. 2014), showed nine isolates to be closely related to *Penicillium citrinum* (Fig. 3). Isolate 6038FS was grouped with *Penicillium sizovae*, *P. steckii*, *P. tropicalis* and *P. tropicum*, which are not distinguishable by ITS sequences. Isolate 6003FS, which belongs to the section *Exilicaulis*, showed 100% sequence identity with the type strain of *Penicillium parvum*.

Eight isolates were identified as belonging to the genus *Cladosporium*. Isolates 6051RS, 6053RS and 6057RS were identified as belonging to the *Cladosporium cladosporioides* complex. Isolates 6018FS, 6031RS, 6032RS, 6033RS and 6047FS were identified as belonging to the *C. sphaerospermum* complex.

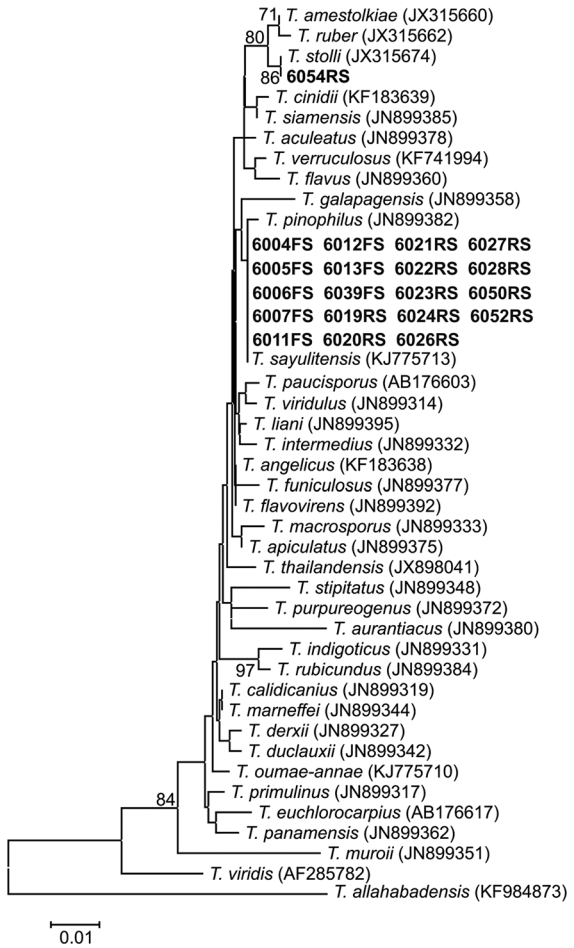

Fig. 2 Neighbor-joining tree reconstructed from the ITS sequence of *Talaromyces* section *Talaromyces* isolated from the samples of fine shale particles and retorted shale (*bold*) aligned with corresponding sequences of *Talaromyces* section *Talaromyces* deposited in public databases. *Numbers at branch nodes* refer to bootstrap values (1000 replicates). Only values >70% are shown

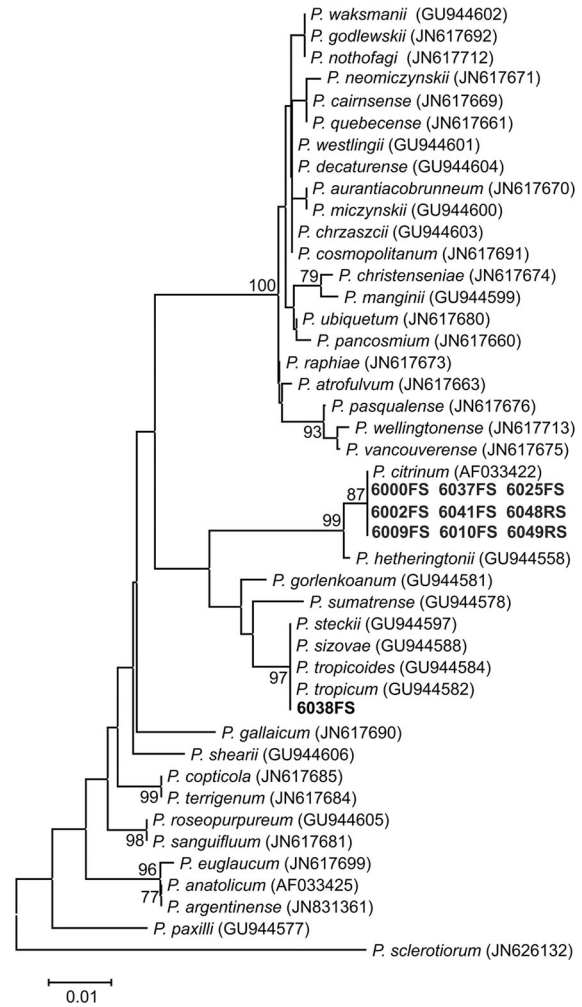

Fig. 3 Neighbor-joining tree reconstructed from the ITS sequence of *Penicillium* section *Citrina* isolated from the samples of fine shale particles and retorted shale (*bold*) aligned with corresponding sequences of *Penicillium* section *Citrina* deposited in public databases. *Numbers at branch nodes* refer to bootstrap values (1000 replicates). Only values >70% are shown

Three *Aspergillus* isolates were obtained from the samples studied herein. Isolates 6001FS, 6029RS, and 6017FS were identified as *Aspergillus flavus*, *A. niger/welwitschiae* with 100% sequence identity and *A. sydowii* with 99%, respectively.

Two isolates, 6055RS and 6056RS, were identified as *Trichoderma atroviride* with 100% identity.

Finally, the ITS sequence of isolate 6042FS was found to be distantly related to that of *Ochroconis mirabilis* with only 87% sequence identity (accessed 18 Jan, 2017).

Discussion

This is the first work carried out in Brazil on the isolation of microorganisms from shale by-products. No fungal growth was observed in Martin medium. Considering that this medium is generally used for isolation and relative enumeration of fungi in soils, we suggest that isolates obtained in this study originate from the shale samples and rather not from soil surrounding the storage piles. In addition, some of the

detected genera are common to shale samples from other localities in the world.

The number of isolates was higher in FS than RS samples maybe due to acidity, low pH and presence of aluminium; hence, we suggest that the composition of by-products alters the fungal diversity. There was a relationship between selected chemical characteristics and genus diversity in these oil shale by-products. For example, *Acidiella* was the predominant (90%) genus found in FS samples, which is a highly acidic substrate. This genus was also found living in highly acidic environments (Hujslová et al. 2013, 2017; Vázquez-Campos et al. 2014). *Fodinomyces*, another acid tolerant genus, was recognised as a synonym of *Acidiella* based on the high similarity in rDNA sequences, ecology, physiology and morphology (Kolařík et al. 2015).

Comparing the two shale by-products, there was a prevalence of *Talaromyces* in RS samples. The occurrence of *T. sayulitensis, T. diversus* and *T. stolli* in oil shale is described here for the first time. The genus *Talaromyces* was described as a sexual state of *Penicillium*, but with the recent adoption of the 'one fungus, one name' concept, in combination with some phylogenetic studies, *Penicillium* subgenus *Biverticillium* was transferred to the genus *Talaromyces* (Visagie et al. 2014; Yilmaz et al. 2014). The prevalent species in our study, *T. sayulitensis* was described based on isolates from house dust in Mexico (Visagie et al. 2014). The genus *Talaromyces* has been considered important for biotechnological purposes, based on its species' ability to produce enzymes and soluble pigments (Yilmaz et al. 2014). A study by our group on biofertilizer production showed that inoculation and incubation of RS and FS particles with *T. sayulitensis* (6004FS) increased the concentrations of carbon, phosphorus, potassium, calcium, magnesium and sulphur (unpublished data). Recently, Orencio-Trejo et al. (2016) reported that a Mexican strain of *T. stollii* has the potential for the production of balanced enzymatic complexes, with biotechnological use in the deconstruction of the lignocellulosic biomass.

In our study, the genus *Penicillium* was isolated more often from FS compared to RS with *P. citrinum* as the most representative species for this genus occurring in both RS and FS. This species was already reported from shale samples in Pakistan (Anjum et al. 2015). Based on the ITS region, an unidentified *Penicillium* isolate clustered with four *Penicillium* type species (Fig. 3). This was due to the lack of variability of the ITS region among these closely related *Penicillium* species (Visagie et al. 2014). Jiang et al. (2016) studied three main oil shale production sites from different areas in China, and observed that *Penicillium* was found in all three mines. These authors suggested that environmental and climatic conditions of each mine significantly affected the diversity of fungal genera, whereby two mines with temperate climate had higher diversity of fungi while a mine with subtropical climate allowed the isolation of only six genera (Jiang et al. 2016). In our study, the store piles of RS and FS were under the same environmental subtropical climatic conditions, hence, our hypothesis is that the differences in fungal diversity between two by-products samples were due mainly to chemical composition.

The genus *Penicillium* shows potential for the biotransformation of black shale, in particular the bioleaching of copper, cobalt and zinc, a process in which *Penicillium notatum* showed a good potential for generating organic acids effective in metal solubilization (Anjum et al. 2009).

The genus *Cladosporium* has been intensively investigated over the past decade resulting in the resolution of three major species complexes (*C. cladosporioides, C. herbarum* and *C. sphaerospermum*) based on morphology and DNA phylogeny (Bensch et al. 2015). The ITS region has limited resolution for many species in *Cladosporium* (Zalar et al. 2007). Consequently, our ITS sequence data were insufficient to discriminate further than to the *Cladosporium* species complexes. *Cladosporium* is a genus that has been found on many substrates and regions throughout the world, including oil shale from the Green River Formation, USA (Bradley 1931). *Cladosporium* species are also able to produce some secondary metabolites such as long-chain and α,β-unsaturated aldehydes with antibiotic activity (Gallo et al. 2004) and *C. cladosporioides* was identified as an extracellular enzyme producer (Aslam et al. 2012). Furthermore, some *Cladosporium* species are efficient biological insecticides (Abdel-Baky and Abdel-Salam 2003).

Aspergillus flavus, *A. sydowii,* and *A. niger/welwitschiae* were the only *Aspergillus* species found in our samples. Anjum et al. (2015) also reported the presence of *A. niger,* and *A. flavus* in black shale samples in Pakistan. *A. niger* and *A. flavus* are

predisposed to produce a variety of organic acids (citric and oxalic) for effectively solubilizing rare earth elements (REEs). Amin et al. (2014) tested the ability of some fungi isolated from Sinai Peninsula carbonaceous shales to leach REEs from these rocks by microbial means and found that *A. niger* and *A. flavus* were the most efficient microorganisms.

Trichoderma species are fungi that occur in nearly all soils and other natural habitats. The genus comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on multiple mechanisms (Sharma and Gothalwal 2017).

In our study, *Trichoderma atroviride* was found only in RS. The genus *Trichoderma* has been reported to be able to solubilize coal (Hölker and Höfer 2002; Oboirien et al. 2008) and to be tolerant to a range of recalcitrant pollutants, including heavy metals, pesticides, and polyaromatic hydrocarbons (Tripathi et al. 2013).

The isolate 6042FS could not be identified to the species level by ITS sequence analysis. To determine whether 6042FS represents a new species of the genus *Ochroconis*, analyses of additional sequence data, such as actin, β-tubulin, and translation elongation factor 1-α genes, morphological characters and metabolite profiling will be necessary.

The genus *Ochroconis* was reviewed by Samerpitak et al. (2014) and it was recognized to contain thirteen species. After this review, a further eight new species were proposed (Crous et al. 2014; Samerpitak et al. 2015a, b). Several species of the genus *Ochroconis* show unusual high DNA sequence heterogeneity compared to most other ascomycetous fungi (Samerpitak et al. 2015a, 2016). Not only ITS but also nuclear LSU showed significant sequence variation for species recognition (Samerpitak et al. 2015a). The species of the genus *Ochroconis* have a cosmopolitan distribution and can be isolated from various sources, including cave rock and ants (Duarte et al. 2014; Martin-Sanchez et al. 2012; Nováková 2009).

Conclusions

In conclusion, combining morphology and DNA sequence data, we have described for the first time a snapshot of the diversity of filamentous fungi colonizing oil shale by-products from the Irati Formation in Brazil. *T. sayulitensis* was the species most frequently found in retorted shale, and *A. bohemica* in fine shale particles.

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Conflict of interest The authors declare that they have no conflict of interest.

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