Ecotoxicology and Environmental Safety 151 (2018) 28–34

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Biodegradation and detoxification of aliphatic and aromatic hydrocarbons by new yeast strains

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ARTICLE INFO

Keywords:
Environment
Contamination
Octane
Pyrene
Yeasts
Bioremediation

ABSTRACT

Seeking new efficient hydrocarbon-degrading yeast stains was the main goal of this study. Because microorganisms are greatly affected by the environmental factors, the biodegradation potentiality of the microorganisms varies from climatic area to another. This induces research to develop and optimize the endemic organisms in bioremediation technology. In this study, 67 yeast strains were tested for their growth potentiality on both aliphatic and aromatic hydrocarbons. The most efficient six strains were identified using sequence analysis of the variable D1/D2 domain of the large subunit 26S ribosomal DNA. The identity of these strains was confirmed as *Yamadazyma mexicana* KKUY-0160, *Rhodotorula taiwanensis* KKUY-0162, *Pichia kluyveri* KKUY-0163, *Rhodotorula ingeniosa* KKUY-0170, *Candida pseudointermedia* KKUY-0192 and *Meyerozyma guilliermondii* KKUY-0214. These species are approved for their ability to degrade both aliphatic and aromatic hydrocarbons for the first time in this study. Although, all of them were able to utilize and grow on both hydrocarbons, *Rhodotorula taiwanensis* KKUY-0162 emerged as the best degrader of octane, and *Rhodotorula ingeniosa* KKUY-170 was the best degrader of pyrene. GC-MS analysis approved the presence of many chemical compounds that could be transitional or secondary metabolites during the utilization of the hydrocarbons. Our results recommend the application of these yeast species on large scale to approve their efficiency in bioremediation of oil-contamination of the environment. Using these yeasts, either individually or in consortia, could offer a practical solution for aquatic or soil contamination with the crude oil and its derivatives in situ.

1. Introduction

Environmental pollution can be caused by spills during the industrial production process or disposal of toxic compounds. Industrial wastes such as aliphatic and aromatic hydrocarbons are derived from petroleum, charcoal, and wood, as well as natural products, halogenated solvents, pesticides, herbicides and explosives (Csutak et al., 2010). Environmental pollution by petroleum hydrocarbons and its adverse effects are among the most threatening problems that the world is facing today (Tanaka and Yamada et al., 2012; Ghosal et al., 2016).

Many physicochemical techniques including incineration, base-catalyzed dechlorination, UV oxidation, fixation and solvent extraction are used for the clean-up of oil contamination, but the interest in using of microbial biodegradative activity is growing (Gargouri et al., 2015; Ghosal et al., 2016). Bioremediation of petroleum hydrocarbon has many advantages compared to the conventional decontamination techniques. These advantages include maintaining of the ecological equilibrium and the low cost. In addition, the bioremediation could be used in combination with other treatment techniques (Csutak et al., 2010; Iheanacho et al., 2014). It was approved that several compounds present in petroleum hydrocarbons are consumed by microorganisms (Tanaka and Yamada et al., 2012). Many microorganisms were tested for bioremediation of different hydrocarbons due to their ability to assimilate them. Recently, hydrocarbon-utilizing yeasts emerged as significant microbes that could oxidize the spills on land and in aquatic environments (Garapati et al., 2012). The common yeast species that were recorded as hydrocarbon-degraders are belonged to the genera: *Candida, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Stephanoascus, Trichosporon* and *Yarrowia* (Kannan et al., 1990; Csutak et al., 2010; Kumari and Abraham, 2011; Jain and Bajpai, 2012; Gargouri et al., 2015; Okerentugba et al., 2016). *Yarrowia lipolytica* strain was among the most common yeasts that have a positive influence on crude oil degradation (Horakova and Nemec, 2000; Iheanacho et al., 2014). However, these microorganisms are affected by several environmental factors such as energy sources, nutrients, pH, temperature (Csutak et al., 2010). This makes the efficiency of microorganisms vary widely from ecological area to another. In addition, the

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https://doi.org/10.1016/j.ecoenv.2017.12.064
Received 17 July 2017; Received in revised form 29 December 2017; Accepted 29 December 2017

bioremediation potentiality could be varied from strain to strain within the same species depending on the enzyme producing capacity and other factors (Okerentugba et al., 2016). Consequently, seeking and adaptation of new yeast species or strains that could degrade a wide range of hydrocarbons represent a prospective achievement from the practical point of view.

2. Materials and methods

2.1. Yeast organisms

The yeast species, used in this study, were obtained during our other works (data not published). Sixty-seven yeast species were isolated, purified and preserved on Yeast-Malt agar medium (YMA) that contains yeast extract (0.3%), malt extract (0.3%), glucose (1.0%) and agar (1.5%).

2.2. Tested hydrocarbons

Octane as an aliphatic hydrocarbon and pyrene as an aromatic hydrocarbon were selected to tests their degradability by different yeast strains in vitro. Octane is an aliphatic alkane hydrocarbon with the chemical formula C_8H_{18}, and its condensed structural formula is $CH_3(CH2)_6CH_3$. Its molecular weight is 114.23 and the density is 0.703 g/ml. Pyrene is a polycyclic aromatic hydrocarbon (PAH) consisted of four fused benzene rings, resulting in a flat aromatic system. Its chemical formula is $C_{16}H_{10}$ and the molecular weight is 202.25. The two hydrocarbons were obtained from Fluka (Steinheim, Germany).

2.3. Identification of yeasts

Six yeasts strains, which emerged as the best hydrocarbon degraders, were subjected to the molecular identification via sequence analysis of the variable D1/D2 domain of the large subunit 26 S ribosomal DNA. Genomic DNA of the target yeasts was isolated using a protocol escribed by Cardinali et al. (2001). The DNA was amplified using primers NL1 (5′GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′ GGTCCGTGTTTCAAGACGG –3′) following the method described by Kurtzman and Robnett (1998). The amplified DNA was purified using the GFXTM – PCR DNA and gel band purification kit (Amersham, Biosciences) according to the manufacturer's protocol. The purified PCR was sequenced at the Macrogen Company (Seoul, Korea). The DNA sequences were analyzed using the DNA Blast and the obtained nucleotide sequences were deposited in the GenBank under specific accession numbers.

The 26S rDNA sequences of the yeast strains were aligned with those of neighboring taxa based on secondary structure information using the PHYDIT program. Phylogenetic trees were constructed with Kimura's 2-parameter distance model (Kimura, 1980) and the neighbor-joining method (Saitou and Nei, 1987) using the PHYLIP 3.57c package. Confidence levels for the individual branches of the resulting tree were assessed by bootstrap analysis (Felsenstein, 1985). The resultant phylogenetic trees were visualized using the TreeView program. Yeast strains were identified by the 100–97% similarity criteria of 26 S rDNA D1/D2 domain (Kurtzman and Robnett, 1998) with reference to the phylogenetic relationships (Hong et al., 2002).

2.4. The preliminarily screening of hydrocarbon degradation

Degradation of both aliphatic and aromatic hydrocarbons by 67 yeast strains was tested preliminarily on solid medium using yeast nitrogen base (YNBA) (Fluka) which contains: ammonium sulfate 5 g/l, biotin 2 µg/l, calcium pantothenate 400 µg/l, folic acid 2 µg/l, inositol 2000 µg/l, nicotinic acid 400 µg/l, p-aminobenzoic acid 200 µg/l, pyridoxine HCl 400 µg/l, riboflavin 200 µg/l, thiamine HCl, 400 µg/l, citric acid 0.1 g/l, trace Elements boric acid 500 µg/l, copper sulfate 40 µg/l, potassium iodide 100 µg/l, ferric chloride 2000 µg/l, manganese sulfate 400 µg/l, sodium molybdate 200 µg/l, zinc sulfate 400 µg/l, potassium phosphate monobasic 1 g/l, magnesium sulfate 0.5 g/l, sodium chloride 0.1 g/l, calcium chloride 0.1 g/l, agar 15 g/l and the final pH was 5.4 ± 0.2 at 25 °C. For each yeast strain, 15 ml of the YNBA, inoculated with the desired yeast isolate (about 10^8 /ml), was poured into Petri plates (3 plates per strain). The yeast inoculum was prepared by growing each yeast isolate on YMA plates at 25 °C for 2 days. After solidification, a hole in the agar was made by a cork borer (0.5 cm in diameter). This hole was filled with either octane or pyrene (50 µl of 1% concentration) as a sole carbon source. The plates were incubated at 25 °C for one week. During the incubation period, the growth of the yeast around the hole containing the hydrocarbon was visually examined daily. The result was recorded qualitatively as +ve or –ve result. Control plates were inoculated with sterilized distilled water instead of the hydrocarbons.

2.5. Degradation of hydrocarbon by the most active yeast strains

Hydrocarbon degradation was carried out over 15 days in 250-ml conical flasks according to Singh and Lin (2008). Each conical flask contained 50 ml of MSM (minimal salts medium). This medium composed of KH_2PO_4 1.0 g/l, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCl_2{\cdot}2H_2O$ 0.02 g/l, K_2HPO_4 1.0 g/l, $FeCL_2$0.02 g/l, NH_4NO_3 1.0 g/l, 0.1% Tween 80 in 1 l of distilled water. Flasks containing the medium were autoclaved at 121 °C for 20 min prior to the addition of the tested hydrocarbons. The hydrocarbon (octane or pyrene) was added as 1% of the quantity of the medium. The yeast inocula were prepared by growing them on malt extract agar plates at 25 °C for 2 days and suspending the yielding mass in sterilized distilled water. One ml of yeast inoculum (OD 600 equivalent) was transferred into each conical flask. The flasks were incubated in a rotatory shaker incubator programmed at 150 rpm and 30 °C for 15 days. A control devoid of the yeast isolate was prepared for each set of the experiments. All experiments were performed in triplicate. The evaluation of the biodegradation of the hydrocarbons was carried out spectrophotometry within 3-days intervals (0, 3, 6, 9, 12 and 15 days of experiment, respectively). The optical density (OD) was measured 600 nm (Miranda et al., 2007).

2.6. Gas chromatography - mass spectrometry (GC-MS) analysis

The evaluation of the hydrocarbons biodegradation by yeast strains was carried out after the process of 9 days using gas chromatography with mass spectrometry GC/MS. After 9 days of incubation (end of the exponential phase), the yeast suspension was centrifuged at 10,000 rpm for 15 min under cooling and the supernatant was filtered through cellulose membrane filter (0.45 µm), and then was extracted by chloroform solution. An aliquot of one µl extract (chloroform extract) of cell-free extract was analyzed by HP-5890 GC-MS equipped with HP-5972 mass detector. The analysis was performed by using the HP-innowax column 30 m × 0.25 mm id × 0.25 µm film thickness. Mobile phase: helium, flow rate 1 ml/min. Oven temperature was initiated at 50 °C for 2 min up to 200 °C at a rate of 10 °C/min., 200 °C for 15 min, 200–250 °C at a rate of 15 °C/min. The mass detector temperature was 300 °C. The fragmentation pattern of the obtained mass spectra was analyzed by Wiley 7 N mass library software (Khaled et al., 2012).

2.7. Statistical analysis

All experiments were carried out in triplicate. The standard error was calculated for each experiment and cited as a bar on the histogram.