Screening and molecular characterization of polycyclic aromatic hydrocarbons degrading yeasts

[Polisiklik aromatik hidrokarbonları parçalayan mayaların taranması ve moleküler karakterizasyonu]

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ABSTRACT

Objective: Disasters such as leakages and accidental falls are the main causes of environmental pollution by the petroleum industry product. Since no commercial yeast strain with bio-degradation capacity is available, we aimed to isolate and characterize hydrocarbon degrading yeasts.

Methods: Yeast isolates used in the study were isolated from samples of wastewater, active sludge and crude oil, which was obtained from a petroleum refinery as well as from soil samples, which were contaminated with crude oil. Yeast isolates used in the study were isolated from wastewater, active sludge and petroleum samples obtained from petroleum refinery and soil samples contaminated with petroleum. Degradation of naphthalene, phenanthrene, pyrene and crude petroleum by yeasts were determined using a microtiter plate-based method. Molecular characterization was achieved by performing a sequence analysis of the ITS1-5.8S rRNA-ITS2 and 26S rRNA regions.

Results: In total, 100 yeast isolates were obtained from four different samples. Following the incubation in media containing different polycyclic aromatic hydrocarbon compounds (naphthalene, phenanthrene, pyrene) and crude petroleum, 12 yeast isolates were detected to degrade more than one polyeatomic hydrocarbon. Sequence analyses of rRNA regions revealed that the identified yeasts represented 10 species belonging to 6 genera. The isolates were identified as Candida parasilopsis, Candida sinolaborantium, Cryptococcus albidus, Cryptococcus diffluens, Cryptococcus uzbekistanensis, Pichia kudriavzevi, Rhodotorula diobovatum, Rhodotorula glutinis, Rhodotorula mucilaginosa and Saccharomyces cerevisiae.

Conclusion: Yeast strains that are capable of degrading more than one polycyclic aromatic hydrocarbon compound have the potential of being utilized in future research.

Key Words: Identification, yeasts, polycyclic aromatic hydrocarbon, 26S rRNA, ITS1-5.8S rRNA-ITS2

Conflict of Interest: The authors have no conflict of interest.)

ÖZET

Amaç: Petrol endüstrisi ürünleri ile olушуランる 산출물과 카자라기 외래 격어, 경계기리 등이 없는 관리, 행적에 의한 유해물질의 발생은 주된 원인이다. 환경 오염에 대한 자원이 희소한 상황이므로, 우리는 이 요인에 대해 원활한 수단을 제공할 필요가 있다.

Metod: 야생생물과 같은 생물체는 수소소조도를 포함한 다기능적 화합물을 첨적하고 있다. 이에 따라 이 분야에 대한 연구는 중요하다. 이에 따라, 이 연구에서는 폐수, 활성 슬루지 및 기름을가진 환경에서 분리한 100여 개의 모세조종을 분석하였다. 이들 중 12개의 모세조종이 다수기능적 화합물을 첨적할 수 있었다.

Result: 총 100개의 모세조종이 4개의 샘플에서 분리되었다. 이들 중 12개의 모세조종이 다수기능적 화합물을 첨적할 수 있었다. 이들 중 10개의 종이 6개의 개체를 구성하였다. 이들 중 12개의 모세조종이 다수기능적 화합물을 첨적할 수 있었다.

Conclusion: 이 연구에서 얻은 결과는 이들의 활용가치를 높일 수 있는 가능성을 보여주었다. 이들 중 12개의 모세조종이 다수기능적 화합물을 첨적할 수 있었다. 이들 중 10개의 종이 6개의 개체를 구성하였다. 이들 중 12개의 모세조종이 다수기능적 화합물을 첨적할 수 있었다.

Key Words: Identification, yeasts, polycyclic aromatic hydrocarbon, 26S rRNA, ITS1-5.8S rRNA-ITS2

Conflict of Interest: The authors have no conflict of interest.
Introduction

Petroleum-based products are the main source of energy for industry and daily life [1]. Release of these compounds into the environment is a main cause of water and soil pollution [1,2]. Leakages and accidental spills occur frequently during surveying, production, refining, transport, and storage of petroleum and petroleum-based products [1].

Petroleum is a complex mixture of numerous compounds, which can be separated into four main groups: alkanes, aromatics, resins, and asphaltenes. The alkane fraction is the most biodegradable, whereas the resins and asphaltenes are resistant to biological degradation. The aromatic compounds, particularly the polycyclic aromatic hydrocarbons (PAHs), are compounds composed of two or more bonded benzene rings [3]. Since some PAHs are harmful, mutagenic and carcinogenic, they impose a potential risk to human health. The US Environmental Protection Agency (USEPA) has recognized sixteen PAH compounds as important pollutants, whose levels in industrial wastes require monitoring [4,5].

Reducing PAHs in a contaminated environment is a considerable challenge. Mechanical and chemical techniques have limited efficiency and can be expensive [6]. Biodegradation via microorganisms is generally accepted as the ideal and principal way of PAH removal from the polluted environment owing to some characteristic advantages including cost effectiveness and comparatively high clean-up efficiency [6-10]. Microorganisms, mainly bacteria and fungi, were detected to reduce PAHs via either metabolism or co-metabolism [8,11,12]. More than 80% of the studies conducted on microbial biodegradation of oil-related contaminants are devoted to bacterial biodegradation. Bacteria are the most studied microorganisms and the contribution of bacteria during hydrocarbon mineralization in soil has been widely studied [13-15], however, a small number of fungal species capable of degrading hydrocarbons were reported [8,10-12,14,16].

Hydrocarbon pollutants have similar or analogous molecular structures, which allow the fungi to act on them as well. Fungi possess degrading capabilities to handle a series of hydrocarbon compounds that serve as potential carbon sources. Also, fungi have numerous stress responses that generate further phenotypic and genetic variety and thus, they can adapt to a new environmental condition. When an environment is contaminated, residents that are better able to break down the pollutant are stimulated [17]. The capability of yeasts to transform PAHs seems to be widespread in diverse environments. As PAH-degrading yeasts are well adapted to their environments, isolates acquired from different locations may have diverse degradative potentials [18]. Therefore, the selection of microorganism strains with improved degrading capabilities is one of the most important factors that affect the success of microbial biodegradation studies.

In the present study, we aimed to isolate and characterize hydrocarbon degrading yeasts.

Materials and Methods

Sampling and isolation of yeasts

In the study, the active sludge (1) and petroleum samples (1) were obtained from İzmir Tüpraş petroleum refinery and the two soil samples contaminated with petroleum were obtained from Mersin harbor from Turkey. Yeast isolation was performed on Yeast Malt (YM) agar composed of (g/L); 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 15 g agar. Inoculated plates were incubated at 27°C for 5-7 days [19].

Determination of degradation capabilities of yeasts by microtiter plate technique

The screening of yeasts for their capability of utilizing hydrocarbons was performed in microtiter plates (flat bottom, 96 wells), using 200 µl Bushnell-Haas medium (pH 7.2), per well. Each well received 10 µl of each stock solution of phenanthrene, pyrene or naphthalene (>96% purity, Fluka) dissolved in acetone to give a final concentration of 10 mg PAH/l. The acetone was allowed to evaporate at room temperature. Afterwards, the PAH-containing medium was inoculated with 10 µl of a dense suspension of microbial cells. Each yeast isolate was inoculated in four different microtiter plates containing phenanthrene, pyrene, naphthalene and crude petroleum. Plates were incubated for 21 days at 27°C. At the end of the period, 50 µl of p-iodonitrotetrazolium violet (INT) indicator was added to each well. Plates were re-incubated for 24 hours and each microtiter plate was scored for positive results. INT is one of the growth indicator reagents and thus, microbial growth was observed easily as colored precipitate. For medium to low chain alkanes a positive result was indicated by red precipitate; crude oil samples were scored for brown precipitate [20,21].

Phenotypic characterization of the yeast isolates

Individual yeast isolates with degradation capacity were characterized based on their physiological and morphological properties according to Kurtzman et al. [22].

Sequencing of ribosomal RNA regions

In our study, yeast isolates showing PAH-degrading capabilities were also characterized by sequencing two different rRNA genes. DNA isolation was performed as described by Liu et al. [23]. Oligonucleotide primers that were used for the amplification of gene sequences and the sequencing of the genes are given in the following sections. The amplification reactions were performed with Corbett Cool Gradient Palm Cycler CGI-96, under the conditions described elsewhere [19]. Both strands of the DNA were sequenced using an ABI 3130XL automated sequencer (Applied Biosystems, USA) following the manufacturer’s instructions.

ITS1-5.8S rRNA-ITS2. PCR amplicons were synthesized
using primers ITS1 (5’-TCC GTA GGT GAA CCT GCG G-3’) and ITS4 (5’-TCC TTC CC CC GCT TAT TGA TAT GC-3’) (Fermentas, Germany). For the amplification was performed for 40 PCR cycles with denaturation at 95°C for 2 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min, and final extension for 10 min.

Sequence of D1/D2 domain of 26S rRNA. The following primers were used amplifications: NL1 (5’-GCA TATCAA TAA GCG GAG GAA AAG–3’) and NL4 (5’-GGTCGC TGT TTC AAG ACG G-3’). Amplification was performed for 35 PCR cycles with denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min, and final extension for 10 min. The sequences were compared pairwise using a BLASTN search and were aligned with sequences of related species retrieved from GenBank using the multiple alignment CLUSTAL W version 2.0 software. A phylogenetic tree was constructed using the Tamura-Nei neighbor joining method by MEGA software version 5.0 [24]. Confidence levels of the clades were estimated by bootstrap analysis (1,000 replicates) [25].

Results

In the presented study, different environments polluted with petroleum were sampled for the isolation of yeasts and in total one hundred yeast isolates were obtained. Then, the degradation of two-ringed naphthalene, three-ringed phenanthren, four-ringed pyrene hydrocarbons and crude petroleum by the yeast isolates were investigated using microtiter plate-based technique. Among all tested yeast isolates; twelve (numbered as TEM12, 13, 14, 15, 17, 22, 27, 29, 39, 44, 66 and 79) exhibited red and brown precipitate and was capable of degrading more than one polyaromatic hydrocarbon (Table 1).

Table 1. Some properties and degradation capacities of the yeast isolates

| Isolate No. | Source     | Phenotypic identification | Naphthalene | Phenanthren | Pyrene | Petroleum |
|-------------|------------|---------------------------|-------------|-------------|--------|-----------|
| TEM12       | Active sludge | *Candida* sp.              | +           | +           | +      | –         |
| TEM13       | Petroleum   | *Rhodotorula* sp.          | –           | +           | +      | +         |
| TEM14       | Active sludge | *Candida* sp.              | –           | +           | +      | +         |
| TEM15       | Active sludge | *Cryptococcus* sp.         | +           | +           | +      | –         |
| TEM17       | Soil        | *Rhodotorula* sp.          | +           | +           | +      | +         |
| TEM22       | Soil        | *Candida* sp.              | +           | +           | +      | +         |
| TEM27       | Soil        | *Pseudozyma* sp.           | –           | +           | –      | +         |
| TEM29       | Soil        | *Rhodotorula* sp.          | +           | +           | +      | –         |
| TEM39       | Soil        | *Pseudozyma* sp.           | –           | –           | +      | +         |
| TEM44       | Petroleum   | *Rhodotorula* sp.          | +           | +           | +      | +         |
| TEM69       | Soil        | *Candida* sp.              | +           | +           | +      | +         |
| TEM79       | Soil        | *Kondoa* sp.               | +           | –           | +      | +         |

Table 2. Molecular characterization of the yeasts

| Isolate no. | ITS1-5.8S rRNA-ITS2 | D1/D2 domain of 26S rRNA | Species               |
|-------------|---------------------|--------------------------|-----------------------|
|              | Accession No.       | Homology (%)             | Accession No.         | Homology (%)             | Species               |
| TEM12       | KC182124.1          | 100                      | JQ779970.1            | 99                      | *Pichia kudriavzevii* |
| TEM13       | KC182125.1          | 99                       | JQ779971.1            | 99                      | *Rhodotorula glutinis*|
| TEM14       | KC182126.1          | 99                       | JQ779972.1            | 99                      | *Saccharomyces cerevisiae*|
| TEM15       | KC182127.1          | 100                      | JQ779973.1            | 99                      | *Rhodosporidium diobovatum*|
| TEM17       | KC182128.1          | 98                       | JQ779974.1            | 99                      | *Rhodotorula mucilaginosa*|
| TEM22       | KC182129.1          | 99                       | KC182118.1            | 98                      | *Candida sinolaborantium*|
| TEM27       | KC182130.1          | 99                       | JQ277254.1            | 99                      | *Cryptococcus albidus*|
| TEM29       | KC182131.1          | 99                       | JQ779975.1            | 96                      | *Rhodotorula mucilaginosa*|
| TEM39       | KC182132.1          | 99                       | KC182119.1            | 98                      | *Candida sinolaborantium*|
| TEM44       | KC182134.1          | 99                       | JQ277259.1            | 99                      | *Cryptococcus diffluens*|
| TEM69       | KC182136.1          | 99                       | KC182121.1            | 99                      | *Candida parapsilosis*|
| TEM79       | KC182137.1          | 99                       | JQ779981.1            | 99                      | *Cryptococcus uzbekistanensis*|
Physiological and morphological characteristics of the yeasts

Investigation of the physiological and the morphological characteristics of twelve yeast isolates revealed that yeast isolates from five different genera were represented in the samples. These genus are Candida sp. (TEM12, 14, 22 and 69), Rhodotorula sp. (TEM13, 17, 29 and 44), Cryptococcus sp. (TEM15), Pseudozyma sp. (TEM27 and 39) and Kondoa sp. (TEM79).

Sequencing of ITS region

In our study, PCR amplification of the ITS1-5.8S rRNA-ITS2 regions using primers ITS1 and ITS4 rendered products varying in size from 424 to 719 bp. The smallest fragment was produced by the Pichia kudriavzevii isolate and the largest by isolates included in the Saccharomyces cerevisiae. For twelve isolates, accession numbers were reserved and the sequences obtained were compared with GenBank database, using the BLASTN tool presented in Table 2.

Sequencing of D1/D2 domain of 26S rDNA region

As a result of the amplification of D1/D2 domain of 26S rRNA regions, a single fragment with a molecular size, ranging from 489 to 582 bp, was obtained. The sequences were compared with GenBank database using the BLASTN tool and accession numbers were taken.

Sequencing of ITS1-5.8S rRNA-ITS2 and 26S rRNA regions of twelve yeast isolates revealed the presence of ten species belonging to six different genera. The 7 of the species were characterized as basidiomycetes and 5 of the species were characterized as ascomycetes (Table 2 and Fig. 1).

Discussion

Using the physiological and morphological characteristics for the identification of yeasts at the species level [22,26], does not yield consistent results with those determined through the molecular biological methods [27,28]. Therefore, the strains phenotypically identified at the genus level in our study were also subjected analyses using to molecular biological methods. The variances in the rRNA internal transcribed spacer (ITS) were used to classify yeast species [29]. The ITS region is relatively short (500-800 bp) and can be readily amplified by PCR using universal single primer pairs that are complementary to conserved regions within the rRNA subunit genes [30]. Also, Kurtzman and Robnett [31] determined the extent of divergence in the variable D1/D2 domain of 26S rDNA in about 500 species of ascomycetous yeasts. The results indicated that the strains displaying greater than 1% substitution in proximity of the 600-nucleotide D1/D2 domain were likely to be different species, and that strains with 0-3 nucleotide differences are either conspecific or sister species. Therefore, this technique was reported to be effective for the
identification and classification of yeasts [31,32].

Eco-friendly biological methods have been preferred in the removal of environmental contaminants in recent years, owing to the performance of assemblies of novel and effective genetically engineered organisms. This study aimed for the isolation and identification of yeast strains, which were effective in PAH degradation. Currently, no commercial yeast strain having biodegradation capacity is available. Therefore, determination of yeast strains with improved degradation activity plays an important role in the management of pollution resulting from environmental disasters such as tanker accidents.

The potential for yeasts to metabolize PAHs is well-known. Yeasts were found to oxidize PAHs via either metabolism or co-metabolism [8,11,12,16]. Also, in a previous study [33], some yeast isolates were determined to possess more than one route to degrade PAHs. For decades, members of the genera Candida, Cryptococcus, Pichia, Rhodosporidium, Rhodotorula and Saccharomyces used in the study are considered potential degraders for harmful organic contaminants, such as aliphatic and PAHs [1,10,12,18,34,35]. Biodegradation capacities of most of the yeast species isolated in the study has been revealed for the first time. As seen in Table 1, the yeasts were determined to be capable of biodegrading more than one polyaromatic hydrocarbon. Since strains capable of degrading at least three PAH compounds have the potential of being used as mixed cultures in future researches, it is expected that researchers interested in this field can make use of these strains.

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Conflict of Interest

There are no conflicts of interest among the authors.

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