Biodiversity of oil-degrading microorganisms in the North-west Pacific

太平洋北西部における

石油分解微生物の多様性

Doan Dang Phi Cong

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ABA	: autochthonous bioaugmentation
bbl/d	: barrels per day
BHA	: Bushnell-Haas mineral salt agar
BHB	: Bushnell-Haas mineral salt broth
BLAST	: The Basic Local Alignment Search Tool
CD1	: Dam Trau Beach
CD2	: Vong Beach
CD3	: An Hai Port
CD4	: Ben Dam Port
CDA	: Czapek-Dox malt extract agar
CFU	: colony-forming units
DCM	: dichloromethane
DDBJ	: DNA Data Bank of Japan
dw	: distilled water
EIA	: US Energy Information Administration
ESI	: Environmental Sensitivity Index
GC-MS	: gas chromatography–mass spectrometry
INT	: *p*-iodonitrotetrazolium
Ir1	: Urauchi mangrove forest
Ir2	: Uehara Port

Ir3	: Funaura Bay
Ir4	: Ohara Port
ITS	: internal transcribed spacer
MSA	: mineral salt agar
MSB	: mineral salt broth
NCBI	: The National Center for Biotechnology Information, U.S. National Library of Medicine
ODB	: oil-degrading bacteria
ODF	: oil-degrading fungi
PAHs	: polycyclic aromatic hydrocarbons
PCR	: polymerase chain reaction
PDA	: potato dextrose agar
S	: sediment samples
S1	: sediment samples in subtidal zone
S2	: sediment samples in intertidal zone
SBA	: Sabouraud agar
SLEB	: Seria Light Export Blend
TCA	: tricarboxylic acid
TSA	: tryptic soy agar
W	: seawater samples

Abstract

The islands located in the North-west Pacific are facing dangers of oil spillage, because they are considered predicted contamination sites on a nearby busy transportation route. So far, there is few report on the distribution of oil-degrading microorganisms in tropical and subtropical areas compared with temperate areas. The aim of this study was to select indigenous oil-degrading microorganisms from the two islands, subtropical Iriomote, Japan and tropical Con Dao, Vietnam, and to compare their distributions and degradation abilities.

I used three kinds of samples, sediments from supratidal and intertidal zones, and seawater. Selecting media isolated bacteria and fungi, and the media with *p*-iodonitrotetrazolium determined oil-degradation ability by color changes. The DNA methods identified the microorganisms which showed the degradation abilities. For the bacterial isolates, the degradation experiments using crude oil, *n*-alkanes and recalcitrant polycyclic aromatic hydrocarbons (PAHs), and temperature effect examination were conducted.

As the result, there were no difference between Iriomote and Con Dao in the counts of the total bacteria and oil-degrading bacteria (ODB) in the sediment samples, while those in the seawater samples were significantly lower for Iriomote than for Con Dao. Total 45 ODB isolates, 25 from Iriomote and 20 from Con Dao, were collected. The dominant genera were *Achromobacter*, *Pseudomonas*, and *Ochrobactrum* on Iriomote and *Pseudomonas* and *Microbacterium* on Con Dao. Some bacteria that are close relatives of

the isolates previously reported as pathogens were not used in the further experiments. Among the remaining 41 isolates, the counts of the isolates that degraded the crude oil, *n*-alkanes, and PAHs in a high level were higher for Iriomote (3, 11, and 2, respectively) than for Con Dao (2, 4, and 1, respectively). It is noteworthy that the isolates, *Acinetobacter* sp., *Pseudomonas* sp., *Pseudomonas putida* from Iriomote and *Pseudomonas mendocina, Pseudomonas putida, Microbacterium* sp. from Con Dao, showed the highest degradability for crude oil, *n*-alkanes, and PAHs, respectively, and survived at 42℃.

For the fungi, the counts of the total and oil-degrading fungi (ODF) in the sediment samples on Iriomote were significantly higher than on Con Dao. The seawater samples, however, showed the same result for the two islands. In total, 23 ODF including 11 isolates in 5 genera from Iriomote and 12 isolates in 4 genera from Con Dao were isolated. One *Candida* isolate from Iriomote and two *Aspergillus*, one *Penicillium* and one *Trichoderma* isolates from Con Dao had high potential of oil-degradation, and grew and survived at 42℃.

These results suggest that the diversities on both Iriomote and Con Dao were higher comparing previous reports. The obtained notable isolates, six bacteria and five fungi, showed the potential for autochthonous bioaugmentation application for the treatment of oil contamination in these areas. Further examinations are needed to clarity the abilities for degradation of other crude oil components by these selected isolates. The combined treatment techniques with bacterial and fungal isolates are necessary.

Chapter 1

General introduction

Worldwide industrialization and daily life have expanded the consumption of oil and its products as the primary energy source. The oil production and transportation to support that demand have increased the risk of oil spill accidents. Several noticeable oil spill accidents from oil and gas activities were occurred and have impacted significantly to environment and human activities.

Bioremediation, the treatment that uses microorganisms to break down hazardous substances into less toxic or nontoxic substances, have been proved as an efficient method for degrading oil contamination. Bacteria and fungi are considered as the most importance oil degraders in the environment. Up to now, more than 175 genera of bacteria (Prince et al. 2010), and 103 genera of fungi (Sardrood et al. 2013) which can utilize hydrocarbons as carbon source in the world were known. However, these microorganisms have mainly been isolated and studied from temperate and boreal zones, and information regarding tropical oil-degrading microorganisms is relatively scarce (Prince 1993; Elshafie et al. 2007; Harwati et al. 2007).

In this study, I carried out researches about the oil-degrading microorganism community in sub-tropical Iriomote Island, Japan and tropical Con Dao Island, Vietnam. Those islands are the conservation areas in which 70–80% were covered by the national parks with sensitive ecosystems including jungle, mangrove forests, seagrass meadows and coral reefs. In addition, they are both predicted contamination sites locating on a busy maritime route which transports and supplies more than 80% of crude oil and oil products to Japan and East Asian countries (EIA 2014).

1.1 Oil spill accidents and its impacts to coastal environment

Oil is a significant source of energy for both industry and daily life worldwide. It remains the world's leading fuel, with 33% of global energy consumption (BP Group 2014). During the oil and gas activities including exploration, production, refining and transportation, leaks and accidental spills occur regularly. Among that, transportation is considered as the serious pollution source to the environment. Oil is transported across the world from production sites to refineries and from refineries to consumers by different modes of transportation such as maritime routes using barges and tankers, and inland routes using pipelines, trucks and trains. Several noticeable oil spill accidents from oil transportation were occurred such as Exxon Valdez in Alaska (1989), Prestige oil-tanker off the coast of north-west Spain (2002) and Hebel Spirit off the coast of South Korea (2007). In 2013, total world crude oil and other liquid fuel production was about 90 million barrels per day (bbl/d). It is estimated that 63% of this amount traveled via a maritime route (EIA 2014). Annual oil spillage from waterborne transportation has been estimated at 4 million bbl of crude oil seepage (Kvenvolden and Cooper 2003; Das and Chandran 2011), and accounts for 70% of pollution from oil and gas activities (Weis 2015).

Oil spills can seriously affect to the marine environment and human activities. The serious of impact generally depends on several factors, including the amount and type of oil spilt, the type of human activities, the environmental conditions and the sensitivity of the affected organisms and their habitats to the oil. Because oil contains a huge of components which are toxic to organisms, the levels of oil pollution in all accidents reached lethal limits for marine fauna, mainly for sea animals, birds and plants. They were

also killed by physical smothering from the oil, as well as from physical damage and dispersant toxic by the clean-up operations (Saadoun 2015). Additionally, oil spills in the environment cause long-term damage to aquatic and soil ecosystems, human health and natural resources (McGenity et al. 2012). After being exposed to the oil, the surviving planktonic organisms showed the abnormalities of development and reproduction (Jiang 2010); plant showed the chlorosis, yellowing, growth reduction and perturbation in developmental parameters (Meudec et al. 2007); and the early stage of fish showed the melanosis, erratic swimming, loss of equilibrium, reduced mobility and startled response (Barron et al. 2005). Furthermore, PAHs can be accumulated in the high trophic level through the food chain such as large pelagic fish and seals, polar bears, killer whales. They are accumulated into the brain and liver and causing neurological disorders and liver damage (Neff 1988).

1.2 Bioremediation by microorganisms for treating oil contamination

There are three ways of treating oil contamination: physical, chemical and biological. In comparison with physical and chemical methods, the biological method, bioremediation, is considered a non-destructive, cost-effective treatment. Bioremediation can be divided into two basic types: (1) biostimulation, which is the technique relies on increasing the activity of the resident microorganisms by adding the limit factors such as nutrients or air (Ueno et al. 2007), and (2) bioaugmentation , which is the survival and degrading ability of microorganisms introduced to a contaminated site (Gentry et al. 2004). The advantage of biostimulation is the indigenous microorganisms are the best adapted to the environment of the treated site (Rahman et al. 2003). However, the bioaugmentation methods give a shorter treatment time to the sites which are scarcity of indigenous microorganisms capable of degrading hydrocarbons or high concentrations of the pollutants (Hosokawa et al. 2009). In recent years, a new concept in bioaugmentation which called autochthonous bioaugmentation (ABA) is proposed and developed in oil spill bioremendiation (Ueno et al. 2007). In ABA, indigenous microorganisms from the contaminated site or predicted contamination site which are potentially capable of degrading oils and well-characterized are used. This method has a number of advantages for the decontamination of oil-polluted land with shorter treatment time, greater potential efficiency, lower impact on the environment, and relative ease in obtaining public support (Hosokawa et al. 2009). And it also can be combined with biostimulation method and given an effective treatment (Nikolopoulou et al. 2013).

It is because that oil is a complex mixture of hydrocarbons, other organic compounds and organometallic constituents, a vast array of microbial species (bacteria, fungi, algae, and cyanobacteria) have diverse mechanisms to degrade it (Hamme et al. 2003). Among them, bacteria and fungi are considered as the most importance oil degraders in the environment. They have been extensively studied for their degradability for environmental pollutants originated from spilled oil. Depend on the degrading mechanisms of microorganisms for the contaminants, bioremediation strategies are divided into three general categories including (1) xenobiotic degradation, the contaminant is used as a carbon source, (2) cometabolism, the contaminant is enzymatically attacked but not used as a carbon source, and (3) bioaccumulation, the contaminant is not metabolized at all but is taken up and concentrated within the organisms (Bennet et al. 2002). Although microorganisms participate in all three strategies, bacteria are often more proficient at xenobiotic degradation, and fungi are more efficient at cometabolism and bioaccumulation.

The rate of microbial degradation of oil contaminants depend on a variety of factors including the physical conditions, the oil characteristics and the properties of the biological system (Hamme et al. 2003; McGenity et al. 2012; Okere and Semple 2012). Temperature and nutrients in the soil are the important environmental conditions which affect to the biodegradation of the oil. At higher temperatures, the degradation rate generally increases because of the increase in activity of microorganisms and bioavailability of the oil components, and the optimum temperatures range from 30 to 40℃ in soil environments (Atlas 1981; Das and Chandran 2011). The nutrients in the soil,

particularly nitrogen and phosphorous, influence to the growth ability of the microorganisms. When a major spill occurred, the supply of carbon was significant increased and the low concentration of nitrogen and phosphorous generally became the limiting factor for the oil degradation. Therefore, addition of nutrients were necessary to enhance the biodegradation of oil pollutants (Vyas and Dave 2010; Wu et al. 2016; Das and Chandran 2011). However, the excessiveness of nutrient concentrations can also inhibit the biodegradation activity (Chaillan et al. 2006). The general theoretically C/N/P ratio is about 100/10/1 allowed the best microbial activity and biodegradation of oil contaminants (Leys et al. 2005; Leung et al. 2006; Ubalua 2011). Biodegradation rate was also influenced by the oil properties. Crude oil is a complex mixture of hydrocarbons, and their compositional and physical properties vary widely from different reservoirs. Because the susceptibility of hydrocarbons differ to microbial attack, the biodegradation rate of crude oil are differ. The susceptibility of hydrocarbons have been generally ranked in the following order of decreasing susceptibility: linear alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes > high molecular weight aromatic (Chikere et al. 2011; Das and Chandran 2011). McKew et al. (2007) observed that the hydrocarbon degradation was significant within 10 weeks, ranging from > 99% of low molecular weight alkanes (C_{10} – C_{18}), 41 – 84% of high molecular weight alkanes (C_{20} – C_{32}) and pristance, and 32 – 88% of polycyclic aromatic hydrocarbons (PAHs). Additionally, the biological system, especially the hydrocarbon-metabolizing microorganisms present in the contaminated area, have been a significant role in the biodegradation rate of the oil contamination. Hydrocarbon-degrading microorganisms are widely distributed in marine, freshwater and soil habitats; and their quantity and composition vary from environmental

habitats. For example, the microbial communities in sediments are much more concentrated in cells and greater community evenness than those from open water (McGenity et al. 2012). Because oil consists of a variety of chemically distinct hydrocarbons, it requires several microorganisms with variety of suitable metabolic pathways to activate and degrade them.

1.2.1 Bioremediation by bacteria

Bacteria are the most active agents and primary degraders of oil contaminated environment (Das and Chandran 2011). Because of the versatility characteristics, bacteria may play a greater role than other oil degraders during biodegradation of hydrocarbons (Chikere et al. 2011). Up to now, more than 175 genera of bacteria were published as the oil degraders by several researchers (Prince et al. 2010). Several important oil degrading bacterial genera have been studied and reported including *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter, Bacillus*, *Burkholderia*, *Collimonas*, *Corynebacterium*, *Dietzia*, *Flavobacterium*, *Gordonia*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*,*Sphingomonas*, *Variovorax* and other unculturable bacterial clones (McGenity et al. 2012; Chikere et al. 2011; Das and Chandran 2011). Moreover, they have been studied for genetic diversity in degradation functions and gene modification and used as biosensors and bioremediation agents (Palleroni et al. 2010).

Among the bioremediation strategies, bacteria are often more proficient at xenobiotic degradation. They have metabolic pathways to use oil as a carbon and energy source. Except some bacteria such as *Pseudomonas* and *Rhodococcus* strains have been shown to possess pathways for both aliphatic and PAH compounds, most hydrocarbonoclastic typically degrade a small range of either aliphatic or aromatic compounds (McKew et al. 2007). For *n*-alkanes, there are two metabolic pathways degrade them completely to carbon dioxide and water. The most common pathway which is specific for *n*-alkanes depends on the action of monooxygenase enzymes (Leung et al.

2006; Chikere et al. 2011). The monooxygenase enzymes attacks the terminal methyl group and a primary alcohol is formed. The alcohol is further oxidized to the corresponding aldehyde and fatty acid. In the second pathway, a dioxygenase acts on the terminal methyl group and adds two oxygen atoms to forms a peroxide. The peroxide is converted to fatty acid and then further metabolized via the β-oxidation pathway, a common catabolic pathway found in most living cells, to form acetyl-CoA or propionyl-CoA. These compounds are subsequently metabolized via the tricarboxylic acid cycle (TCA cycle) to carbon dioxide and water (Chikere et al. 2011). For alkenes, the biodegradation pathway is similar to that of alkanes. The monooxygenase generally starts the degradation process by attacking to the methyl groups in the chain. Depending on the location of action and the bonds being attacked, the breakdown products could be either alcohols (when acted on terminal or sub-terminal methyl groups) or epoxides (when acted on the double bond). These intermediate products are further catabolized to fatty acids and degraded via the β-oxidation pathway and TCA cycle, as described above. For aromatic hydrocarbons, the key step required for degradation is cleavage of the aromatic ring. This step is carried out by dioxygenase enzymes which convert the aromatic ring to the catechol aromatic ring. The catechol aromatic ring is then cleaved, again by dioxygenase, via two pathways including the ortho-cleavage, in which the ring is cleaved between the two carbon atoms with hydroxyl groups; and the meta-cleavage, in which the ring is split between adjacent carbon atoms with and without a hydroxyl group. The breakdown metabolites such as acetate, succinate, pyruvate or acetaldehyde subsequently degraded via the TCA cycle. For alicyclic hydrocarbons, an important group of petroleum components, it has been difficult to isolate and identify pure strain of bacteria which are

capable of degrading these compounds. And the their degradative pathways are not clear up to now (Hamme et al. 2003; Leung et al. 2006). The summarized diagram of degradative pathways of these hydrocarbons were presented in Figure 1-1. In addition, hydrocarbon-degrading microbes can produce biosurfactants to enhance the water solubility and the removal of petroleum hydrocarbon compounds (Hamme et al. 2003; Chikere et al. 2011). Biosurfactants can be as part of cell surface or as molecules released extracellularly.

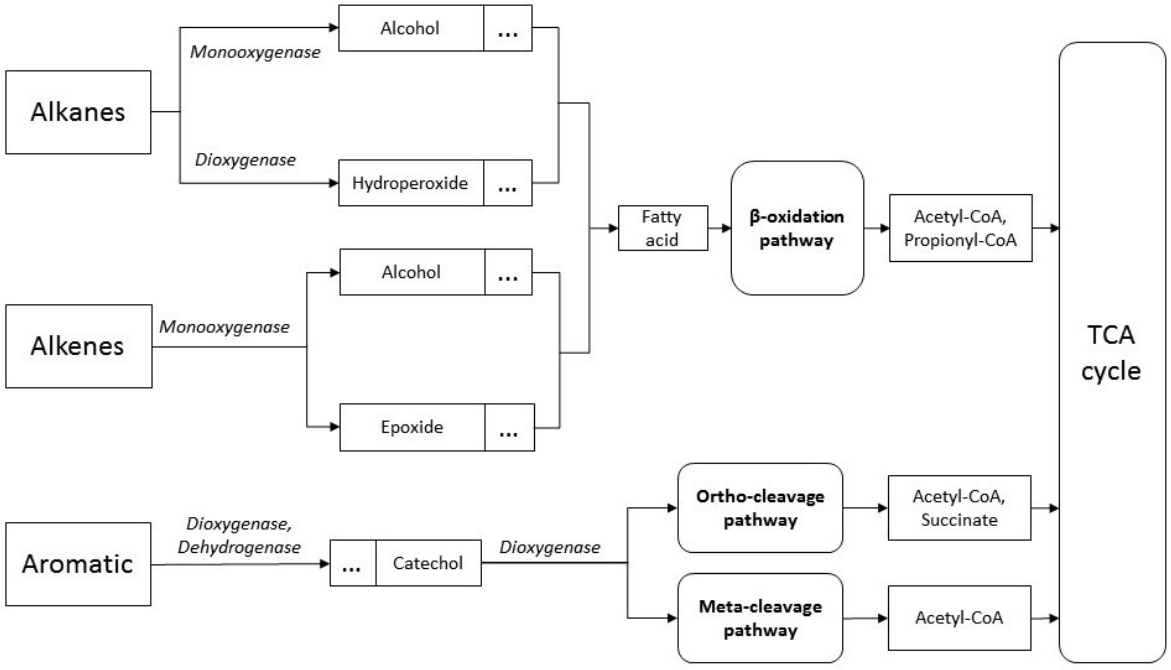

Figure 1-1. Summarized biodegradation pathways of petroleum hydrocarbons by bacteria

1.2.2 Bioremediation by fungi (Mycoremediation)

Fungi are microscopic eukaryotic organisms which are the most diverse and ubiquitous in the environment. They have been widespread used in human life and play important roles in the ecosystems. One of the most important function of fungi is the remediation of pollutants, called mycoremediation. Mycoremediation is a form of bioremediation which is use fungi to degrade or sequester contaminants in the environment. Fungi can participate in all three bioremediation strategies, however, they are often more proficient at cometabolism and bioaccumulation than at xenobiotic degradation. Up to now, numerous species from 103 genera of fungi, including well-known ODF such as *Aspergillus, Mortierella, Penicillium* and *Trichoderma*, were reported (Sardrood et al. 2013). Some fungal genera such as *Aspergillus* and *Penicillium* show greater ability to degrade oil contaminants than bacteria such as *Arthrobacter*, *Brevibacterium*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas* (Sardrood et al. 2013; Elshafie et al. 2007).

Filamentous fungi possess attributes that make them good potential agents for oil degradation (Bennet et al. 2002; Al-Nasrawi 2012; Sardrood et al. 2013; Elshafie et al. 2007). Firstly, the mycelium structure of fungi enables them to rapidly spread and penetrate through substrates. The high surface-to-cell ratio characteristic of filaments maximizes both mechanical and enzymatic contact with the environment. Secondly, the extracellular degradative enzymes enable fungi to tolerate high concentrations of toxic chemicals, environmentally stressed conditions and to degrade the complex and insoluble compounds. Finally, the relevant enzymes can act independently of the concentration of

substrate because they are usually activated by nutritional signals independent of the target compound during secondary metabolism. Therefore, they can scale up degradative activities more than their nutritional demand. Moreover, like bacteria, some fungi can also absorb and degrade contaminants by the intracellular enzymes. The Cytochrome P-450 oxidase enzymes play a major role in the degradative mechanisms of fungi (Bennet et al. 2002). Cytochrome P-450s, a superfamily of mixed-function monooxygenases which are involved in many steps of petroleum degradation and in the metabolism of a variety of environmental pollutants. They are more similar to mammalian enzymes than to bacterial cytochromes. The degradation process of contaminants are also contributed by the organic acids and chelators excreted by the fungus (Barr and Aust 1994; Bennet et al. 2002). They have been shown to serve as the electron donors in the reducing metabolism of pollutants by the fungal monooxygenase.

1.3 Positions of Iriomote and Con Dao islands, and the risk of oil spill from the transportation route

Iriomote and Con Dao islands are both predicted contamination sites locating on a busy maritime route which transported 15 million bbl/d in 2013 and supplies 83% of crude oil and oil products to Japan and East Asian countries (EIA 2014). The subtropical Iriomote Island is the largest of the Yaeyama Islands located in the southernmost region of Japan in the East China Sea. The tropical Con Dao Island is also the largest of the same name archipelago located in the southeast of Vietnam in the South China Sea (Figure 1-2). They are listed as the highest priority conservation areas in which the national parks cover 70–80% by sensitive ecosystems including jungle, mangrove forests, seagrass meadows and coral reefs (Kelleher et al. 1995). Iriomote is a national park which contribute 78% of mangrove forest land area of Japan (Alsaaideh et al. 2013). Mangrove is classified as the highest Environmental Sensitivity Index (ESI) shoreline type which is the most affected area by the oil spill (Laflamme 2012). Con Dao is also a national park and the nation's sixth Ramsar site in Vietnam. Along the coast, there are shallow marine waters, coral reefs, seagrass beds, intertidal flats and mangrove which is the habitat of the critically endangered species such as mangrove *Bruguiera hainesii*, leatherback turtle *Dermochelys coriacea*, hawksbill turtle *Eretmochelys imbricate* and sea mammal dugong *Dugong dugon* (RAMSAR 2014, Cox 2002). Moreover, Con Dao is also surrounded by busy oil fields in the southern of Vietnam.

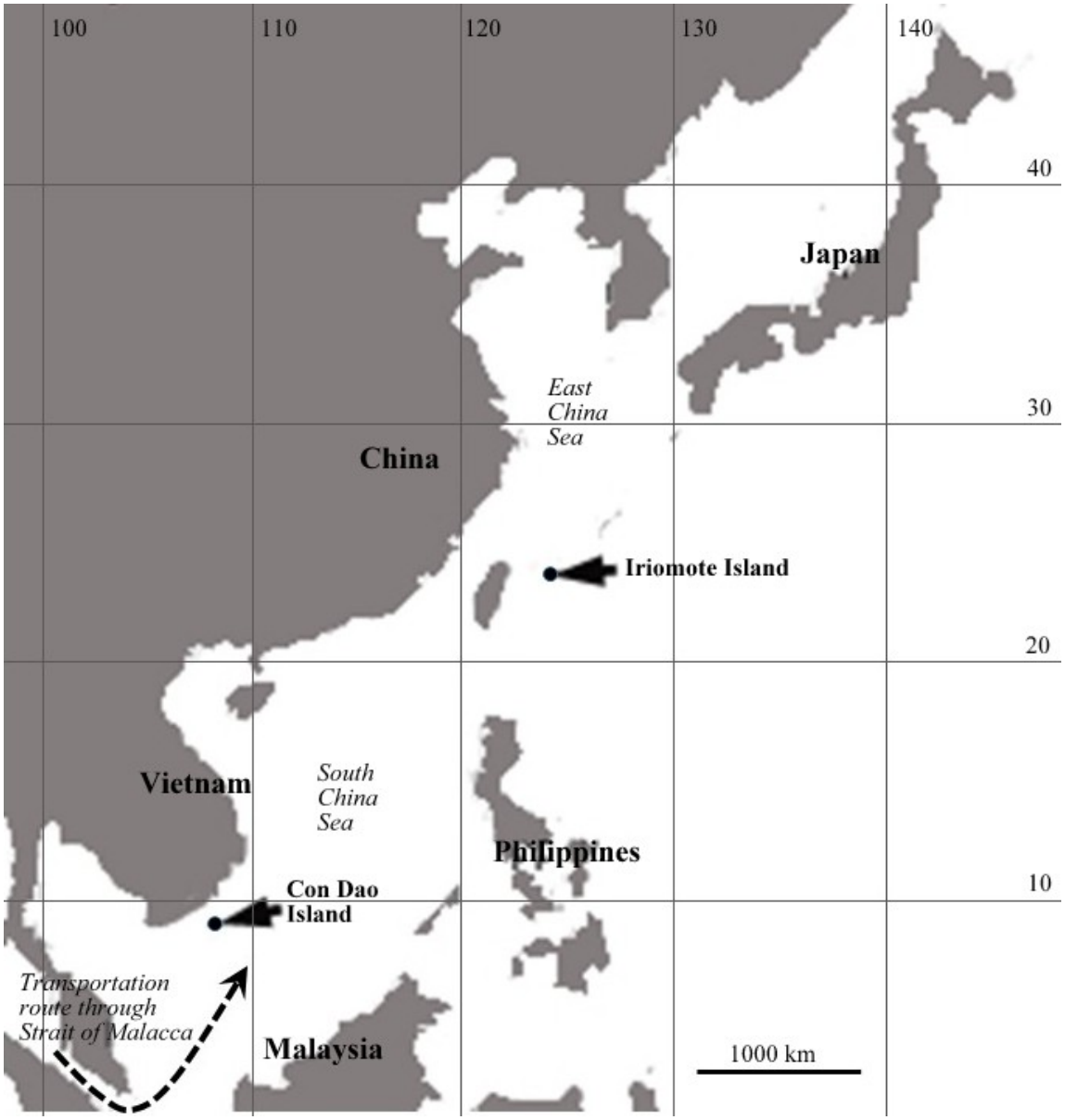

Figure 1-2. Locations of the study areas on the Iriomote and Con Dao islands along the transportation route through the Strait of Malacca.

1.4 Outline of the studies

1. A comparative investigation of the distribution of the indigenous oil-degrading bacteria (ODB) on the two islands was carried out. The ODB were isolated and identified, and their distribution and potential pathogenicity to other living organisms of the isolates was determined (Chapter 2).

2. The degradation abilities of bacterial isolates from the two islands were studied. The degradability of isolates of two alkanes, heptadecane and octadecane, and two PAHs, fluoranthene and pyrene, was representatively determined and compared between the two islands (Chapter 3).

3. A comparative investigation of the distribution of the indigenous oil-degrading fungi (ODF) on the two islands was carried out. The ODF were isolated and identified, and their distribution and potential pathogenicity to other living organisms of the isolates was determined (Chapter 4).

Chapter 2

Isolation and identification of oil-degrading bacteria on Iriomote and Con Dao islands

2.1 Introduction

Oil spillage from waterborne transportation is considered a significant pollution source, which accounts for four million barrels of crude oil discharged through seepage per year and for 70% of pollution due to oil and gas activities (Weis 2015). Marine and coastal areas are seriously affected and exposed to enormous environmental damage caused by oil spillage.

Bioremediation is a biological method, which is considered an effective technique for treating oil contamination. In recent years, a new concept, called autochthonous bioaugmentation, has been introduced that promises the most effective remediation method for oil contamination. In this method, indigenous microorganisms are used, which are well characterized as oil degraders from contaminated sites or predicted contamination sites. This method showed a number of advantages for remediating oil-polluted land, with shorter treatment times, a greater potential efficiency, lower impact on the environment, and easier public acceptance (Hosokawa et al. 2009).

In this study, I selected two islands, which are considered predicted contamination areas because of oil spillage on a nearby busy transportation route. One is the subtropical Iriomote Island, the largest of the Yaeyama Islands located in the southernmost region of Japan in the East China Sea. The other is the tropical Con Dao Island located southeast of Vietnam in the South China Sea. These islands are designated as national parks, 70–80% of which are covered with sensitive ecosystems, including jungles, mangroves, and coral reefs, and inhabited by several endangered species. However, the islands are located on a busy maritime route, by which 15 million barrels of oil are transported per day. This route

supplies 83% of oil and oil products to Japan and East Asian countries (EIA, 2014). In addition, the Con Dao Island is located on the biggest oil fields in Vietnam.

The aim of this study was to compare the distribution of indigenous ODB on two islands. Furthermore, potential pathogenicity of the isolates to other living organisms was assessed to compare isolates from the two islands.

2.2 Materials and methods

2.2.1 Study areas and sample collection

Four study sites were selected on each island, Iriomote and Con Dao (Figure 2-1). On Iriomote, the study sites were Urauchi mangrove forest (Ir1), Uehara Port (Ir2), Funaura Bay (Ir3) and Ohara Port (Ir4), and on Con Dao, Dam Trau Beach (CD1), Vong Beach (CD2), An Hai Port (CD3) and Ben Dam Port (CD4). These sites stretch along the shoreline from the northern to southern part of each island. On Iriomote, Ir1 is in a mangrove forest along the Urauchi river, which is the largest river in Okinawa Prefecture, Ir2 and Ir4 are in busy ports on the main transport routes to the island, and Ir3 is in a bay with seagrass and mangroves. On Con Dao, CD1 is on a tourist beach, CD2 is on a beach with seagrass, CD3 is in a port and residential area with high population density, and CD4 is in a main, busy port of the island.

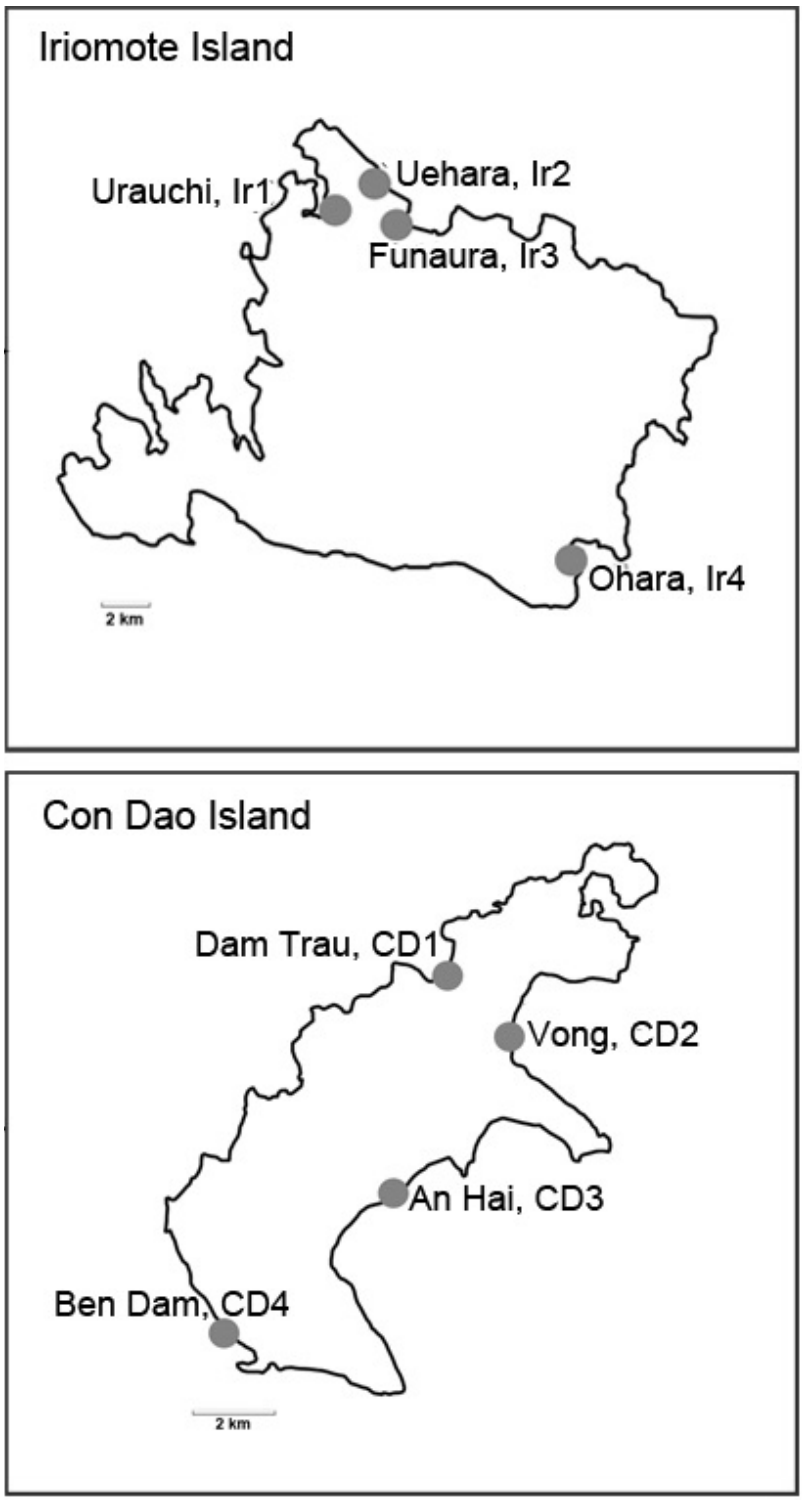

Figure 2-1. Sampling sites on Iriomote and Con Dao islands

The coordinates of the sampling sites are shown in Table 2-1. In each site, two S samples, from supratidal and intertidal zones, respectively, and one W sample were taken during the dry season, from January to May (Figure 2-2). Sediment samples with 10-20 cm depth under sediment surface were collected by a core sampler with sterilized replaceable liners and tips, then transferred to sterilized glass bottles by a sterilized spatula. Each sample was collected from four random locations in each sampling site and transferred to the laboratory in a cool box at 4-8℃ to be analyzed within 48 h. Environmental parameters, pH, salinity of seawater, and air temperature were determined in each sampling site.

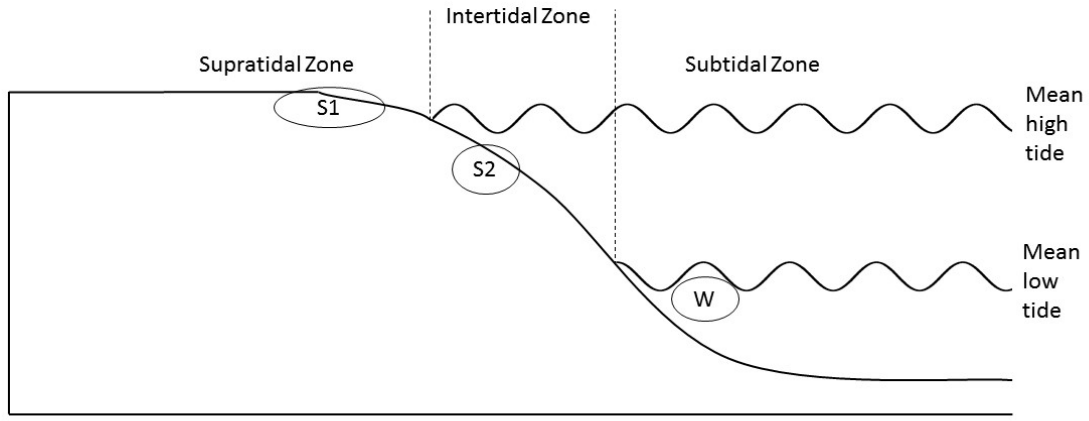

Figure 2-2. Positions of S1, S2 and W on the coast

2.2.2 Medium preparation

For culturing total bacteria and ODB, tryptic soy agar (TSA; Wako Ltd., Japan) and mineral salt agar (MSA) were used, respectively (Chaillan et al., 2004). TSA consisted of Trypton, 15 g; Soytone, 5 g; NaCl, 5 g; and agar, 15g in 1 l of distilled water. MSA consisted of KH_2PO_4, 0.68 g; Na_2HPO_4, 1.79 g; $MgSO_4$, 0.35 g; NH_4NO_3, 1 g; $CaCl_2$, 0.02 g; $FeSO_4$, 0.4 mg; and agar, 15 g in 1 l of distilled water and was supplemented with 0.1 ml of a solution containing 100 mg/l of each element H_3BO_3, $MnSO_4$, $ZnSO_4$, $CuSO_4$, and $CoCl_2$. The medium was supplemented with 1% (v/v) of weathered Seria Light Export Blend (SLEB) crude oil supplied by the Brunei Shell Petroleum Company, Brunei Darussalam, as a sole carbon source. The oil was weathered by heating at 100℃ for 48 h to evaporate the light components, sterilized by membrane filters (47 mm diameter, 0.2 μm pore size), and spread on the surface of MSA plates (Venkateswaran et al. 1991). Cycloheximide (0.1 g/l) was aseptically added as an antifungal agent to the media after sterilization and cooling to 50℃.

For ODB enrichment and degradation experiments, mineral salt broth (MSB) was used, which had the same composition as MSA, except agar. The weathered oil (10 ml/l) and cycloheximide (0.1 g/l) were added to the sterilized medium as a sole carbon source and an antifungal agent, respectively (Venkateswaran et al. 1991).

2.2.3 Enumeration of total bacteria and oil-degrading bacteria

The S samples were treated aseptically. They were weighed and homogenized in a 50% (w/v) sodium chloride solution (0.85%, w/v), and the adhered microorganisms were dissociated by vortexing. A decimal serial dilution of a sample was spread on TSA and MSA plates for the enumeration of total bacteria and ODB, respectively. Each dilution was inoculated in triplicate. The grown colonies were counted, and the results were expressed as colony-forming units (CFU) per gram of dry weight of an S sample.

Bacteria in the W samples were enumerated by the membrane filter method (Venkateswaran et al. 1991). A volume of 100 ml of a W sample was filtered through a membrane (47 mm diameter, 0.2 μm pore size) in duplicate. One membrane was placed on the top of a TSA plate, and the other was placed on the top of an MSA plate for the enumeration of total bacteria and ODB, respectively. The developed colonies were counted, and the results were expressed as CFU per 100 ml of a W sample. All incubations were carried out at 25℃ up to 7–10 d.

2.2.3 Isolation of oil-degrading bacteria

ODB were directly isolated by picking up the colonies grown on the MSA medium in the enumeration step and repeatedly streaked on new TSA plates. ODB were also isolated by the enrichment medium method (Chaerun et al. 2004) described below. One gram of an S sample was inoculated in an Erlenmeyer flask containing 50 m*l* of MSB medium, and the flask was incubated at 120 rpm, 25℃ for 5–20 d. After every 5 days of incubation, the culture was diluted, and an aliquot was spread on MSA medium. The formed colonies were isolated and repeatedly streaked on new TSA plates.

2.2.4 Identification of isolates

2.2.4.1 Morphological and biochemical characteristics

Morphological characteristics of bacterial colonies including form, elevation, margin, surface, opacity, color and size were determined by microscope. The isolates were spread on TSA plates and incubated at 25℃ for 48 hours. A single colony grew on the medium were used for identification (Collins et al. 2004).

Other bacterial characteristics including cell mobility, cell shape, Gram stain and cytochrome oxidase test were determined by common methods as follows. The cell mobility were determined by the "Sloppy Agar" Craigie Tubes method (Collins et al. 2004). 5 ml of TSA broth containing 0.1 − 0.2% agar were added to a 12 ml screw-capped bottles. And a piece of glass tubing of dimensions 50 × 5mm were added to the bottle. The top of the tube must be separately higher than the surface of the medium so that a meniscus bridge does not formed and the only connection between the fluid in the inner tube and outside is through the bottom. A drop of bacterial inoculum were cultured to the inner glass tube and incubated stably at 25℃ for 7 days. The growth of mobile bacteria in outer tube were observed because they can move through the sloppy agar.

The Gram stain were determined by Jensen's method and microscope (Collins et al. 2004). A thin layer of bacteria was spread on a glass slide and dried by waving over a bunsen. The bacteria were stained with methyl violet solution for 20s and washed off by distilled water; replaced with iodine solution for 1 min and washed off by 95% alcohol solution, left for a few second and washed with water; and counterstain with safranin for

2 min, washed and dried. The Gram stain result was determined by microscope. Gram-positive cells will appear blue to purple, while Gram-negative cells will appear pink to red. Moreover, the cell shape was also determined by microscope based on the Gram stain results.

Cytochrome oxidase activities of the isolates were determined by spreading cell pellets on oxidase test papers (Nissui Phamaceutical, Tokyo). Oxydase-positive isolate will appear blue color within 10 s.

2.2.4.2 Biochemical and molecular identification

The generic identities of the isolates were determined biochemically using API kits (bioMérieux, Japan) following the manufacturer's protocols. The API kits contained dehydrated substrates in microtubes to detect enzymatic activities for the assimilation or fermentation of sugars. Following the presumptive characteristics which were determined above, 4 kinds of API kits including API 20 NE, API 20 E, API 20 Strep and API NH were used for bacterial identification.

The species identification was performed by a molecular method using 16S rRNA gene sequences. The isolates were cultured on TSA slants at 25℃ for 2 days, and DNA was extracted by the alkaline wash/lysis method (Millar et al., 2000). Briefly, bacteria was mixed with 1 ml of alkali wash solution (0.5 M NaOH and 0.05 M sodium citrate) for 10 min by a horizontal shaker and centrifuged at 13000 rpm for 5 min. The pellet containing microbial DNA was washed twice by resuspending in 0.5 ml Tris-HCL pH 8.0 and centrifuged at 13000 rpm for 5 min. The resulting pellet was resuspended in 0.1 ml TE pH

8.0 and heated at 100℃ for 1 h in a heating block. The sample was freezed/thawed twice and centrifuged 13000 rpm for 15 min, the supernatant containing DNA was transferred to a clean tube and stored at −20℃ prior to PCR.

DNA was amplified by polymerase chain reaction (PCR) using the bacterial universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Chaerun et al. 2004). PCR reaction mixtures were subjected to 30 amplification cycles at 94℃ for 30 sec, 55℃ for 1 min, and 72℃ for 2 min; and a final extension cycle at 72℃ for 5 min in a PCR Thermal Cycler MP (TaKaRa) (Tokuda et al. 2000). The PCR samples were directly sequenced on an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA, USA) using the primers 27f and 1492r. The DNA sequences were aligned using the ATGC Ver.7 Sequence Assembly software for Windows (GENETYX Corporation) and MEGA Ver. 6 (Tamura et al., 2013). The sequences were analyzed by the National Center for Biotechnology Information BLAST tool to identify closely related bacterial sequences. The isolates were identified to a certain species if the BLAST results showed similarity values of higher than 97%.

2.2.5 Screening for oil degradation of the isolates

Oil degradation abilities of the isolates were verified using a modified technique based on the redox indicator *p*-iodonitrotetrazolium (INT) (Wrenn and Venosa 1996). The isolates were cultivated in 5 ml of MSB supplemented with oil. After 7 days, 100 µl of filter-sterilized INT (5 g/l) was added, and the color change of the mixture was observed after overnight incubation. The color change of INT from light yellow to pink or red was scored as + or ++, respectively. The darker medium color indicated better bacterial growth in the medium with oil.

2.2.6 Temperature effect on the isolates

Temperature resistance is a characteristic that shows bioremediation application and pathogenic potentials of isolates. Our isolates were cultured on TSA slants in duplicate at temperatures of 15, 20, 30, 35, 37, 39, and 42°C, and their growth was recorded after 7 days. After the incubation, the test tubes with no growth were kept at 25°C for 7 days to check for bacterial survival.

2.3 Results

2.3.1 Environmental parameters of sampling sites

The environmental parameters of the sampling sites are shown in Table 2-1. The pH and salinity values were not much different among all sampling sites on Iriomote and Con Dao, except Ir1. This site had the lowest values of pH (7.9) and salinity (2.5%). The air temperatures ranged between 15.1 and 16.8℃ on Iriomote and 34.2 and 35.5℃ on Con Dao.

Table 2-1. Coordinates and environmental parameters of sampling sites on Iriomote and Con Dao islands

Sampling sites	Abbreviation	Coordinates	pH	Salinity (%)	Air temp. (ºC)	Seawater temp. (ºC)
Iriomote						
Urauchi mangrove forest	Ir1	24.4042 N 123.7774 E	7.9	2.5	15.3	12.5
Uehara Port	Ir2	24.4142 N 123.8030 E	8.1	3.5	15.1	12.1
Funaura Bay	Ir3	24.4007 N 123.8117 E	8.0	3.3	16.4	13.5
Ohara Port	Ir4	24.2757 N 123.8820 E	8.0	3.2	16.8	12.8
Con Dao						
Dam Trau Beach	CD1	08.7328 N 106.6220 E	8.2	3.5	34.2	26.1
Vong Beach	CD2	08.7130 N 106.6382 E	8.1	3.4	34.8	25.5
An Hai Port	CD3	08.6813 N 106.6088 E	8.1	3.5	35.1	25.7
Ben Dam Port	CD4	08.6603 N 106.5698 E	8.1	3.5	35.5	26.5

2.3.2 Distribution of total bacteria

Comparing the average numbers of total bacterial counts obtained from the S and W samples taken on Iriomote and Con Dao, there were no differences in the results for the S samples, whereas the numbers for the W samples taken on Con Dao were statistically larger than those for the W samples taken on Iriomote. The bacterial count distributions for the sampling sites are shown in Table 2-2 and Figure 2-2. On Iriomote, the total numbers of bacteria ranged from 6.5×10^4 (Ir3) to 5.7×10^5 CFU g^{-1} (Ir1) in the S samples and from 3.3×10^2 (Ir3) to 1.2×10^3 CFU 100 ml^{-1} (Ir4) in the W samples. The total numbers of bacteria on Con Dao ranged from 2.3×10^4 (CD1) to 2.4×10^6 CFU g^{-1} (CD4) in the S samples and 4.0×10^3 (CD1 and CD3) to 6.3×10^3 CFU 100 ml^{-1} (CD4) in the W samples. On Iriomote, Ir1 gave the highest total number of bacteria among the S samples, whereas Ir4 gave the highest total number of bacteria among the W samples. On Con Dao, CD4 gave the highest total number of bacteria among both S and W samples.

Table 2-2. The average numbers of total bacteria and oil-degrading bacteria obtained in S and W samples from the sampling sites on Iriomote and Con Dao islands

Site	Total bacteria		ODB		Percentage of ODB	
	S sample (CFU/g)	W sample (CFU/100ml)	S sample (CFU/g)	W sample (CFU/100ml)	S sample	W sample
Iriomote						
Ir1	$5.7 \times 10^5 \pm 2.4 \times 10^5$	$1.1 \times 10^3 \pm 2.3 \times 10^2$	$2.7 \times 10^4 \pm 5.0 \times 10^3$	$7.0 \times 10^1 \pm 1.9 \times 10^1$	4.7	6.4
Ir2	$9.4 \times 10^4 \pm 1.1 \times 10^5$	$5.3 \times 10^2 \pm 1.0 \times 10^2$	$1.6 \times 10^4 \pm 1.6 \times 10^4$	$7.3 \times 10^1 \pm 2.3 \times 10^1$	17.0	13.8
Ir3	$6.5 \times 10^4 \pm 2.3 \times 10^4$	$3.3 \times 10^2 \pm 3.2 \times 10^1$	$1.8 \times 10^4 \pm 7.0 \times 10^3$	$6.8 \times 10^1 \pm 2.2 \times 10^1$	27.7	20.6
Ir4	$1.2 \times 10^5 \pm 4.9 \times 10^4$	$1.2 \times 10^3 \pm 2.5 \times 10^2$	$2.2 \times 10^4 \pm 1.0 \times 10^4$	$1.6 \times 10^2 \pm 3.3 \times 10^1$	18.3	13.3
Con Dao						
CD1	$2.3 \times 10^4 \pm 1.2 \times 10^4$	$4.0 \times 10^3 \pm 2.8 \times 10^2$	$3.1 \times 10^2 \pm 6.3 \times 10^2$	$3.2 \times 10^2 \pm 1.3 \times 10^2$	1.3	8.0
CD2	$3.6 \times 10^5 \pm 3.5 \times 10^5$	$5.3 \times 10^3 \pm 4.2 \times 10^2$	$1.6 \times 10^4 \pm 1.8 \times 10^4$	$7.0 \times 10^2 \pm 2.4 \times 10^2$	4.4	13.2
CD3	$3.2 \times 10^4 \pm 6.2 \times 10^3$	$4.0 \times 10^3 \pm 2.8 \times 10^2$	$5.5 \times 10^3 \pm 5.3 \times 10^3$	$7.8 \times 10^2 \pm 2.1 \times 10^2$	17.2	19.5
CD4	$2.4 \times 10^6 \pm 1.8 \times 10^6$	$6.3 \times 10^3 \pm 4.2 \times 10^2$	$1.7 \times 10^5 \pm 1.6 \times 10^5$	$1.1 \times 10^3 \pm 9.6 \times 10^2$	7.1	17.5

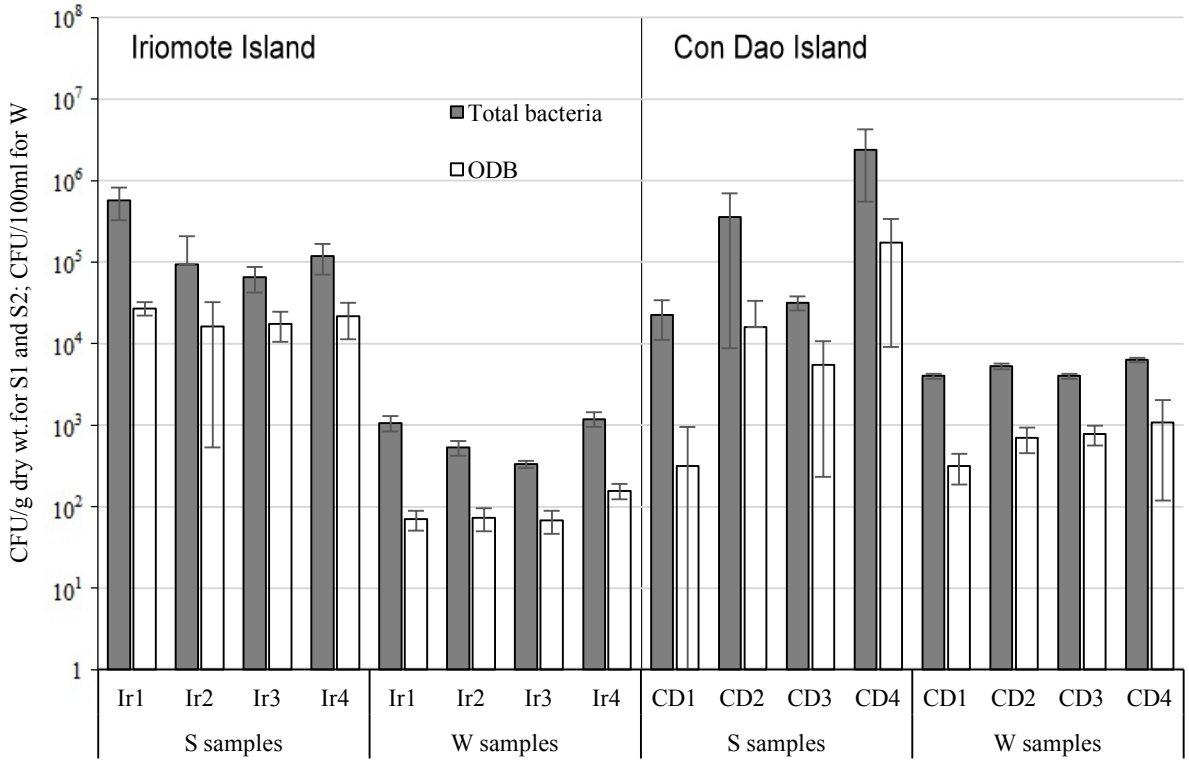

Figure 2-3. The counts of the total bacteria and oil-degrading bacteria in S and W samples from the four sites on the Iriomote and Con Dao islands.

Ir1-4 and CD1-4 are shown in Table 2-1.

The bars represent standard deviations.

2.3.3 Distribution of oil-degrading bacteria

Comparing the two study areas, the numbers of ODB showed the same distribution tendency as the total numbers of bacteria. On Iriomote, the numbers of ODB in the S samples ranged from 1.6×10^4 (Ir2) to 2.7×10^4 CFU g^{-1} (Ir1), and those in the W samples ranged from 6.8×10^1 (Ir3) to 1.6×10^2 CFU 100 ml^{-1} (Ir4). The numbers of ODB in the S samples from Con Dao ranged from 3.1×10^2 (CD1) to 1.7×10^5 CFU g^{-1} (CD4), and those in the W samples ranged from 3.2×10^2 (CD1) to 1.1×10^3 CFU 100 ml^{-1} (CD4). On Iriomote, Ir4 was characterized by the highest number of ODB in the W samples among the four sites. On Con Dao, CD4 gave significantly higher numbers of ODB for the S and W samples. On Iriomote, the highest percentages of ODB in the total bacterial population were found at Ir3, 27.7% in the S samples and 20.6% in the W samples. The highest percentages of ODB in the total bacterial population on Con Dao were obtained at CD3, 17.2% in the S samples and 19.5% in the W samples.

2.3.4 Isolation and identification of oil-degrading bacteria

A total of 45 ODB including 25 from Iriomote and 20 from Con Dao, which could grow in the medium with oil, were isolated. Morphological and biochemical characteristics of bacterial are showed in Appendix 1.

The 25 isolates from Iriomote were identified as belonging to 11 genera, *Achromobacter* (five isolates), *Acinetobacter* (three isolates), *Alcaligenes* (one isolate), *Burkholderia* (two isolates), *Dietzia* (one isolate), *Gemella* (one isolate), *Ochrobactrum* (four isolates), *Paenibacillus* (one isolate), *Pseudomonas* (five isolates), *Vibrio* (one isolate), and a Gram-negative coccus (one isolate). The 20 isolates from Con Dao were identified as members of 11 genera, *Achromobacter* (one isolate), *Brachybacterium* (one isolate), *Gemella* (two isolates), *Granulicatella* (one isolate), *Halomonas* (one isolate), *Klebsiella* (two isolates), *Microbacterium* (three isolates), *Ochrobactrum* (one isolate), *Pseudomonas* (six isolates), *Yersinia* (one isolate), and a Gram-positive bacillus (one isolate). The sequences of the isolates were submitted to the genetic sequence database at DDBJ/EMBL/GenBank with the accession numbers shown in Table 2-3.

Table 2-3. List of bacterial isolates from Iriomote and Con Dao islands and their characteristics

No.	Isolate	Species	Accession No.	Oil-degradation screening[1)]	Maximum growth temp.[2)] (°C)	Maximum survival temp.[2)] (°C)
Iriomote						
1	Ue-1.2	*Achromobacter denitrificans*	LC125138	+	42	42
2	Ue-2.2	*Achromobacter xylosoxidans*	LC125142	+	42	42
3	Oh-1.13	*Achromobacter* sp.	LC125139	++	42	42
4	Ir-1	*Achromobacter* sp.	LC125140	+	42	42
5	Ir-12.2	*Achromobacter* sp.	LC125141	+	42	42
6	Oh-2.10.0	*Acinetobacter* sp.	LC125144	+	42	42
7	Ue2-1.1	*Acinetobacter* sp.	LC125143	++	42	42
8	Ur-2.10.2	*Acinetobacter* sp.	LC125145	++	42	42
9	Fu-2.2	*Alcaligenes* sp.	LC125146	+	42	42
10	Oh-1.14	*Burkholderia cepacia*	–[3)]	++	42	42
11	Ir-5	*Burkholderia* sp.	−	+	42	42
12	Fu2-3.2	*Dietzia* sp.	LC125147	+	42	42
13	Ur-2.5.1	*Gemella* sp.	−	+	42	42
14	Oh-1.14.2	Gram −ve cocci	−	+	42	42
15	Fu-3.1.1	*Ochrobactrum anthropi*	−	+	37	42
16	Ue-1.1	*Ochrobactrum anthropi*	LC125148	+	42	42
17	Ir-2	*Ochrobactrum anthropi*	LC125149	+	39	42
18	Oh-1.15.2	*Ochrobactrum* sp.	LC125150	+	37	42
19	Fu2-1.4	*Paenibacillus* sp.	LC125151	++	42	42
20	Oh2-1.3	*Pseudomonas putida*	LC125152	++	42	42
21	Oh-1.0.2	*Pseudomonas* sp.	LC125154	+	35	35
22	Oh-1.15	*Pseudomonas* sp.	LC125155	++	42	42
23	Ue2-1.2	*Pseudomonas* sp.	−	++	42	42
24	Ue2-2.5.2	*Pseudomonas* sp.	LC125153	++	42	42
25	Fu2-2.1	*Vibrio* sp.	LC125156	++	42	42

Table 2-3 (Continued)

No.	Isolate	Species	Accession No.	Oil-degradation screening[1)]	Maximum growth temp.[2)] (℃)	Maximum survival temp.[2)] (℃)
Con Dao						
26	BD-2.3	*Achromobacter* sp.	LC125157	+	42	42
27	VB-2.1.2	*Brachybacterium rhamnosum*	LC125158	++	42	42
28	VB-1.8	*Gemella haemolysans*	LC125159	+	42	42
29	DT-2.15.1	*Gemella* sp.	−	++	42	42
30	VB-1.11	Gram +ve baccillus	−	+	39	42
31	DT-2.3.1	*Granulicatella adiacens*	−	++	42	42
32	AH-1.15.1	*Halomonas* sp.	LC125160	+	35	37
33	VB-1.5	*Klebsiella pneumoniae*	LC125161	+	42	42
34	VB-1.1	*Klebsiella pneumoniae*	LC125162	++	42	42
35	BD-2.4.2	*Microbacterium* sp.	LC125163	++	39	42
36	VB-1.14.2	*Microbacterium* sp.	LC125164	+	37	42
37	DT-2.3.2	*Microbacterium* sp.	LC125165	+	42	42
38	BD-2.5.3	*Ochrobactrum anthropi*	LC125166	++	42	42
39	BD-1.5.1	*Pseudomonas mendocina*	LC125167	++	42	42
40	AH-2.5.1	*Pseudomonas pseudoalcaligenes*	LC125168	+	42	42
41	BD-2.5.6	*Pseudomonas putida*	−	++	42	42
42	AH-2.1	*Pseudomonas stutzeri*	−	+	42	42
43	BD-2.2.1	*Pseudomonas stutzeri*	LC125170	+	42	42
44	BD-2.2	*Pseudomonas* sp.	LC125169	+	42	42
45	DT-1.15.2	*Yersinia* sp.	−	++	42	42

[1)] The oil degradation ability was surveyed by the color change of the incubation medium with INT: +, low; ++, high.

[2)] The growth and survival of the isolates were examined after 7 days of incubation at the temperatures of 15, 20, 30, 35, 37, 39, and 42℃.

[3)] The species were identified biochemically using the API kits.

2.3.5 Screening the oil degradation of the isolates

A total of 45 isolates had the ability to grow in the medium with oil as the sole carbon source (Table 2-3). The ability of the isolates to degrade oil was rated at two levels with INT. On Iriomote, 10 isolates, Oh-1.13 from the genus *Achromobacter*, Ue2-1.1 and Ur-2.10.2 from *Acinetobacter*, Oh-1.14 from *Burkholderia*, Fu2-1.4 from *Paenibacillus*, Oh2-1.3, Ue2-1.2, Ue2-2.5.2, and Oh-1.15 from *Pseudomonas*, and Fu2-2.1 from *Vibrio* showed high abilities to degrade oil (++). On Con Dao, nine isolates, VB-2.1.2 from the genus *Brachybacterium*, DT-2.15.1 from *Gemella*, DT-2.3.1 from *Granulicatella*, VB-1.1 from *Klebsiella*, BD-2.4.2 from *Microbacterium*, BD-2.5.3 from *Ochrobactrum*, BD-1.5.1 and BD-2.5.6 from *Pseudomonas*, and DT-1.15.2 from *Yersinia* showed high abilities to degrade oil (++).

2.3.6 Temperature effect and pathogenic potential

The growth and survival of the isolates at the temperatures from 15 to 42℃ are shown in Table 2-3. In the range from 15 to 35℃, there was no temperature effect on the growth of the isolates. Among the Iriomote isolates, one isolate, Oh-1.0.2, could grow or survive up to 35℃. Two isolates, Oh-1.15.2 and Fu-3.1.1, grew up to 37 but survived at 42℃, and one isolate, Ir-2, grew up to 39 but survived at 42℃. Among the Con Dao isolates, one isolate, AH-1.15.1, grew and survived up to 35℃ and 37℃, respectively. Isolate VB-1.14.2 grew up to 37 but survived at 42℃, and two isolates, VB-1.11 and BD-2.4.2, grew up to 39 but survived at 42℃.

Based on the results of the species identification and the temperature effects (Govan et al. 1996; Podschun and Ullmann 1998; Cargill et al. 2012), one isolate, *Burkholderia cepacia* Oh-1.14, from Iriomote and three isolates, *Granulicatella adiacens* DT-2.3.1, *Klebsiella pneumoniae* VB-1.1, and *K. pneumoniae* VB-1.5, from Con Dao were determined as potentially pathogenic. I assigned numbers to the rest of the isolates and used them for further studies (Table 2-3).

2.4 Discussion

The percentages of the number of ODB out of the total number of bacterial counts on Iriomote and Con Dao were 27.1 and 17.4%, respectively, in the S samples and 20.5 and 19.4%, respectively, in the W samples. It has been reported that oil-degrading bacteria accounted for 1–10% of the total number of bacteria in heterotrophic bacterial communities in vegetated soils (Atlas, 1981). Because the sampling sites used in this study were exposed to no oil spill accidents during the collection period, the percentages of ODB (17.4–27.1%) indicate suitable application conditions for autochthonous bioaugmentation for the treatment of oil contamination in these areas. In addition, the relatively large number of ODB might also show chronic oil-contamination (Atlas 1981; Chikere et al. 2011) in the study islands from oil seepage of human activities or busy oil transportation.

The oil-degrading screening by INT based on the transfer of electron from NADH to the tetrazolium dye *p*-iodonitrotetrazolium violet. During the active growth of bacteria, an electron is transferred from NADH (colorless) to *p*-iodonitrotetrazolium violet resulting in a formazan dye (purple color). The darker medium color indicated better bacterial growth in the medium with oil.

The 45 ODB isolates, 25 from Iriomote and 20 from Con Dao, belonged to 18 genera. The isolate from the genus *Granulicatella* and the two unidentified isolates, a Gram-negative coccus and a Gram-positive bacillus, were isolated for the first time as ODB in this study. A *Granulicatella* isolate was identified by Al-Mailem et al. (2014) using a molecular method in man-made biofilms used for the bioremediation of hydrocarbons contaminating a sewage effluent. The prevalent genera of ODB are *Achromobacter*,

Acinetobacter, *Alcaligenes*, *Burkholderia*, *Dietzia*, *Pseudomonas*, and *Vibrio* (Atlas 1981; Chikere et al. 2011). The uncommon genera are *Brachybacterium, Halomonas, Klebsiella, Microbacterium, Ochrobactrum*, *Paenibacillus*, and *Yersinia* (Daane et al. 2001; Hamme et al. 2003; Mnif et al. 2009; Rodrigues et al. 2009; Hassanshahian et al. 2012; Lily et al. 2013; Bhattacharya and Biswas 2014). The genus *Gemella* was first reported as ODB in 2015 (Kok Kee et al. 2015).

Among the 11 genera detected on Iriomote, the three dominant genera were *Achromobacter*, *Pseudomonas*, and *Ochrobactrum*. Among the 11 genera detected on Con Dao, the two dominant genera were *Pseudomonas* and *Microbacterium*. Three common genera, *Achromobacter*, *Ochrobactrum*, and *Pseudomonas*, were present in both study areas. *Pseudomonas* spp. are most frequently isolated and studied as hydrocarbon-degrading bacteria (Mittal and Singh 2009). Among the isolates from both study areas, those belonging to *Pseudomonas* species were not only dominant in quantity but also in their degradation efficiency. There were three members of *Pseudomonas* species that gave more than 50% of oil degradation. Because *Pseudomonas* species play a significant role in oil degradation due to their wide distribution and ability to degrade a wide range of hydrocarbon compounds, they are studied for genetic diversity in degradation functions and gene modification and used as biosensors and bioremediation agents (Palleroni et al., 2010).

Chapter 3

Biodegradability of oil-degrading bacteria on Iriomote and Con Dao islands

3.1 Introduction

The aim of this study was to compare the degradation abilities of bacterial isolates from two different study areas, subtropical Iriomote Island and tropical Con Dao Island, for crude oil, *n*-alkanes, and polycyclic aromatic hydrocarbons (PAHs). Because oil is a complex mixture of hydrocarbons and no single species can completely degrade all components (Chikere et al. 2011), degradation of two alkanes, heptadecane and octadecane, and two PAHs, fluoranthene and pyrene, was representatively used.

3.2 Materials and methods

3.2.1 Biodegradation of crude oil and *n*-alkanes

The degradation of oil and the *n*-alkanes, heptadecane and octadecane, by the isolates was determined by the gravimetric method (Chaillan et al. 2004). The isolates were grown in duplicate without shaking in 250-ml Erlenmeyer flasks containing 100 ml of MSB medium and 500 mg of oil or 100 mg of each *n*-alkane as the sole carbon source. Sterile controls were used to quantify abiotic losses due to evaporation. After 30 days of incubation at 28 ± 2℃, microbial activities were stopped by adding 1 ml of 1 N HCl to the flasks. The residual oil was recovered by dichloromethane extraction (Chaillan et al. 2004). The extracted solvent was evaporated by a vacuum rotary evaporator at 35℃. Subsequently, the residual degraded oil products were re-dissolve in 5 ml DCM and transferred to a pre-weighed vial. The vials were kept underhood until the DCM has completely evaporated and the residual crude oil were quantified gravimetrically. The degradation rate was determined as $B\ (\%) = 100 \times (W_I - W_C)/W_I$, where W_I is the residual mass in the control and W_C is that in the culture.

3.2.2 Biodegradation of PAHs

The two PAHs, fluoranthene and pyrene, were used for determining the degradation abilities of the isolates. A solution of fluoranthene or pyrene in N,N-dimethylformamide was added at a final concentration of 100 mg/l to 100 ml of MSB medium in 250-ml Erlenmeyer flasks. The incubation and solvent extraction were carried out under the same conditions as in the oil degradation experiments described above. After the extraction, residual PAH concentrations were determined by a gas chromatography–mass spectrometry (GC-MS) method (Mineki et al. 2015). The degradation rate was calculated using the same formula as described above for the oil degradation rate.

3.3 Results

3.3.1 Biodegradation rates of crude oil by the isolates

The results of the oil degradation rates shown by the isolates are presented in Table 3-1 and Figure 3-1. Among the Iriomote isolates, three isolates, No. 6 (Ue2-1.1, 71.2%), No. 21 (Ue2-2.5.2, 53.0%), and No. 23 (Oh-1.15, 55.1%) could degrade more than 50% of the oil present in the media. Among the Con Dao isolates, two isolates, No. 35 (BD-1.5.1, 66.3%) and No. 37 (BD-2.5.6, 62.9%) showed more than 50% degradation rates.

Table 3-1. Biodegradation rate of the bacterial isolates for crude oil, *n*-alkanes and PAHs after 30 days of incubation

No.	Species	Isolate	Crude oil (%)	*n*-alkane		PAH	
				Heptadecane (%)	Octadecane (%)	Fluoranthene (%)	Pyrene (%)
Iriomote							
1	*Achromobacter denitrificans*	Ue-1.2	34.9 ± 7.5	52.9 ± 10.9	55.2 ± 5.7	24.8 ± 10.7	18.2 ± 6.1
2	*Achromobacter xylosoxidans*	Ue-2.2	30.4 ± 13.7	12.7 ± 8.0	10.6 ± 0.4		
3	*Achromobacter* sp.	Oh-1.13	32.0 ± 7.8	64.0 ± 2.7	59.7 ± 8.4		
4	*Achromobacter* sp.	Ir-1	26.2 ± 2.0	2.7 ± 1.0	5.4 ± 1.5		
5	*Achromobacter* sp.	Ir-12.2	26.4 ± 4.4	5.2 ± 3.2	5.5 ± 3.2		
6	*Acinetobacter* sp.	Oh-2.10.0	71.2 ± 2.9	88.5 ± 1.2	78.4 ± 8.0		
7	*Acinetobacter* sp.	Ue2-1.1	27.4 ± 1.9	10.3 ± 5.1	11.0 ± 4.1		22.0 ± 1.2
8	*Acinetobacter* sp.	Ur-2.10.2	44.1 ± 7.5	67.3 ± 3.5	63.9 ± 7.9	29.8 ± 9.2	18.6 ± 5.5
9	*Alcaligenes* sp.	Fu-2.2	27.3 ± 4.5	6.3 ± 1.7	5.6 ± 3.5		
10	*Burkholderia* sp.	Ir-5	26.8 ± 5.9	2.6 ± 1.8	6.2 ± 3.4	21.3 ± 5.5	11.3 ± 9.7
11	*Dietzia* sp.	Fu2-3.2	35.4 ± 2.7	56.6 ± 7.2	48.5 ± 14.5	32.0 ± 11.8	20.3 ± 9.0
12	*Gemella* sp.	Ur-2.5.1	27.1 ± 2.9	7.2 ± 6.5	9.7 ± 4.1		12.8 ± 3.8
13	Gram −ve cocci	Oh-1.14.2	27.0 ± 0.3	14.9 ± 2.4	11.4 ± 4.5	25.1 ± 2.2	16.5 ± 1.5
14	*Ochrobactrum anthropi*	Fu-3.1.1	28.5 ± 2.5	14.6 ± 0.2	18.3 ± 6.0		
15	*Ochrobactrum anthropi*	Ue-1.1	32.7 ± 6.6	13.5 ± 6.3	7.2 ± 4.0		
16	*Ochrobactrum anthropi*	Ir-2	27.6 ± 2.1	18.1 ± 1.1	20.2 ± 5.8		
17	*Ochrobactrum* sp.	Oh-1.15.2	27.3 ± 0.3	13.8 ± 6.2	15.0 ± 4.2	26.4 ± 14.1	33.1 ± 11.3
18	*Paenibacillus* sp.	Fu2-1.4	44.1 ± 7.9	74.3 ± 4.3	78.0 ± 4.2	22.4 ± 6.0	21.1 ± 3.5
19	*Pseudomonas putida*	Oh2-1.3	46.0 ± 2.4	66.5 ± 3.5	69.7 ± 9.6	25.4 ± 2.0	39.0 ± 9.3
20	*Pseudomonas* sp.	Oh-1.0.2	45.4 ± 3.9	71.4 ± 4.6	65.8 ± 4.6	22.7 ± 1.1	17.4 ± 4.6
21	*Pseudomonas* sp.	Oh-1.15	55.1 ± 2.1	84.0 ± 2.9	87.8 ± 2.7	21.7 ± 0.3	15.2 ± 1.3

Table 3-1 (Continued)

No.	Species	Isolate	Crude oil (%)	*n*-alkane		PAH	
				Heptadecane (%)	Octadecane (%)	Fluoranthene (%)	Pyrene (%)
22	*Pseudomonas* sp.	Ue2-1.2	35.1 ± 8.0	59.7 ± 10.1	62.5 ± 9.6	20.0 ± 2.1	20.4 ± 3.9
23	*Pseudomonas* sp.	Ue2-2.5.2	53.0 ± 2.1	92.0 ± 1.7	93.1 ± 0.9	22.2 ± 3.0	12.0 ± 0.9
24	*Vibrio* sp.	Fu2-2.1	43.7 ± 2.1	63.0 ± 7.9	59.1 ± 9.4	22.1 ± 6.3	24.9 ± 7.9
Con Dao							
25	*Achromobacter* sp.	BD-2.3	31.8 ± 1.8	16.2 ± 3.4	14.4 ± 4.6	17.3 ± 8.4	15.6 ± 0.9
26	*Brachybacterium rhamnosum*	VB-2.1.2	34.8 ± 8.9	20.9 ± 13.4	24.5 ± 5.5	22.0 ± 0.3	19.1 ± 2.1
27	*Gemella haemolysans*	VB-1.8	27.5 ± 3.3	29.7 ± 4.9	27.1 ± 4.0		
28	*Gemella* sp.	DT-2.15.1	30.0 ± 0.9	71.7 ± 17.8	78.0 ± 5.6	20.7 ± 5.4	16.0 ± 1.6
29	Gram +ve baccillus	VB-1.11	34.7 ± 9.9	82.9 ± 2.2	78.3 ± 8.6	18.9 ± 2.1	18.9 ± 7.0
30	*Halomonas* sp.	AH-1.15.1	28.1 ± 13.6	5.0 ± 3.7	6.9 ± 1.5	22.6 ± 3.4	14.9 ± 4.8
31	*Microbacterium* sp.	BD-2.4.2	34.9 ± 1.5	25.2 ± 5.5	17.6 ± 0.4	16.8 ± 0.6	33.3 ± 5.2
32	*Microbacterium* sp.	VB-1.14.2	28.8 ± 0.6	4.7 ± 1.6	12.0 ± 3.8	19.3 ± 2.7	13.5 ± 2.2
33	*Microbacterium* sp.	DT-2.3.2	27.7 ± 3.8	12.8 ± 4.6	10.3 ± 1.7	23.0 ± 0.7	14.6 ± 3.2
34	*Ochrobactrum anthropi*	BD-2.5.3	43.6 ± 0.3	46.3 ± 8.2	52.4 ± 12.3	28.4 ± 5.1	
35	*Pseudomonas mendocina*	BD-1.5.1	66.3 ± 1.6	77.0 ± 5.3	82.4 ± 14.5		
36	*Pseudomonas pseudoalcaligenes*	AH-2.5.1	48.6 ± 4.2	47.3 ± 9.1	41.6 ± 6.7	21.5 ± 2.1	17.0 ± 5.4
37	*Pseudomonas putida*	BD-2.5.6	62.9 ± 2.6	93.9 ± 0.8	87.3 ± 4.8		16.7 ± 5.6
38	*Pseudomonas stutzeri*	AH-2.1	29.2 ± 0.1	12.9 ± 1.4	18.3 ± 5.4		
39	*Pseudomonas stutzeri*	BD-2.2.1	30.2 ± 2.5	21.8 ± 5.2	22.1 ± 0.1	20.1 ± 2.1	12.9 ± 3.3
40	*Pseudomonas* sp.	BD-2.2	27.4 ± 2.3	21.9 ± 4.2	34.5 ± 0.8	19.8 ± 7.5	
41	*Yersinia* sp.	BD-2.2.1	45.6 ± 6.5	49.2 ± 4.4	56.6 ± 8.1	20.4 ± 2.2	18.3 ± 3.4

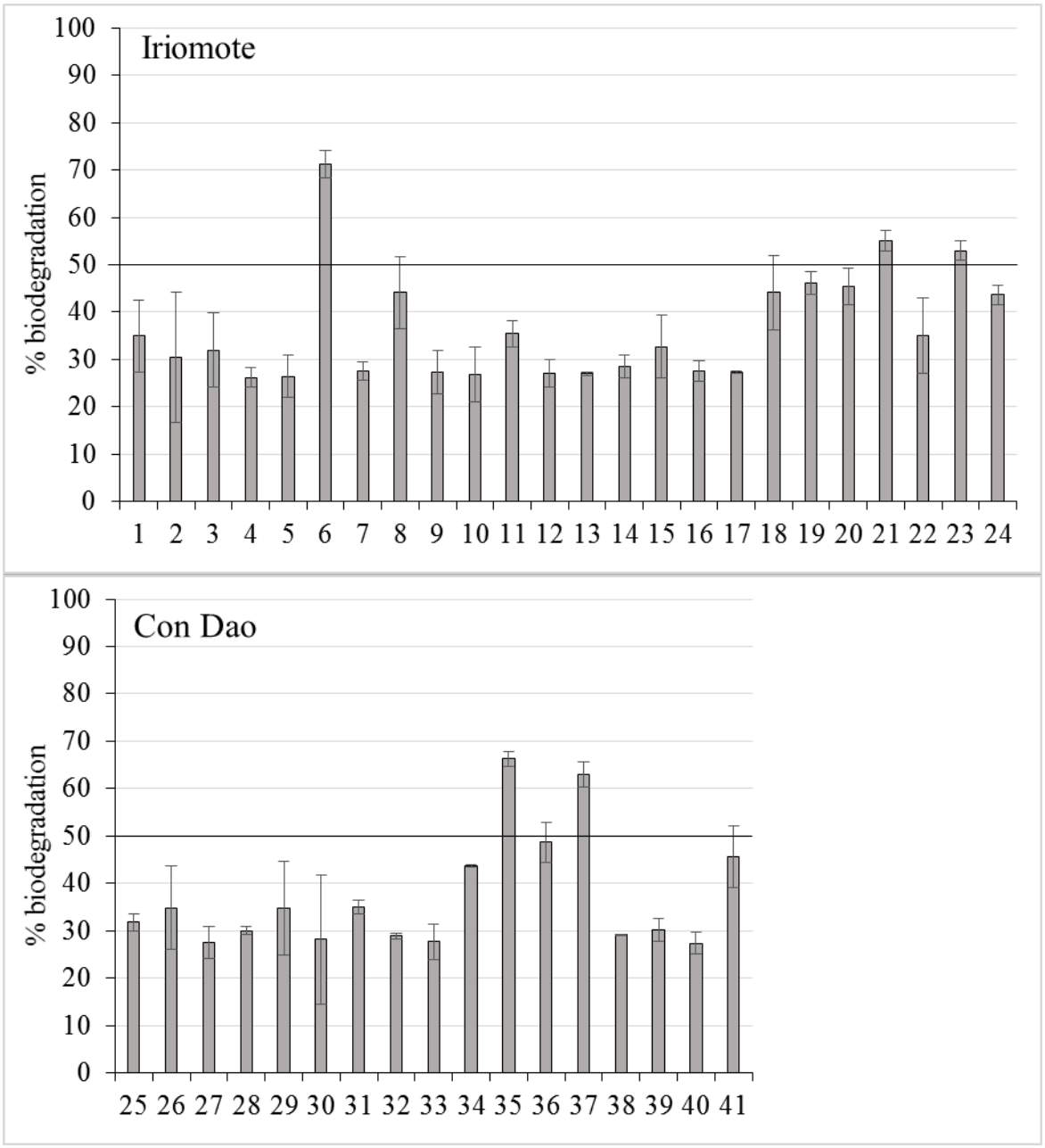

Figure 3-1. Biodegradation rates of crude oil after 30 days of incubation with the isolates from Iriomote and Con Dao islands.

The Iriomote isolates were numbered from 1 to 24 and those from Con Dao were numbered from 25 to 41. The numbers are the same as in Table 3-1.

The bars represent standard deviations.

3.3.2 Biodegradation rates of *n*-alkanes by the isolates

The degradation rates of the *n*-alkanes, heptadecane and octadecane, by the isolates are shown in Table 3-1 and Figure 3-2. All isolates could grow in the medium with the pure *n*-alkanes as the sole carbon sources. Among the 24 Iriomote isolates, 11 isolates showed degradation abilities of more than 50% for both chemicals. The highest degradation rates of heptadecane and octadecane were 92.0 and 93.1% for No. 23 (Ue2-2.5.2), followed by 88.5 and 78.4% for No. 6 (Oh-2.10.0) and 84.0 and 87.8% for No. 21 (Oh-1.15), respectively. Among the 17 Con Dao isolates, four isolates showed degradation abilities of more than 50%. The highest degradation rates of heptadecane and octadecane were 93.9 and 87.3% for No. 37 (BD-2.5.6), followed by 82.9 and 78.3% for No. 29 (VB-1.11) and 77.0 and 82.4% for No. 35 (BD-1.5.1), respectively.

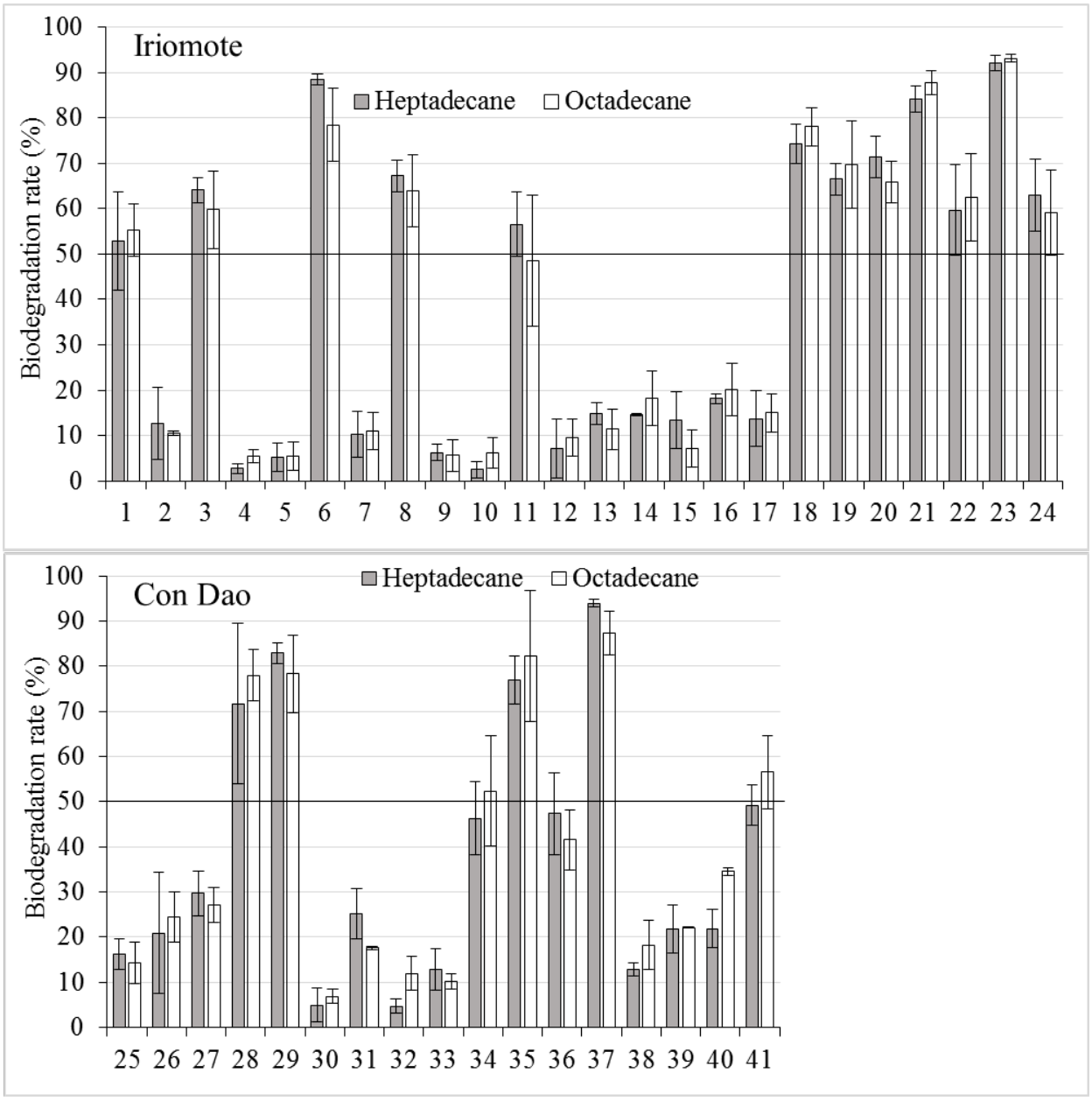

Figure 3-2. Biodegradation rates of the *n*-alkanes after 30 days of incubation with the isolates from Iriomote and Con Dao islands.

The isolate numbers are the same as in Table 3-1.

The bars represent standard deviations.

3.3.3 Biodegradation of PAHs by the isolates

Table 3-1 and Figure 3-3 show the degradation rates of the PAHs, fluoranthene and pyrene, for the isolates. Figure 3-4 and Figure 3-5 show the GC-MS chromatograms of Pyrene and Fluoranthene, respectively. The degradation data for fluoranthene and pyrene showed different tendencies. Among the 24 isolates from Iriomote, 13 isolates could grow in the medium with fluoranthene. The highest degradation rate of 32.0% was shown by No. 11 (Fu2-3.2), followed by 29.8% shown by No. 8 (Ur-2.10.2) and 26.4% shown by No. 17 (Oh-1.15.2). Among the 17 Con Dao isolates, 13 isolates could grow in the medium with fluoranthene. The highest degradation rate of 28.4% was obtained with No. 34 (BD-2.5.3), followed by 23.0% obtained with No. 33 (DT-2.3.2), and 22.6% obtained with No. 30 (AH-1.15.1).

Among the 24 Iriomote isolates, 15 isolates could grow in the medium with pyrene. The highest degradation ability of 39.0% was shown by No. 19 (Oh2-1.3), followed by 33.1% shown by No. 17 (Oh-1.15.2). Among the 17 Con Dao isolates, 12 isolates could grow in the medium with pyrene, and the highest degradation rate of 33.3% was shown by No. 31 (BD-2.4.2).

Isolates No. 34 (BD-2.5.3) and No. 40 (BD-2.2) could degrade fluoranthene but not pyrene. On the other hand, No. 7 (Ue2-1.1) and No. 12 (Ur-2.5.1) could not degrade fluoranthene but degraded pyrene.

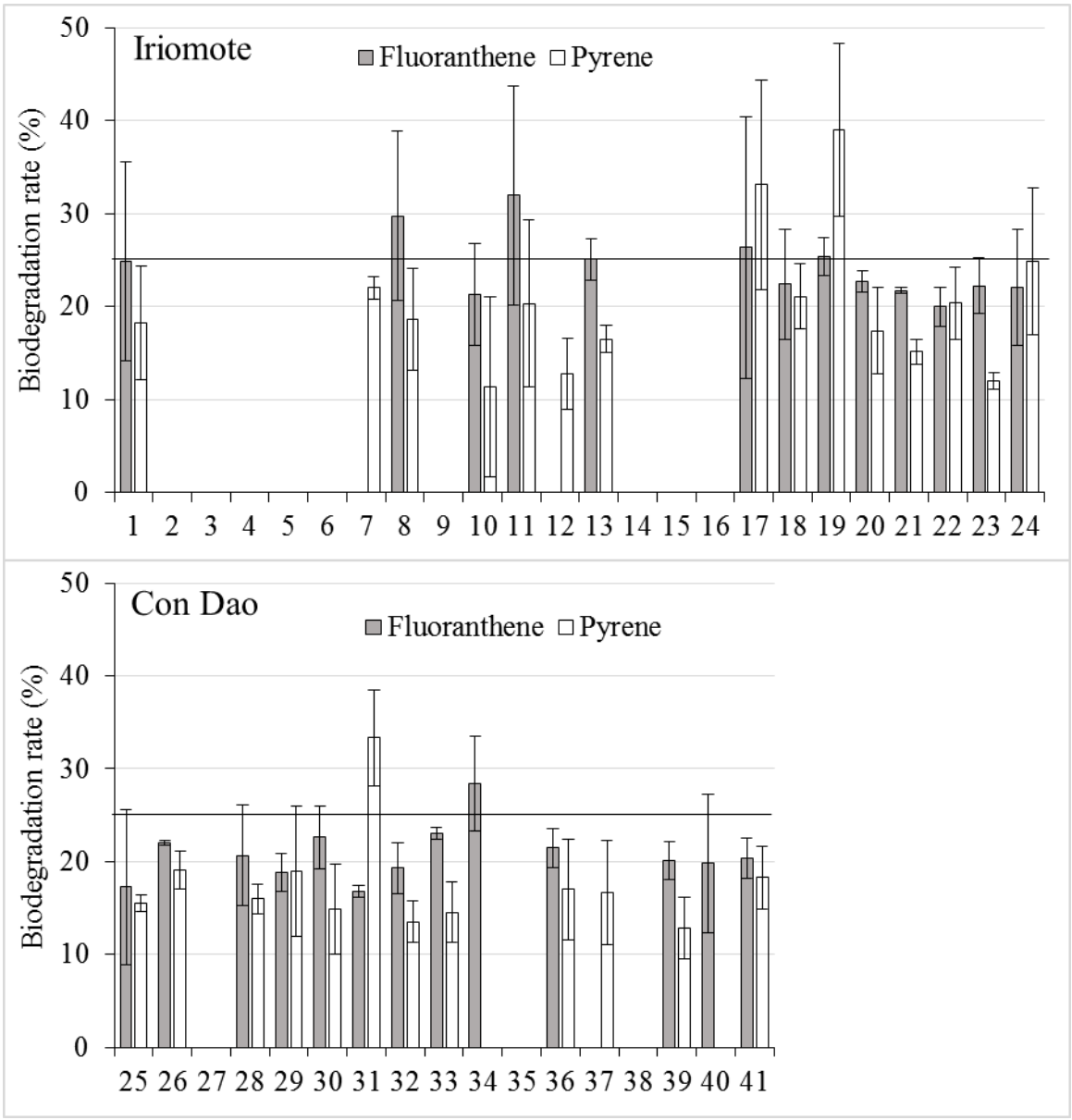

Figure 3-3. Biodegradation rates of the PAHs after 30 days of incubation with the isolates from Iriomote and Con Dao islands.

The isolate numbers are the same as in Table 3-1.

The bars represent standard deviations.

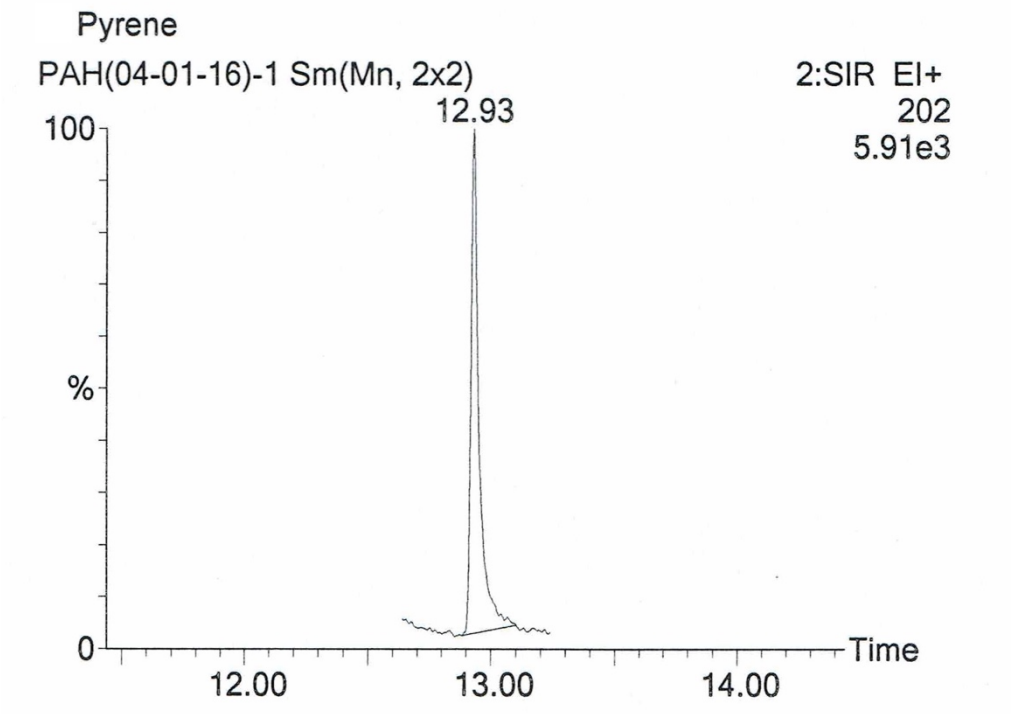

Figure 3-4. GC-MS chromatogram of Pyrene

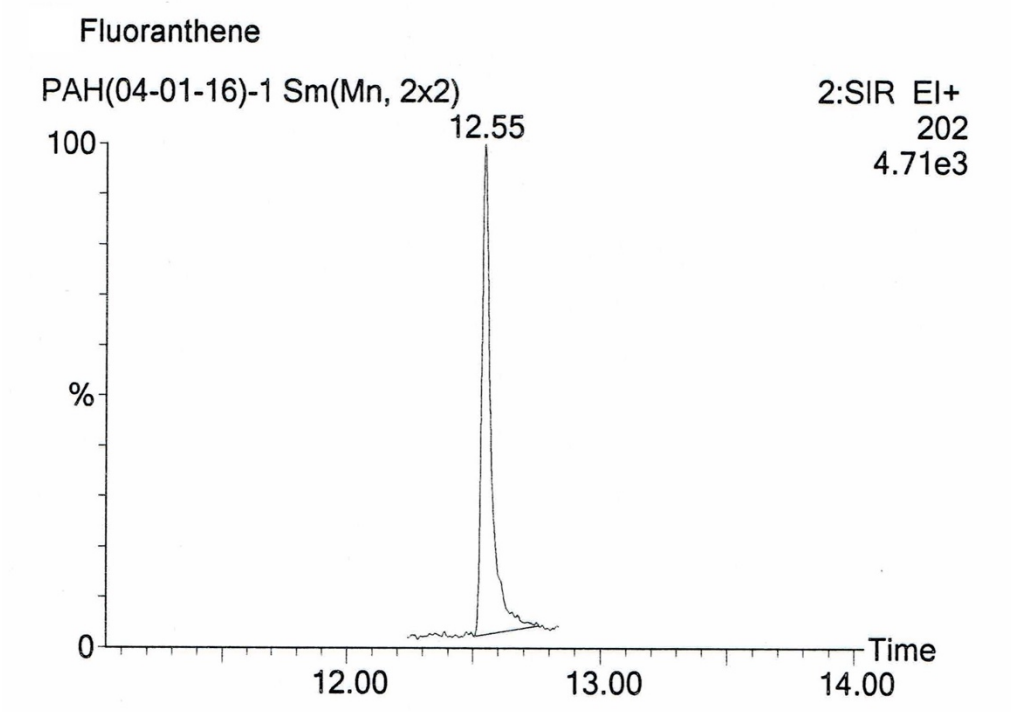

Figure 3-5. GC-MS chromatogram of Fluoranthene

3.4 Discussion

The oil degradation rates shown by the bacterial isolates ranged from 26.2 to 71.2% for the Iriomote isolates and from 27.4 to 66.3% for the Con Dao isolates. The number of the isolates that degraded more than 50% of crude oil was higher on Iriomote (three isolates) than on Con Dao (two isolates). In comparison with other studies, the oil degradation rates shown by our isolates were higher during the same incubation time than those obtained for other tropical areas such as Indonesia (4.9–22.0%; Chaillan et al. 2004) and India (11.6–52.8%; Mittal and Singh 2009). In comparison with the oil-degradation screening results in Chapter 2, some bacteria including samples No. 3, 26, 28 and 31 did not show high biodegradability of crude oil (Figure 3-1), though they indicated high activity in oil degradation screening (Table 2-3). The reason is that, in some cases, high microbial growth were not indicative of higher degradation extents (Aldrett et al. 1997).

n-Alkanes are major components in oil and readily degraded in the environment via several pathways (Hassanshahian et al. 2012). Among crude oil components, *n*-alkanes are the preferred carbon source for bacteria (Delille et al. 2004). This is the reason why the degradability of the *n*-alkanes by the isolates (Figure 3-2) was more distinct than the one of crude oil (Figure 3-1). The degradation rate of an *n*-alkane depends on the chain length. Short-chain alkanes ($< C_9$) are volatized and rapidly released into the atmosphere. Intermediate-chain alkanes (C_{10}–C_{24}) are readily degraded by microorganisms, and longer-chain alkanes are generally resistant to degradation. The results of this study showed that the isolates with a high degrading ability for oil also showed a high degrading

ability for heptadecane (C_{17}) and octadecane (C_{18}). The number of the isolates that degraded more than 50% of the *n*-alkanes present in the media was greater on Iriomote (11 isolates) than on Con Dao (four isolates). The members of the genera *Pseudomonas* and *Acinetobacter* were good *n*-alkane degraders, which has also been reported previously (Chikere et al. 2011).

PAHs belong to most hazardous organic pollutants, which is due to their toxicogenic, mutagenic, and carcinogenic properties (Darmawan et al. 2015). PAHs are present in oil, are highly resistant to biodegradation, and are degraded by fewer microbial species as compared with oil or alkane compounds (Fernandes 2006; Chaerun et al. 2004). In this study, only 13 and 15 isolates out of the 24 isolates from Iriomote and 13 and 12 isolates out of the 17 isolates from Con Dao could grow in the medium with fluoranthene and pyrene, respectively. The number of the isolates that degraded more than 25% of the fluoranthene and pyrene present in the media was greater on Iriomote (two isolates each) than on Con Dao (one isolate each). The isolates that showed high abilities to degrade the PAHs were different from those with high abilities to degrade oil or the alkanes. Because of the variety and complexity in the structure of PAHs, there are diverse catabolic pathways according to microbial species. The major principle of aromatic hydrocarbon biodegradation include two steps: 1) activating a thermodynamically stable benzene ring from structurally diverse aromatics by adding oxygen atoms of molecular oxygen as hydroxyl groups to the aromatic ring; and 2) subsequent cleavage, ring-cleavage dioxygenases decyclize aromatic compounds allowing the products to be channeled into the cell's central metabolic pathways (Pérez-Pantoja et al. 2010; Haddock 2010). Key

enzymes in aerobic aromatic degradation are oxygenases. In cases of fluoranthene and pyrene, there are 54 and 27 enzymes, respectively, present in *Mycobacterium vanbaalenii* involve in the degradation of those PAHs (Kweon et al. 2010).

Isolates No. 6 (Oh-2.10.0) and No. 35 (BD-1.5.1) showed high abilities to degrade oil and the alkanes, but they could not grow in the medium with the PAHs. Isolates No. 17 (Oh-1.15.2) from Iriomote and No. 30 (AH-1.15.1) and No. 33 (DT-2.3.2) from Con Dao showed high degradation rates for fluoranthene but low degradation rates for oil and the alkanes. Isolates No. 17 (Oh-1.15.2) from Iriomote and No. 31 (BD-2.4.2) from Con Dao showed high abilities to degrade pyrene but low abilities to degrade oil and the alkanes. A number of hydrocarbon-degrading enzymes from bacteria participate in the degradation of different groups of hydrocarbon compounds in oil. Each bacterial group has only one or a few enzymes that can degrade one or a group of hydrocarbon compounds. It is noteworthy that the isolates of *Acinetobacter* sp., *Pseudomonas* sp., *Pseudomonas putida* from Iriomote and *Pseudomonas mendocina, Pseudomonas putida, Microbacterium* sp from Con Dao showed the highest degradability for crude oil, *n*-alkanes, and PAHs, respectively, and survived at 42℃.

Chapter 4

Isolation and identification of oil-degrading fungi on Iriomote and Con Dao islands

4.1 Introduction

Oil is a significant source of energy for both industry and daily life worldwide. It remains the world's leading fuel, with 33% of global energy consumption (BP Group 2014). Oil is transported across the world from production sites to refineries and from refineries to consumers by different modes of transportation such as maritime routes using barges and tankers, and inland routes using pipelines, trucks and trains. In 2013, total world crude oil and other liquid fuel production was about 90 million barrels per day (bbl/d). It is estimated that 63% of this amount traveled via a maritime route (EIA 2014). Annual oil spillage from waterborne transportation has been estimated at 4 million bbl/d of crude oil seepage per year (Kvenvolden and Cooper 2003; Das and Chandran 2011), and accounts for 70% of pollution from oil and gas activities (Weis 2015). Oil spills in the environment cause long-term damage to aquatic and soil ecosystems, human health and natural resources (McGenity et al. 2012).

There are three ways of treating oil contamination: physical, chemical and biological. In comparison with physical and chemical methods, the biological method, bioremediation, is considered a non-destructive, cost-effective treatment. In this study, I followed the newly introduced bioremediation method by Ueno et al. (2007) called autochthonous bioaugmentation. In this method, indigenous microorganisms which are potentially capable of degrading oils and well-characterized from the contaminated sites or predicted contamination sites are used. This method has a number of advantages for the decontamination of oil-polluted land with shorter treatment time, greater potential efficiency, lower impact on the environment, and relative ease in obtaining public support

(Hosokawa et al. 2009). Iriomote and Con Dao islands are both predicted contamination sites locating on a busy maritime route which transported 15 million bbl/d in 2013 and supplies 83% of crude oil and oil products to Japan and East Asian countries (EIA 2014). Because these islands are covered 70–80% by ecologically sensitive national parks that include jungle, mangrove (Alsaaideh et al. 2013), coral reef ecosystems and several endangered species (Cox 2002, RAMSAR 2014), I have screened the indigenous microorganisms from these islands to minimize disturbance to their ecosystems.

Because oil is a complex mixture of hydrocarbons, other organic compounds and organometallic constituents, a vast array of microbial species (bacteria, fungi, algae, and cyanobacteria) have diverse mechanisms to degrade it (Hamme et al. 2003). Filamentous fungi possess attributes that make them good potential agents for oil degradation such as quick spread in the environment, digestion of substrates by extracellular enzymes and ability to grow under stressful environmental conditions, especially due to their tolerance to high concentrations of polluting chemicals (Cerniglia and Sutherland 2001, Elshafie et al. 2007; Al-Nasrawi 2012; Sardrood et al. 2013). In addition, some fungal genera such as *Aspergillus* and *Penicillium* show greater ability to degrade oil contaminants than bacteria such as *Arthrobacter*, *Brevibacterium*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas* (Elshafie et al. 2007; Sardrood et al. 2013).

Oil-degrading microorganisms in general and fungi in particular are not restricted to a particular ecosystem, country or continent and have been found in the Arctic, Antarctic and temperate regions (Jones 1993); however, there are few reports on their distribution in high temperate ecosystems (Oudot et al. 1993; Prince 1993; Elshafie et al. 2007).

Moreover, most works focus on the biodegradation of oil by bacteria, whereas fewer papers deal with the degradation of oil by fungi (Chaîneau et al. 1999). Recently, comparative research was carried out in subtropical areas such as the Gulf of Mexico (Al-Nasrawi 2012) and Arabian Gulf region (Hashem 2007), and in tropical areas such as Oman (Elshafie et al. 2007), Indonesia ((Chaillan et al. 2004), and India (Saravanan and Sivakumar 2013), which identified a number of fungal genera in subtropical and tropical areas that can degrade oil, such as *Aspergillus*, *Eupenicillium*, *Fusarium*, and *Penicillium*.

Iriomote, Japan, has a subtropical climate, whereas Con Dao, Vietnam, has a tropical climate. The aim of this study was to compare the distribution of indigenous ODF on two islands, which have sensitive ecosystems.

4.2 Materials and methods

4.2.1 Study sites and sample collection

The study sites, sample collections and parameter measurements were the same as Chapter 2.

4.2.2 Medium preparation

For enumerating total fungi, Czapek-Dox malt extract agar (CDA) was used (Al-Nasrawi 2012). The composition of CDA medium was 30 g sucrose, 2.0 g $NaNO_3$, 0.5 g magnesium glycerolphosphate, 0.5 g KCl, 0.35 g K_2SO_4, 0.01 g $FeSO_4$, 30 g malt extract, 15 g agar in 1 l of distilled water (dw). Streptomycin (500 mg/l) was added to the media after sterilization as an antibacterial agent.

To enumerate ODF, Bushnell-Haas mineral salt agar (BHA) was used (Al-Nasrawi 2012). BHA consisted of 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.05 g $FeCl_3$, 1 g NH_4NO_3, 20 g NaCl, 20 g agar in 1 l of dw. The streptomycin (500 mg/l) was added. SLEB crude oil supplied from Brunei Shell Petroleum Company, Brunei Darussalam, was heated at 100℃ for 48 h and sterilized by filter membranes (0.2 μm pore size). One percent (v/v) of this weathered crude oil was spread on the surface of BHA medium as the sole carbon source.

For ODF enrichment and oil-degradability screening, Bushnell-Haas mineral salt broth (BHB) was used. BHB has the same composition as BHA except that agar, streptomycin (500 mg/l) and 1% (v/v) weathered crude oil were added to the sterilized medium (Venkateswaran et al. 1991; Al-Nasrawi 2012).

For characteristic identification of isolated fungi, potato dextrose agar (PDA, Wako Ltd., Japan) and Sabouraud agar (SBA) were used. SBA consisted of 40 g dextrose, 10 g peptone and 20 g agar in 1 l of dw.

4.2.3 Enumeration of total and oil-degrading fungi

Two kinds of sediment samples from S1 and S2 were prepared by aseptically homogenized, weighed and mixed with an equal weight of 0.85% (w/v) sodium chloride. The adhering microorganisms were dissociated by a vortex mixer. The microorganisms were spread as 10-fold serial dilutions onto CDA and BHA plates for enumeration of total fungi and ODF, respectively (Al-Nasrawi 2012). Each dilution was inoculated in triplicate. The colonies that developed were counted and expressed as colony forming units (CFU) per gram dry weight of sample.

Fungi in seawater samples W were enumerated by the membrane filter method (Venkateswaran et al. 1991). Each 100 ml seawater sample was filtered through a membrane (47 mm diameter, 0.2 μm pore size) in quadruplicate; two membranes were singularly placed on top of CDA plates and the other two singularly on top of BHA plates for the enumeration of total and ODF, respectively (Al-Nasrawi 2012). The colonies that developed were counted and expressed as CFU per 100 ml water sample. All incubations were carried out at 25℃ up to 7–10 days.

4.2.4 Isolation of oil-degrading fungi

The colonies that formed on BHA during the enumeration steps were isolated by repeated streaking on fresh CDA plates. ODF were also isolated by the enrichment medium method (Chaerun et al. 2004). One gram of each sediment sample was inoculated in an Erlenmeyer flask containing 50 ml autoclaved BHB. The samples were incubated at 120 rpm, 25℃ on an orbital shaker for 5–20 days. After every 5 days of incubation, a sample of culture medium was serially diluted and spread on BHA. The colonies that formed were isolated by repeated streaking on fresh CDA plates.

4.2.5 Oil-degradability screening

Biodegradability of isolates was verified using the modified technique based on the redox indicator *p*-iodonitrotetrazolium (INT) (Wrenn and Venosa 1996; Masoko et al. 2005). The isolate was cultivated in 50 ml Erlenmeyer flasks containing 10 ml BHB. After 7 days of incubation, 1 ml of culture medium was transferred aseptically to a tube and added 100 µl of filter sterilized INT (5 g/l). Color change of the mixture was observed; the mixture in which INT was reduced to change the color from slight yellow to pink or red were scored as + and ++, respectively, after 2-3 days incubation. The darker color of the medium meant the greater fungal growth was in the medium with crude oil.

4.2.6 Morphological and molecular identifications of isolates

Fungal genera were identified based on the taxonomic keys based on morphology (Watanabe 2010). The keys were the color and tint in colony surface and reverse, presence of aerial hyphae, colony surface texture, colony margin, pattern and pigment exudation.

DNA sequencing was used to identify the isolates by species. The internal transcribed spacer (ITS) 1, 5.8S and ITS2 regions of the ribosomal RNA genes were obtained by PCR with primers ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) using sequencing methods described previously (White et al. 1990; Yamaguchi et al. 2014).

DNA was extracted from one piece (0.5 mm^2) of fungal mycelia from a culture incubated at 25℃ for 7 days on a PDA slant using a DEXPAT kit (TaKaRa, Ohtsu, Japan). The reaction mixtures were denatured once at 95℃ for 4 min and amplified for 30 cycles at 94℃ for 1 min, 50℃ for 1 min, and 72℃ for 2 min, with a final extension at 72℃ for 10 min in a PCR Thermal Cycler MP (TaKaRa).

The PCR samples were directly cycle sequenced with BigDye Terminator ver. 1.1 (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 sequencer (Applied Biosystems) using the primers ITS-5, ITS-4, ITS-2 (5′-GCTGCGTTCTTCATCGATGC-3′), and ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) (White et al. 1990). The DNA sequences were aligned using ATGC Ver. 7 - Sequence Assembly Software for Windows (GENETYX Corporation) and the MEGA Ver. 6 tool (Tamura et al. 2013). Sequences were analyzed by the NCBI BLAST tool to classify and identify closely related fungal

sequences. I identified the isolates to the certain species if the BLAST results showed similarity values of 98% or higher.

4.2.7 Effect of temperature on isolates

High-temperature resistance and pathogenic potential of the isolates were examined. The isolates were cultured on PDA slants in duplicate and incubated at temperatures of 15, 20, 30, 35, 37, 39 and 42℃ for 7 days to evaluate growth of the mycelia. After incubation, inoculated mycelia showing no growth were kept at 25℃ for another 7 days to check mycelial survival.

4.2.8 Statistical analysis

The data were analyzed using Excel (Microsoft Office ver. 2013, Redmond, WA, USA). The difference in the means of the total fungi and ODF between Iriomote and Con Dao was each analyzed by Student's *t*-test ($p < 0.05$). The difference in the means of the total fungi and ODF among the 4 sampling sites was analyzed by the analysis of variance (ANOVA, $p < 0.05$).

4.3 Results

4.3.1 Environmental parameters of study sites

The seawater temperature ranged between 12.5 and 13.5℃ on Iriomote and between 25.5 and 26.5℃ on Con Dao (Table 2-1). The average seawater temperature 12.7℃ of the four study sites on Iriomote was 13.3℃ lower than 26.0℃ on Con Dao. The average ambient temperature on Iriomote was 15.9℃, which was 19.0℃ lower than 34.9℃ on Con Dao. The salinity and pH were similar between the sites except for Ir1. This site had the lowest value of pH (7.9) and salinity (2.5%).

4.3.2 Distribution of total fungi

The numbers of the total fungi from the S1 and S2 zones on Iriomote were statistically greater than on Con Dao ($p < 0.05$, Figure 4-1). On the controversy, the numbers of the total fungi from the W zones on Iriomote were less than on Con Dao ($p < 0.05$). Table 4-1 and Figure 4-2 show the number of the total fungi in each study site. At the S1 zones on Iriomote, the number of total fungi ranged from 2.5×10^3 (Ir4) to 7.9×10^3 CFU g^{-1} (Ir1), and at the S2 zones from 0.9×10^3 (Ir2) to 9.2×10^3 CFU g^{-1} (Ir1). At the W zones, the number of total fungi in the seawater ranged from none (Ir2) to 2.2×10^1 CFU 100 ml^{-1} (Ir3). At the S1 zones on Con Dao, the number of total fungi ranged from none (CD1) to 2.4×10^2 CFU g^{-1} (CD2), and at the S2 zones from 1.1×10^1 (CD3) to 1.8×10^3 CFU g^{-1} (CD1), and at the W zones 0.8×10^1 (CD3) to 3.5×10^1 CFU 100 ml^{-1} (CD4). The numbers of total fungi in the four sites from only S2 zone in Iriomote gave the statistical difference ($p < 0.05$).

Table 4-1. The average numbers of total fungi and oil-degrading fungi obtained from S1, S2 and W from the sampling sites on Iriomote and Con Dao islands

Site	Total fungi			ODF			Percentage of ODF		
	S1 (CFU/g)	S2 (CFU/g)	W (CFU/ 100ml)	S1 (CFU/g)	S2 (CFU/g)	W(CFU/ 100ml)	S1	S2	W
Iriomote									
Ir1	$7.9\times10^{3} \pm 1.4\times10^{3}$	$9.2\times10^{3} \pm 3.7\times10^{3}$	$1.2\times10^{1} \pm 3.6$	$7.1\times10^{2} \pm 1.1\times10^{2}$	$9.7\times10^{2} \pm 1.1\times10^{2}$	2.0 ± 1.7	9.0	10.5	16.7
Ir2	$7.3\times10^{3} \pm 2.7\times10^{3}$	$9.1\times10^{2} \pm 1.6\times10^{2}$	0	$2.9\times10^{1} \pm 1.3\times10^{1}$	$1.8\times10^{1} \pm 1.5\times10^{1}$	0	4.0	2.0	0
Ir3	$4.2\times10^{3} \pm 9.4\times10^{2}$	$1.7\times10^{3} \pm 7.7\times10^{2}$	$2.2\times10^{1} \pm 6.4$	$6.1\times10^{2} \pm 3.8\times10^{2}$	$7.3\times10^{2} \pm 6.2\times10^{2}$	4.0 ± 3.0	14.5	42.9	18.2
Ir4	$2.5\times10^{3} \pm 4.2\times10^{2}$	$2.0\times10^{3} \pm 4.3\times10^{2}$	9.0 ± 3.6	$3.0\times10^{2} \pm 5.1\times10^{1}$	$7.7\times10^{2} \pm 3.9\times10^{1}$	1.0 ± 1.0	12.0	38.5	11.1
Con Dao									
CD1	0	$1.8\times10^{3} \pm 1.1\times10^{3}$	$3.0\times10^{1} \pm 5.7$	0	$1.3\times10^{1} \pm 1.1\times10^{1}$	$1.4\times10^{1} \pm 2.8$	0	0.7	46.7
CD2	$2.4\times10^{2} \pm 3.4\times10^{1}$	$1.3\times10^{1} \pm 3.8$	$2.2\times10^{1} \pm 8.5$	2.4 ± 2.2	2.7 ± 2.6	5.0 ± 4.2	1.0	20.8	22.7
CD3	9.1 ± 6.5	$1.1\times10^{1} \pm 1.0\times10^{1}$	8.0 ± 5.7	4.6 ± 0.0	2.7 ± 2.7	2.0 ± 3.0	50.5	24.5	25.0
CD4	$1.5\times10^{1} \pm 9.3$	$2.9\times10^{1} \pm 1.1\times10^{1}$	$3.5\times10^{1} \pm 7.1$	6.6 ± 3.1	$1.0\times10^{1} \pm 7.3$	$1.3\times10^{1} \pm 9.4$	44.0	34.5	37.1

4.3.3 Distribution of oil-degrading fungi

Figure 4-1 also shows the distribution of ODF at the study sites. The numbers of ODF from S1 and S2 were significantly greater ($p < 0.05$) on Iriomote, however those from W zones were less ($p < 0.05$) than on Con Dao; this was the same distribution tendency with the total fungi. At the S1 zones on Iriomote, the number of ODF ranged from 0.3×10^2 (Ir2) to 7.1×10^2 CFU g^{-1} (Ir1), at the S2 zones from 0.2×10^2 CFU g^{-1} (Ir2) to 9.7×10^2 CFU g^{-1} (Ir1), and at the W zones from none (Ir2) to 4.0 CFU 100 ml^{-1} (Ir3). At the S1 zones on Con Dao, the number of ODF ranged from none (CD1) to 6.6 CFU g^{-1} (CD4), at the S2 zones from 2.7 (CD2 and CD3) to 1.3×101 CFU g^{-1} (CD1), and at the W zones from 2.0 (CD3) to 1.4×10^1 CFU 100 ml^{-1} (CD1).

Among the four sites on Iriomote, there were significant differences ($p < 0.05$) only in the samples from S2 zones (Figure 4-2). Ir1 showed the highest number; this was the same tendency with the total fungus number. The percentage of the ODF out of the total fungi was from 0.4% (Ir2) to 14.5% (Ir3) at the S1 zones, 2.0% (Ir2) to 41.7% (Ir3) at the S2 zones, and none (Ir2) to 18.5% (Ir3). In Funaura Bay (Ir3), all percentages of the ODF were the highest, and in Uehara Port (Ir2) the lowest. On Con Dao, there was not clear tendency.

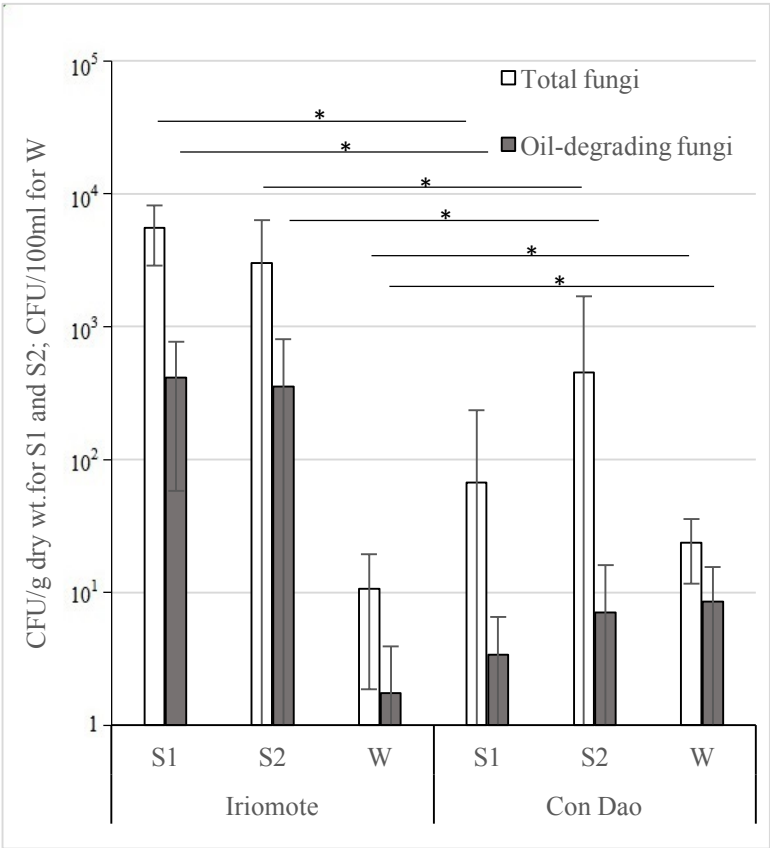

Figure 4-1. The average number of total and oil-degrading fungi in three kinds of samples from Iriomote and Con Dao islands.

Bars represent standard deviations.

* means the statistical difference by t-test ($p < 0.05$).

The kinds of samples, S1, S2 and W, are the supratidal sediment samples, intertidal sediment samples and seawater samples, respectively.

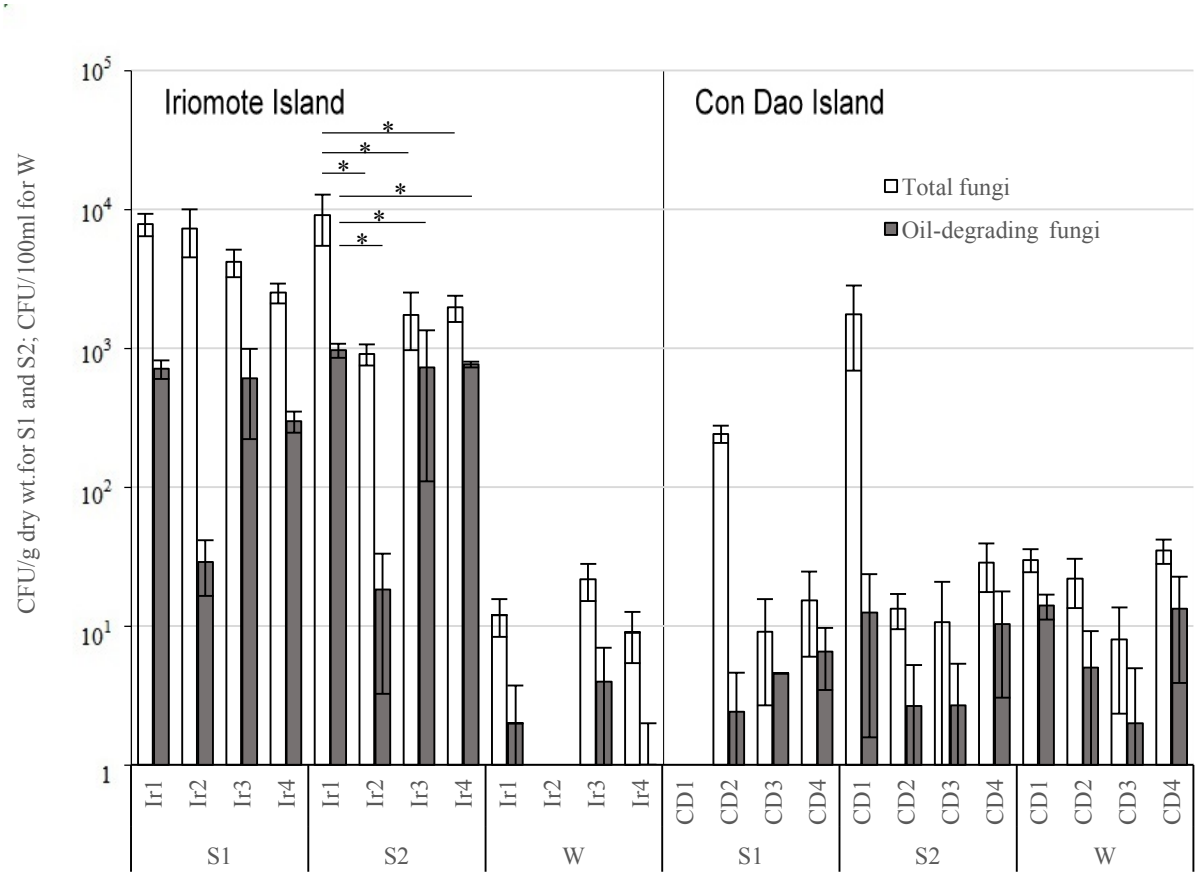

Figure 4-2. The average number of total and oil-degrading fungi in the samples from four sites, in Iriomote and Con Dao islands.

Bars represent standard deviations.

* means the statistical difference by ANOVA ($p < 0.05$).

The study sites, Ir1-4 and CD1-4, are the same as in Table 2-1.

The kinds of samples, S1, S2 and W, are the same as in Figure 4-1.

4.3.4 Identification of isolates

I identified oil-degrading fungi which could grow on the medium with the oil. A total of 23 isolates were identified based on morphology and ITS rRNA gene sequences. Morphology and characteristics of isolated fungi are showed in Appendix 2 and 3. Eleven isolates from Iriomote were identified in 5 genera, *Candida*, *Fusarium*, *Penicillium*, *Scesoporium*, and *Trichoderma*. From Con Dao, 12 isolates were identified in 4 genera, *Aspergillus*, *Cladosporium*, *Penicillium*, and *Tricoderma*. The sequences of the 23 isolates have been submitted to the genetic sequence database at the DNA Data Bank of Japan (DDBJ). The accession numbers of these isolates in DDBJ are shown in Table 4-2.

In terms of the frequency of occurrence of isolates in the study areas, 6 isolates out of 11 from Iriomote were present at more than two sites (Table 4-2). The 9 isolates out of 12 from Con Dao were present at only one site, while 3 isolates were present at more than two sites. This indicates that the species commonness of ODF on Iriomote is higher than on Con Dao.

Table 4-2. List of fungal isolates with characteristics from Iriomote and Con Dao islands

Isolate	Species	Accession No.	Site count founded[1)]	Potential of oil-degradation[2)]	Temperature reduced growth[3)] (℃)	Temperature with no surival[3)] (℃)
Iriomote						
Fu-1.12	*Candida* sp.	LC057428	1	+	>42	>42
Fu-1.11	*Fusarium solani*	LC057427	3	+	37	37
Oh-1.9.2	*Fusarium* sp.	LC057424	3	+	35	37
Oh-1.12	*Fusarium* sp.	LC057429	3	+	37	37
Fu-1.14	*Penicillium citrinum*	LC057420	1	+	37	42
Oh-1.7	*Penicillium sclerotiorum*	LC055789	1	++	35	37
Oh-1.6.1	*Penicillium* sp.	LC057421	3	++	37	37
Fu-1.13	*Scedosporium* sp.	LC057419	1	+	37	42
Oh-2.1	*Trichoderma asperellum*	LC057426	2	+	37	42
Oh-1.10.1	*Trichoderma* sp.	LC057422	2	++	37	37
Oh-1.5	*Trichoderma* sp.	LC057423	1	+	37	37
Con Dao						
BD-3.4/2	*Aspergillus nidulans*	LC057414	1	++	37	42
DT-2.5.2	*Aspergillus nomius*	LC057403	1	++	42	42
DT-3.4	*Aspergillus terreus*	LC057406	2	++	>42	>42
DT-2.5.1/2	*Aspergillus versicolor*	LC057402	2	++	37	>42
DT-2.3	*Cladosporium* sp.	LC057411	1	+	35	39
VB-3.5	*Penicillium citrinum*	LC057408	3	+	37	>42
DT-3.5	*Penicillium coffeae*	LC057404	1	+	35	37
AH-1.1	*Penicillium mallochii*	LC057417	1	+	35	42
DT-2.5.3	*Penicillium* sp.	LC057401	1	++	35	42
AH-3.3/2	*Penicillium* sp.	LC057416	1	+	35	37
AH-3.3/1	*Penicillium* sp.	LC057412	1	+	35	42
DT-3.3	*Trichoderma reesei*	LC057405	1	+	42	>42

[1)] The number of sampling sites, which were the same as in Table 1 and Figure 1, where the isolates were found.

[2)] The potential of mycelial oil-degradation was surveyed based on the color change of the incubated medium with INT; + low, ++ high.

[3)] The growth and survival of the mycelia were examined at the temperatures of 15, 20, 30, 35, 37, 39 and 42℃ for 7 days incubation.

4.3.5 Oil-degradability of isolates

The potentials of oil-degradation of the isolates were rated to 2 levels in Table 4-2. The results showed that total 23 isolates had the ability to grow in the medium with crude oil as the sole carbon source. On Iriomote, *Penicilium* (Oh-1.7 and Oh-1.6.1) and *Trichoderma* (Oh-1.10.1), and on Con Dao, *Aspergillus* (BD-3.4/2, DT-2.5.2, DT-3.4 and DT-2.5.1/2) and *Penicillium* (DT-2.5.3) showed the greatest oil-degradability (++).

4.3.6 Temperature effect on isolates

Table 4-2 also shows the growth and survival of the isolates at different temperatures from 15℃ to 42℃. All isolates were able to grow and survive from 15℃ to 30℃. Among Iriomote isolates, only one isolate of *Candida* sp. Fu-1.12 grew and survived at 42℃. *P. citrinum* Fu-1.14, *Scesoporium* sp. Fu-1.13 and *T. asperellum* Oh-2.1 reduced growth but survived at 37℃. For the isolates from Con Dao, *A. terreus* DT-3.3 continued growth and survived at 42℃. *A. terreus* DT-3.4 and *A. versicolor* DT-2.5.1/2 and *T. reesei* DT-3.3 survived at 42℃. *A. nidullans* BD-3.4/2, *A. nomius* DT-2.5.2, *P. mallochii* AH-1.1, *Penicillum* sp. DT-2.5.3 and AH-3.3/1 showed no survival at 42℃. There was only one strain Fu-1.12 survived at above 42℃ on Iriomote, however 3 isolates on Con Dao had high-temperature resistance.

4.4 Discussion

The numbers of the ODF were from 0.2×10^2 to 9.7×10^2 CFU g^{-1} in the sediment samples from S1 and S2 zones on Iriomote. The number of ODF up to 10^2 CFU/g in the two study areas was similar to the results from the study of Walker and Colwell (1976) in a temperate region, Maryland, USA.

Compared with the results from Con Dao Island, as to both total fungi and ODF, their CFUs of S1 and S2 in Iriomote Island had higher values. During the sample collections, I observed the following characteristics in both samples; the samples from Iriomote were the mixture of sand and mud, whereas samples from Con Dao were fine sand. That meant the organic matter from Iriomote samples was higher than that from Con Dao. That might be the reason why the CFUs of fungi in Iriomote samples were higher than in Con Dao samples.

The site showing the highest CFUs in the zones of S1 and S2 was Urauchi mangrove forest Ir1 on Iriomote. The percentages of the ODF out of total fungi were greatest in Funaura Bay Ir3 on Iriomote. These sampling sites did not have any oil spill accidents during the collection period. These sites are located at the large river mouth and bay with mangrove and seagrass, so the biodiversity are higher than at other sampling sites.

The numbers of isolates of ODF were 11 and 12 from Iriomote and Con Dao, respectively. From Iriomote the dominant species belonged to 5 genera, including *Fusarium*, *Penicillium*, and *Trichoderma*, and from Con Dao 4 genera including *Aspergillus* and *Penicillium*. There was, however, no genus *Aspergillus* isolated from Iriomote and no genus *Fusarium* from Con Dao. The difference in the composition of fungi in the two study areas was depending on water temperature, rather than air temperature (Hyde et al. 1998). Water temperatures near the islands are influenced by the tropical or cold ocean currents which can cross the boundaries of the study areas (Hyde et al. 1998). Iriomote lies within the boundary of the East China Sea and Philippine Sea. This marine area is mainly affected by the Kuroshio Current (Ichikawa and Beardsley 2002). Con Dao lies within the southern South China Sea where complicated currents depending on the season and area are observed (Hu et al. 2000). The main currents in this area include the seasonal circulation, which is affected by the monsoon winds, the South China Sea warm current, and the Kuroshio Branch Current. Although there is water exchange between the South China Sea and the East China Sea, through the Taiwan Strait and between the South China Sea and the Kuroshio through the Luzon Strait, these marine areas are mainly separated by the Kuroshio Current. This contributes to the differences in biogeography of fungi between Iriomote and Con Dao.

In comparison with other studies in subtropical areas, the number of isolates 11 on Iriomote was lower than 16 isolates in 11 genera in the Gulf of Mexico (Al-Nasrawi 2012) and 20 isolates in 9 genera in the Gulf of Arabia (Hashem 2007). The number of isolates 12 on Con Dao was higher than 10 isolates in 4 genera in Oman (Elshafie et al. 2007) and 10 isolates in 6 genera in India (Saravanan and Sivakumar 2013), but lower than 21 isolates in 10 genera in Indonesia (Chaillan et al. 2004). This difference is depending upon variable elements such as environmental parameters and contamination conditions of the study areas. The numbers of ODF isolates in this study were lower than the previous studies in the contaminated areas such as Gulf of Mexico, Gulf of Arabia and Indonesia, because quantity and composition of oil-degrader community in the environment increase rapidly after exposed to hydrocarbon (Chikere et al. 2011).

Oil-degradability and high-temperature resistance are two of the important factors for bioremediation. The numbers of isolates, which had high oil-degradability, were 2 in *Penicillium*and one in *Trichoderma* from Iriomote, while 4 in *Aspergilllus* and one *Penicillum* from Con Dao. Among these isolates, 3 isolates on Iriomote showed no survival at 37℃, and 5 isolates on Con Dao could survive at more than 37℃. This meant that the oil-degradable isolates on Con Dao were higher-temperature resistant than those on Iriomote. This result also supported the previous study, suggesting that *Penicillium* and *Aspergillus* are the most common genera found in marine ecosystem (Hyde et al. 1998) and these genera are more active in ability to degrade crude oil than others (Elshafie et al. 2007; Hashem 2007; Al-Nasrawi 2012; Saravanan and Sivakumar 2013).

Temperature affects not only chemistry, including the solubility of hydrocarbons (Foght et al. 1996) but also the physiology and diversity of microbial flora (Das and Chandran 2011). Thus, it affects the growth and activity of microorganisms and rate of biodegradation by microorganisms. The rate of hydrocarbon degradation depends on temperature and it is optimal at 30–40℃ in soil environments, 20–30℃ in some freshwater environments and 15–20℃ in marine environments (Bartha and Bossert 1984; Cooney 1984). In this study, the majority of isolates could grow at up to 35℃. The isolates *Candida* sp. (Fu-1.12) on Iriomote and *A. nomius* (DT-2.5.2), *T. reesei* (DT-3.3) and *A. terreus* (DT-3.4) on Con Dao could grow at higher temperatures. These isolates showed good attributes for application in oil bioremediation. However, they may have pathogenic potential to humans and other warm-blooded animals.

Chapter 5

General conclusions

The indigenous oil-degrading microorganisms were screened from the two islands, Iriomote, Japan and Con Dao, Vietnam. These islands are considered predicted contamination sites from oil spillage on a nearby busy transportation route. The numbers of the total bacteria and ODB showed the same distribution tendencies in comparison between the two study areas. There were no differences between Iriomote and Con Dao in the counts of the total bacteria and ODB in sediment samples, while those in seawater samples were statistically lower for Iriomote than for Con Dao. The percentages of ODB relative to the total bacterial counts obtained on Iriomote and Con Dao were higher than the general rates of ODB in heterotrophic bacterial communities. It indicate suitable application conditions for autochthonous bioaugmentation for the treatment of oil contamination in these areas. In addition, the relatively large number of ODB might also show chronic oil-contamination in the study islands from oil seepage of human activities or busy oil transportation.

The total 45 ODB isolates, 25 from Iriomote and 20 from Con Dao, were collected. They belonged to 18 genera in which 11 from each island. The isolate from the genus *Granulicatella* and the two unidentified isolates, a Gram-negative coccus and a Gram-positive bacillus, were isolated for the first time as ODB in this study. Among detected genera, the dominant genera were *Achromobacter*, *Pseudomonas*, and *Ochrobactrum* on Iriomote and *Pseudomonas* and *Microbacterium* on Con Dao. The common genera, *Achromobacter*, *Ochrobactrum*, and *Pseudomonas*, were present in both study areas. Some bacteria that are close relatives of the isolates from this study have been listed as pathogens, including *B. cepacia, G. adiacens*, and *K. pneumoniae*. They were not used for

the further experiments because of the safety.

The degradation ability of the isolates was studied for crude oil, two kinds of *n*-alkanes, and two kinds of polycyclic aromatic hydrocarbons (PAHs). The numbers of the isolates that degraded more than 50% of the crude oil and *n*-alkanes and 25% of the PAHs present in the media were higher for Iriomote (3, 11, and 2, respectively) than for Con Dao (2, 4, and 1, respectively). It is noteworthy that the isolates of *Acinetobacter* sp., *Pseudomonas* sp., *Pseudomonas putida* from Iriomote and *Pseudomonas mendocina, Pseudomonas putida, Microbacterium* sp from Con Dao showed the highest degradability for crude oil, *n*-alkanes, and PAHs, respectively, and survived at 42℃.

For the fungi, the counts of total fungi and ODF in sediment samples from supratidal and intertidal zones on Iriomote were statistically higher than on Con Dao. Water samples from seawater, however, gave a reverse result. The numbers of isolates of ODF were 11 and 12 from Iriomote and Con Dao, respectively. From Iriomote the dominant species belonged to 5 genera, including *Fusarium*, *Penicillium*, and *Trichoderma*, and from Con Dao 4 genera including *Aspergillus* and *Penicillium*. There was, however, no genus *Aspergillus* isolated from Iriomote and no genus *Fusarium* from Con Dao.

The numbers of isolates, which had high oil-degradability, were 2 in *Penicillium* and one in *Trichoderma* from Iriomote, while 4 in *Aspergilllus* and one *Penicillum* from Con Dao. *Penicillium* and *Aspergillus* are the most common genera found in marine ecosystem and more active in oil degradability than others. The majority of isolates could grow at up to 35℃. The oil-degradable isolates on Con Dao were higher-temperature resistant than those on Iriomote. The isolates *Candida* sp. (Fu-1.12) on Iriomote and *A. nomius* (DT-

2.5.2), *T. reesei* (DT-3.3) and *A. terreus* (DT-3.4) on Con Dao could grow at higher temperatures. These isolates showed good attributes for application in oil bioremediation. However, they may have pathogenic potential to humans and other warm-blooded animals.

These results suggest that the diversities on both Iriomote and Con Dao were higher comparing previous reports. The obtained notable isolates, six bacteria and five fungi, showed the potential for autochthonous bioaugmentation application for the treatment of oil contamination in these areas. Further examinations are needed to clarity the abilities for degradation of other crude oil components by these selected isolates. The combined treatment techniques with bacterial and fungal isolates are necessary.

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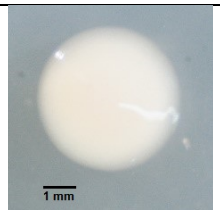
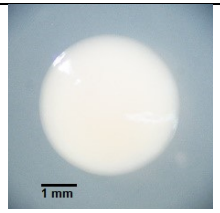
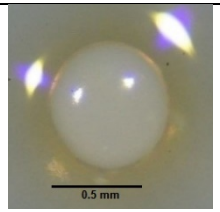
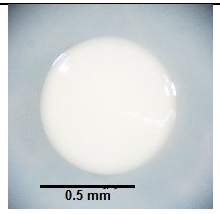
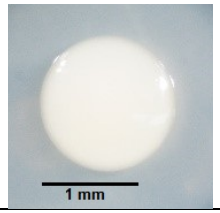
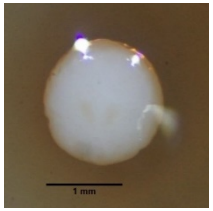
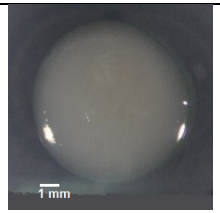
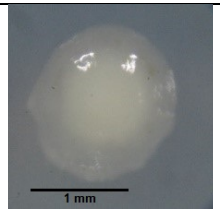
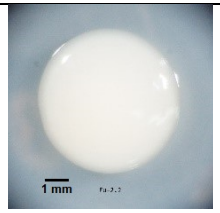
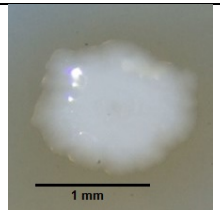
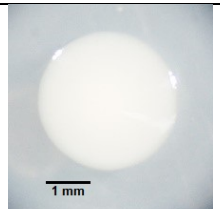
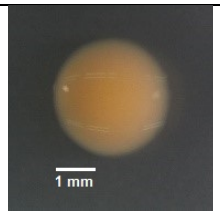
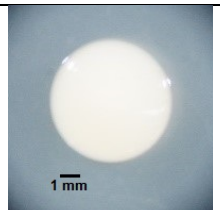
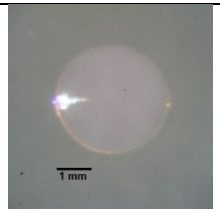
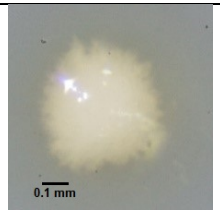
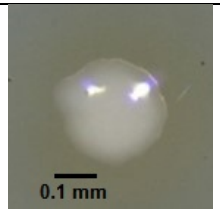
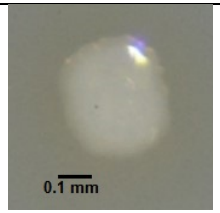
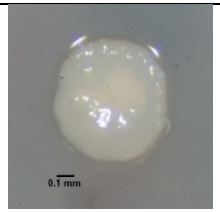
APPENDICES

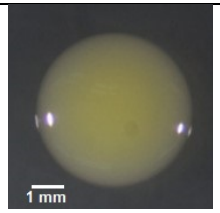
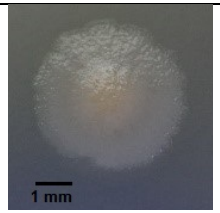
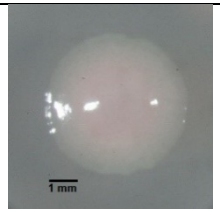
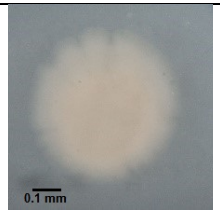
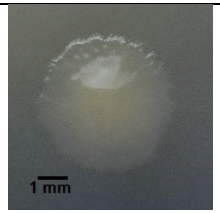
Appendix 1 Characteristics of oil-degrading bacteria

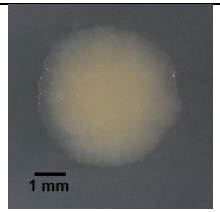
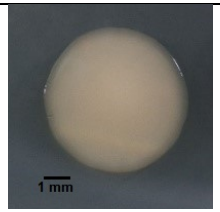
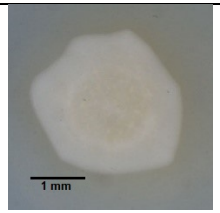
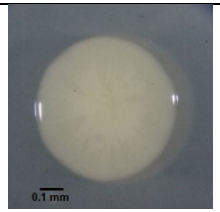
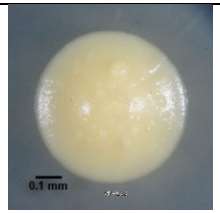
No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
1	Ue-1.2	- Form: round - Elevation: convex - Margin: circular - Surface: dry, smooth - Opacity: opaque - Color: cream-white - Size: 0.3-0.5 cm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	+	*Achromobacter*
2	Ue-2.2	- Form: round - Elevation: raised - Margin: circular - Surface: dry, smooth - Opacity: opaque - Color: cream-white - Size: 0.3-0.5 cm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	+	*Achromobacter*
3	Oh-1.13	- Form: round - Elevation: convex - Margin: circular - Surface: dry, smooth - Opacity: opaque - Color: cream-white - Size: 0.5-1.0 mm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	+	*Achromobacter*
4	Ir-1	- Form: round - Elevation: convex - Margin: entire - Surface: dry, shiny, smooth - Opacity: opaque - Color: cream-white - Size: 0.5 - 1 mm 	- Shape: long bacilli - Arrangement of the cells: separate	–	+	+	*Achromobacter*

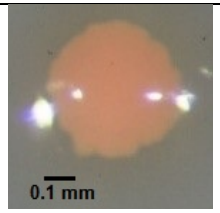
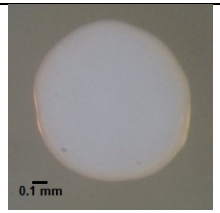
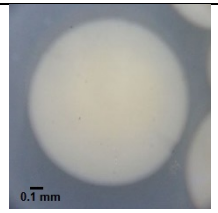
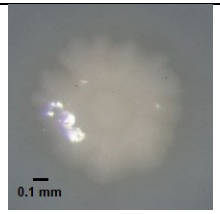
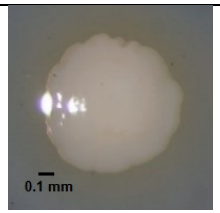
No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
5	Ir-12.2	- Form: round - Elevation: raised - Margin: entire - Surface: dry, smooth - Opacity: opaque - Color: cream-white - Size: 1-1.5 mm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Achromobacter*
6	Oh-2.10.0	- Form: round - Elevation: convex - Margin: circular, occasionally slightly wavy - Surface: smooth, shiny, dry, occasionally rough or unstable cause of overgrowth - Opacity: opaque - Color: cream-white - Size: 1-1.5mm 	- Shape: cocci - Arrangement of the cells: long strings	–	+	–	*Acinetobacter*
7	Ue2-1.1	- Form: round - Elevation: raised - Margin: entire - Surface: smooth, shiny - Opacity: opaque - Color: cream - Size: 0.5-1cm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	–	*Acinetobacter*
8	Ur-2.10.2	- Form: irregular - Elevation: umbonate with a big, high knobby protuberance - Margin: wavy - Surface: moist, viscous, wrinkled - Opacity: slightly transparent - Color: cream - Size: 1.5-2.0mm 	- Shape: cocci - Arrangement of the cells: separate	–	–	–	*Acinetobacter*

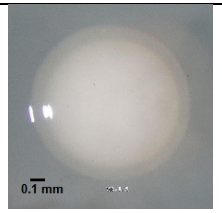
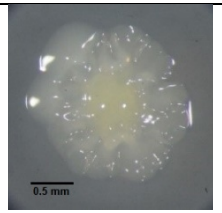
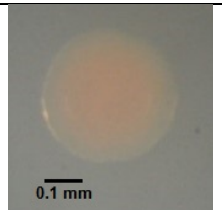
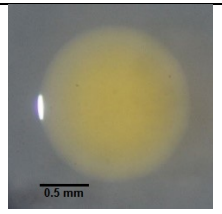
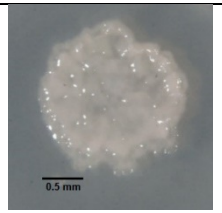
No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
9	Fu-2.2	- Form: round - Elevation: raised - Margin: entire - Surface: smooth, shiny - Opacity: opaque - Color: cream-white - Size: 0.5-1cm 	- Shape: short bacilli - Arrangement of the cells: separate	–	–	+	*Alcaligenes*
10	Oh-1.14	- Form: irregular - Elevation: raised - Margin: wavy - Surface: shiny, dry, wrinkled - Opacity: opaque - Color: cream-white - Size: 1-1.5mm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	+	*Burkholderia*
11	Ir-5	- Form: round - Elevation: raised - Margin: entire - Surface: dry, shiny, smooth - Opacity: opaque - Color: cream-white - Size: 0.3-0.5cm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	+	*Burkholderia*
12	Fu2-3.2	- Form: round - Elevation: raised - Margin: regular/entire - Surface: smooth, shiny - Opacity: opaque - Color: light pink-yellow - Size: 0.3-0.5cm 	- Shape: short bacilli - Arrangement of the cells: separate	+	–	–	*Dietzia*
13	Ur-2.5.1	- Form: round - Elevation: raised - Margin: entire - Surface: smooth, shiny - Opacity: opaque - Color: cream-white - Size: 0.5-1cm 	- Shape: cocci - Arrangement of the cells: long strings	+	–	+	*Gemella*

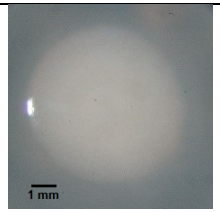
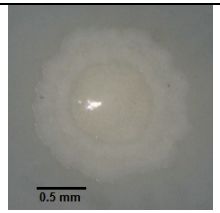
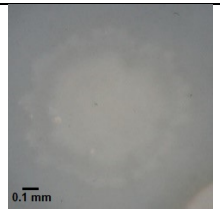
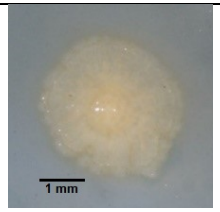
No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
14	Oh-1.14.2	- Form: round - Elevation: flat - Margin: circular - Surface: smooth, dry, shiny - Opacity: slightly transparent - Color: cream-white - Size: 0.3-0.5cm 	- Shape: cocci - Arrangement of the cells: long strings	–	+	+	Gram −ve cocci
15	Fu-3.1.1	- Form: irregular - Elevation: raised - Margin: undulate/wavy - Surface: veined, shiny, dry - Opacity: cloudy - Color: yellow-cream - Size: 0.5 mm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	+	*Ochrobactrum*
16	Ue-1.1	- Form: round - Elevation: raised - Margin: entire - Surface: smooth, shiny, dry - Opacity: cloudy - Color: cream-white - Size: 0.3 – 0.5 mm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	+	*Ochrobactrum*
17	Ir-2	- Form: round - Elevation: raised - Margin: entire - Surface: smooth, shiny, dry - Opacity: translucent to cloudy - Color: cream-white - Size: 0.3 – 0.5 mm 	- Shape: short bacilli - Arrangement of the cells: separate	–	+	+	*Ochrobactrum*
18	Oh-1.15.2	- Form: round - Elevation: slightly crateriform - Margin: circular - Surface: shiny, dry, veined to smooth - Opacity: opaque - Color: cream-white - Size: 0.5-1.0mm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Ochrobactrum*

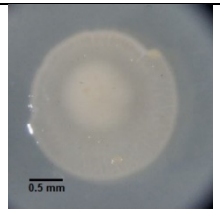
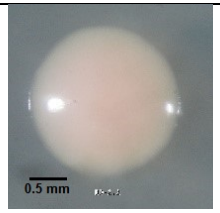
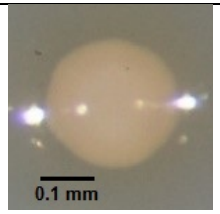
No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
19	Fu2-1.4	- Form: round - Elevation: raised - Margin: regular/entire - Surface: smooth, shiny, dry - Opacity: opaque - Color: light yellow - Size: 0.5cm 		+	–	–	*Paenibacillus*
20	Oh2-1.3	- Form: round - Elevation: flat - Margin: wavy - Surface: rough, shiny - Opacity: slight opaque - Color: light reddish in center - Size: 0.5-1cm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Pseudomonas*
21	Oh-1.0.2	- Form: round - Elevation: raised - Margin: regular/entire - Surface: smooth, shiny, dry - Opacity: opaque - Color: light pink - Size: 0.5cm 	- Shape: long bacilli - Arrangement of the cells: separate	–	+	–	*Pseudomonas*
22	Oh-1.15	- Form: irregular - Elevation: flat - Margin: wavy - Surface: slightly rough, shiny, wet - Opacity: opaque - Color: light yellow - Size: 0.5-1 mm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	–	*Pseudomonas*
23	Ue2-1.2	- Form: round - Elevation: flat - Margin: regular, and wavy after 7 days incubation - Surface: slightly rough, shiny, wet - Opacity: slightly transparent - Color: light yellow - Size: 0.5-1cm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Pseudomonas*

No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
24	Ue2-2.5.2	- Form: round - Elevation: raised - Margin: slightly wavy - Surface: smooth, shiny - Opacity: slightly transparent in outer and opaque in center - Color: light pink - Size: 0.5-1cm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Pseudomonas*
25	Fu2-2.1	- Form: round - Elevation: flat - Margin: regular/entire - Surface: smooth, shiny - Opacity: opaque - Color: light pink - Size: 0.5-1.0 cm 		–	+	+	*Vibrio*
26	BD-2.3	- Form: irregular - Elevation: flat - Margin: irregular - Surface: smooth - Opacity: opaque - Color: cream-white - Size: 2.5mm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Achromobacter*
27	VB-2.1.2	- Form: round - Elevation: raised - Margin: circular - Surface: smooth, dry - Opacity: cloudy - Color: cream-white - Size: 0.5-1mm 	- Shape: cocci - Arrangement of the cells: separate	+	–	–	*Brachybacterium*
28	VB-1.8	- Form: round - Elevation: raised - Margin: circular - Surface: smooth, dry, shiny - Opacity: cloudy - Color: cream - Size: 0.5-1 mm 	- Shape: cocci - Arrangement of the cells: separate	+	–	–	*Gemella*

No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
29	DT-2.15.1	- Form: round - Elevation: raised - Margin: circular - Surface: smooth - Opacity: cloudy - Color: yellow-pink - Size: 0.5mm 	- Shape: cocci - Arrangement of the cells: separate	+	–	–	*Gemella*
30	VB-1.11	- Form: round - Elevation: flat - Margin: circular - Surface: smooth, dry, shiny - Opacity: cloudy - Color: cream-white - Size: 1mm 	- Shape: short bacilli - Arrangement of the cells: separate	+	–	–	Gram +ve baccillus
31	DT-2.3.1	- Form: round - Elevation: flat - Margin: circular - Surface: smooth, wet - Opacity: opaque - Color: cream-white - Size: 1-1.5mm 	- Shape: cocci - Arrangement of the cells: separate	+	–	–	*Granulicatella*
32	AH-1.15.1	- Form: irregular - Elevation: raised with a flat top - Margin: lobate/wavy - Surface: smooth, dry, shiny - Opacity: opaque - Color: cream-white - Size: 1 mm 	- Shape: bacilli - Arrangement of the cells: separate	–	–	+	*Halomonas*
33	VB-1.5	- Form: round - Elevation: convex - Margin: slightly wavy - Surface: smooth, shiny - Opacity: opaque - Color: cream-white - Size: 1mm 	- Shape: short bacilli - Arrangement of the cells: separate	–	–	–	*Klebsiella*

No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
34	VB-1.1	- Form: irregular - Elevation: convex - Margin: wavy - Surface: dry, shiny, smooth - Opacity: opaque - Color: cream-white - Size: 1mm 	- Shape: short bacilli - Arrangement of the cells: separate	–	–	–	*Klebsiella*
35	BD-2.4.2	- Form: irregular - Elevation: umbonate with a small knobby protuberance - Margin: irregular/lobate - Surface: rough - Opacity: slightly transparent - Color: cream-yellow - Size: 1.5-2mm 	- Shape: short bacilli - Arrangement of the cells: separate	+	–	–	*Microbacterium*
36	VB-1.14.2	- Form: round - Elevation: flat - Margin: circular - Surface: smooth, dry, shiny - Opacity: slightly transparent - Color: pink-yellow - Size: 0.3-0.5mm 	- Shape: short bacilli - Arrangement of the cells: separate	+	–	–	*Microbacterium*
37	DT-2.3.2	- Form: round - Elevation: flat - Margin: circular - Surface: smooth, wet - Opacity: cloudy - Color: cream-yellow - Size: 1-1.5mm 	- Shape: short bacilli - Arrangement of the cells: separate	+	+	–	*Microbacterium*
38	BD-2.5.3	- Form: irregular - Elevation: raised - Margin: lobate/wavy - Surface: rough, dry, shiny - Opacity: cloudy - Color: cream-white - Size: 1-2mm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Ochrobactrum*

No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
39	BD-1.5.1	- Form: round - Elevation: convex - Margin: slightly wavy - Surface: smooth - Opacity: slightly transparent with opaque outer - Color: cream-yellow - Size: 0.5-1cm 	- Shape: long bacilli - Arrangement of the cells: separate	–	+	+	*Pseudomonas*
40	AH-2.5.1	- Form: round - Elevation: umbonate with a smooth knobby protuberance - Margin: wavy - Surface: rough, dry - Opacity: slightly transparent - Color: cream-white - Size: 1-1.5mm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Pseudomonas*
41	BD-2.5.6	- Form: round - Elevation: umbonate with a big knobby protuberance - Margin: wavy - Surface: veined, - Opacity: slightly transparent - Color: cream-white - Size: 1mm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Pseudomonas*
42	AH-2.1	- Form: round - Elevation: umbonate with a small knobby protuberance - Margin: slightly wavy - Surface: shiny, veined - Opacity: slightly transparent - Color: yellow - Size: ~3mm 	- Shape: bacilli - Arrangement of the cells: separate	–	+	+	*Pseudomonas*

No.	Isolate	Colony morphology	Cell morphology	Gram stain	Movement	Oxidase test	Genus
43	BD-2.2.1	- Form: round - Elevation: umbonate with a smooth knobby protuberance - Margin: circular - Surface: smooth, wet - Opacity: cloudy - Color: cream-white - Size: 2mm 	- Shape: bacilli - Arrangement of the cells: separate	–	–	+	*Pseudomonas*
44	BD-2.2	- Form: round - Elevation: raised - Margin: circular - Surface: smooth - Opacity: cloudy - Color: cream-yellow - Size: 2mm 	- Shape: short bacilli - Arrangement of the cells: separate	–	–	+	*Pseudomonas*
45	DT-1.15.2	- Form: round - Elevation: umbonate with a knobby protuberance - Margin: circular - Surface: smooth, dry - Opacity: cloudy - Color: cream-yellow - Size: 0.2-0.5mm 	- Shape: bacilli - Arrangement of the cells: separate	–	–	–	*Yersinia*

Appendix 2 Characteristics of oil-degrading fungi

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
1	Fu-1.12	- Color and tint in colony surface: white - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, velvety, powdery - Colony margin: smooth, restricted - Pattern: indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: no	- Size: 1.8-1.9 cm after 7 days incubation - Color*: N9 - Shape: hyphae with septate	- Color and tint in colony surface: white - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, velvety, powdery - Colony margin: smooth, restricted - Pattern: indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: no	- Size: 1.8 cm after 7 days incubation - Color: N9 - Shape: hyphae with septate	*Candida*
2	Fu-1.11	- Color and tint in colony surface: pink - Color and tint in colony reverse: red-purple - Aerial hyphae: yes - Colony surface texture: cottony, elevation, sparse - Colony margin: irregular, spreading - Pattern: flowery - Pigment exuded: change medium color to red	- Size: 3.6-3.8 cm after 7 days incubation - Color: 2.5R 7/3 - Shape: hyphae with septate	- Color and tint in colony surface: pink - Color and tint in colony reverse: red-purple - Aerial hyphae: yes - Colony surface texture: cottony, elevation, sparse - Colony margin: irregular, spreading - Pattern: flowery - Pigment exuded: change medium color to red	- Size: 4.0-4.2 cm after 7 days incubation - Color: 5RP 8/3 - Shape: hyphae with septate	*Fusarium*
3	Oh-1.9.2	- Color and tint in colony surface: white - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, elevation, sparse - Colony margin: smooth, spreading - Pattern: flowery - Pigment exuded: some yellow droplets on surface but unchanged medium color	- Size: 7.0 cm after 7 days incubation - Color: N9 - Shape: hyphae with septate	- Color and tint in colony surface: red-purple - Color and tint in colony reverse: red-purple - Aerial hyphae: yes - Colony surface texture: cottony, elevation, sparse - Colony margin: smooth, spreading - Pattern: flowery - Pigment exuded: some yellow droplets on surface but unchanged medium color	- Size: 7.5 cm after 7 days incubation - Color: 7.5RP 7/4 - Shape: hyphae with septate	*Fusarium*

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
4	Oh-1.12	- Color and tint in colony surface: white and purple - Color and tint in colony reverse: red-purple - Aerial hyphae: yes - Colony surface texture: cottony, elevation, thickness - Colony margin: irregular, spreading - Pattern: zonate to 2 concentric circles, purple in outer and white in inner - Pigment exuded: change medium color to red	- Size: 3.2-3.5 cm after 7 days incubation - Color: 7.5R 5/3 - Shape: hyphae with septate	- Color and tint in colony surface: white and purple - Color and tint in colony reverse: yellow-red - Aerial hyphae: yes - Colony surface texture: cottony, elevation, thickness - Colony margin: irregular, spreading - Pattern: zonate to 2 concentric circles, light purple in outer and white in inner - Pigment exuded: many purple droplets on colony surface and change medium color to yellow	- Size: 4.3-4.5 cm after 7 days incubation - Color: 7.5R 7/6 - Shape: hyphae with septate	*Fusarium*
5	Fu-1.14	- Color and tint in colony surface: light grey-green in outer and light pink in inner area - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, light grey-green in outer and light pink in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: many colorless droplets on inner area but unchanged medium color	- Size: 2.6-2.7 cm after 7 days incubation - Color: 5GY 8/1 for outer and 10YR 9/1 for inner area - Shape: hyphae with septate	- Color and tint in colony surface: white in outer and grey-green in inner area - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, white in outer and grey-green in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: many colorless droplets on inner area but unchanged medium color	- Size: 2.6-2.7 cm after 7 days incubation - Color: 5GY 8/1 for outer and 10YR 9/1 for inner area - Shape: hyphae with septate	*Penicillium*

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
6	Oh-1.7	- Color and tint in colony surface: light pink - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: fan-shaped colony with many furrow-drains from center - Pigment exuded: some yellow droplets on center but unchanged medium color	- Size: 3.0-3.2 cm after 7 days incubation - Color: 10YR 9/2 - Shape: hyphae with septate	- Color and tint in colony surface: small white circle in outer and grey-green in inner area - Color and tint in colony reverse: small yellow circle in outer and orange in inner area - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, white in outer and grey-green in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: some yellow droplets on inner area but unchanged medium color	- Size: 2.5 cm after 7 days incubation - Color: N9 for outer and 5GY 7/1 for inner area - Shape: hyphae with septate	*Penicillium*
7	Oh-1.6.1	- Color and tint in colony surface: white - Color and tint in colony reverse: light yellow - Aerial hyphae: yes - Colony surface texture: cottony, velvety, powdery - Colony margin: smooth, spreading - Pattern: indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: many yellow droplets on surface but unchanged medium color	- Size: 6.0-6.3 cm after 7 days incubation - Color: N9 - Shape: hyphae with septate	- Color and tint in colony surface: white and light grey-green - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, velvety, powdery - Colony margin: smooth, spreading - Pattern: zonate to concentric circles with white and grey-green, and indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: many colorless droplets on surface but unchanged medium color	- Size: 4.5-4.6 cm after 7 days incubation - Color: N9 and 10Y 7/2 - Shape: hyphae with septate	*Penicillium*

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
8	Fu-1.13	- Color and tint in colony surface: white - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, velvety, powdery - Colony margin: smooth, restricted - Pattern: no - Pigment exuded: no	- Size: 2.6 cm after 7 days incubation - Color: N9 - Shape: hyphae with septate	- Color and tint in colony surface: white - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, velvety, powdery - Colony margin: smooth, restricted - Pattern: no - Pigment exuded: no	- Size: 2.0 cm after 7 days incubation - Color: N9 - Shape: hyphae with septate	*Scedosporium*
9	Oh-2.1	- Color and tint in colony surface: light yellow - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, elevation, sparse - Colony margin: smooth, spreading - Pattern: flowery - Pigment exuded: no	- Size: 7.5 cm after 3 days incubation - Color: 2.5GY 7/4 - Shape: hyphae with septate	- Color and tint in colony surface: green - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, elevation, sparse - Colony margin: smooth, spreading - Pattern: flowery - Pigment exuded: no	- Size: 7.0 cm after 3 days incubation - Color: 5G 5/3 - Shape: hyphae with septate	*Trichoderma*
10	Oh-1.10.1	- Color and tint in colony surface: white - Color and tint in colony reverse: orange - Aerial hyphae: yes - Colony surface texture: cottony, powdery, homogenous - Colony margin: smooth, spreading - Pattern: no - Pigment exuded: many colorless droplets on surface and medium turn to orange	- Size: 7.5 cm after 3 days incubation - Color: N9 - Shape: hyphae with septate	- Color and tint in colony surface: white with patchy green - Color and tint in colony reverse: light yellow - Aerial hyphae: yes - Colony surface texture: cottony, powdery, heterogeneous - Colony margin: smooth, spreading - Pattern: zonate with white inner and patchy green outer - Pigment exuded: many colorless droplets on surface but unchanged medium color	- Size: 8.0 cm after 3 days incubation - Color: N9 and 10GY 4/4 - Shape: hyphae with septate	*Trichoderma*

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
11	Oh-1.5	- Color and tint in colony surface: white - Color and tint in colony reverse: orange - Aerial hyphae: yes - Colony surface texture: cottony, powdery, homogenous - Colony margin: smooth, spreading - Pattern: no - Pigment exuded: many colorless droplets on surface and medium turn to orange	- Size: 8.0 cm after 3 days incubation - Color: N9 - Shape: hyphae with septate	- Color and tint in colony surface: white with patchy green - Color and tint in colony reverse: light yellow - Aerial hyphae: yes - Colony surface texture: cottony, powdery, heterogeneous - Colony margin: smooth, spreading - Pattern: zonate with white inner and patchy green outer - Pigment exuded: many colorless droplets on surface but unchanged medium color	- Size: 8.0 cm after 3 days incubation - Color: N9 and 10GY 4/4 - Shape: hyphae with septate	*Trichoderma*
12	BD-3.4/2	- Color and tint in colony surface: white in outer and brown in inner area - Color and tint in colony reverse: orange - Aerial hyphae: yes - Colony surface texture: cottony, velvety - Colony margin: smooth, wavy - Pattern: zonate to concentric circles, white in outer and brown in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: many brown droplets on surface and unchanged medium color	- Size: 3.0-3.3 cm after 7 days incubation - Color: N9 in outer and 7YR 9/1 in inner - Shape: hyphae with septate	- Color and tint in colony surface: white in outer and dark green in inner area - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, restricted - Pattern: zonate to 2 concentric circles, white in outer and dark green in inner, and indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: a big brown droplets on inner area but unchanged medium color	- Size: 2.2 cm after 7 days incubation - Color: N9 for outer and 10G 2/1 for inner area - Shape: hyphae with septate	*Aspergillus*

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
13	DT-2.5.2	- Color and tint in colony surface: light yellow - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, powdery, elevation - Colony margin: smooth, wavy, spreading - Pattern: flowery - Pigment exuded: no	- Size: 7.0 cm after 7 days incubation - Color: 10YR 9/2 - Shape: hyphae with septate	- Color and tint in colony surface: light yellow - Color and tint in colony reverse: light yellow - Aerial hyphae: yes - Colony surface texture: cottony, powdery, elevation - Colony margin: smooth, wavy, spreading - Pattern: flowery - Pigment exuded: no	- Size: 5.7-6.0 cm after 3 days incubation - Color: 7.5Y 9/3 - Shape: hyphae with septate	*Aspergillus*
14	DT-3.4	- Color and tint in colony surface: light yellow - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, velvety, powdery, homogeneous - Colony margin: smooth, spreading - Pattern: flowery - Pigment exuded: many yellow droplets on surface and change medium color to light yellow	- Size: 4.8-5.0 cm after 7 days incubation - Color: 2.5Y 9/2 - Shape: hyphae with septate	- Color and tint in colony surface: white in outer and light brown in inner area - Color and tint in colony reverse: orange - Aerial hyphae: yes - Colony surface texture: cottony, velvety, powdery, homogeneous - Colony margin: smooth, spreading - Pattern: zonate to concentric circles, white in outer and light brown in inner - Pigment exuded: many yellow droplets on surface and change medium color to light yellow	- Size: 3.2-3.3 cm after 7 days incubation - Color: N9 for outer and 10YR 8/4 for inner area - Shape: hyphae with septate	*Aspergillus*

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
15	DT-2.5.1/2	- Color and tint in colony surface: white in outer and light brown in inner area - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to concentric circles, white in outer and light brown in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: many brown droplets on surface and unchanged medium color	- Size: 2.6-2.7 cm after 7 days incubation - Color: N9 in outer and 10YR 7/3 in inner - Shape: hyphae with septate	- Color and tint in colony surface: white in outer and light grey-green in inner area - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, white in outer and light grey-green in inner, and indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: many brown droplets on inner area but unchanged medium color	- Size: 2.2-2.3 cm after 7 days incubation - Color: N9 for outer and 10GY 4/1 for inner area - Shape: hyphae with septate	*Aspergillus*
16	DT-2.3	- Color and tint in colony surface: white and green - Color and tint in colony reverse: dark-green - Aerial hyphae: yes - Colony surface texture: cottony, shrunken - Colony margin: smooth, restricted - Pattern: zonate to 2 concentric circles, white in outer and green in inner, and indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: no	- Size: 2.6 cm after 7 days incubation - Color: N9 for outer and 10Y 6/1 for inner area - Shape: hyphae with septate	- Color and tint in colony surface: white and dark green - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken - Colony margin: smooth, restricted - Pattern: zonate to 2 concentric circles, white in outer and dark green in inner, and indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: no	- Size: 1.8-2.0 cm after 7 days incubation - Color: N9 for outer and 2.5GY 5/2 for inner area - Shape: hyphae with septate	*Cladosporium*

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
17	VB-3.5	- Color and tint in colony surface: light grey-green in outer and light pink in inner area - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, light grey-green in outer and light pink in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: many yellow droplets on inner area and unchange medium color	- Size: 2.5-2.7 cm after 7 days incubation - Color: 10GY 8/1 for outer and 10YR 9/1 for inner area - Shape: hyphae with septate	- Color and tint in colony surface: white in outer and grey-green in inner area - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, white in outer and grey-green in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: many colorless droplets on inner area but unchanged medium color	- Size: 2.0-2.1 cm after 7 days incubation - Color: N9 for outer and 10GY 5/1 for inner area - Shape: hyphae with septate	*Penicillium*
18	DT-3.5	- Color and tint in colony surface: white in outer and grey-green in inner area - Color and tint in colony reverse: light yellow - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, white in outer and grey-green in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: many colorless droplets on inner area but unchanged medium color	- Size: 2.5-2.7 cm after 7 days incubation - Color: N9 for outer and 5GY 5/1 for inner area - Shape: hyphae with septate	- Color and tint in colony surface: white in outer and grey-green in inner area - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, white in outer and grey-green in inner, and fan-shaped colony with many furrow-drains from center - Pigment exuded: many colorless droplets on inner area but unchanged medium color	- Size: 2.0 cm after 7 days incubation - Color: 5GY 8/1 for outer and 10YR 9/1 for inner area - Shape: hyphae with septate	*Penicillium*

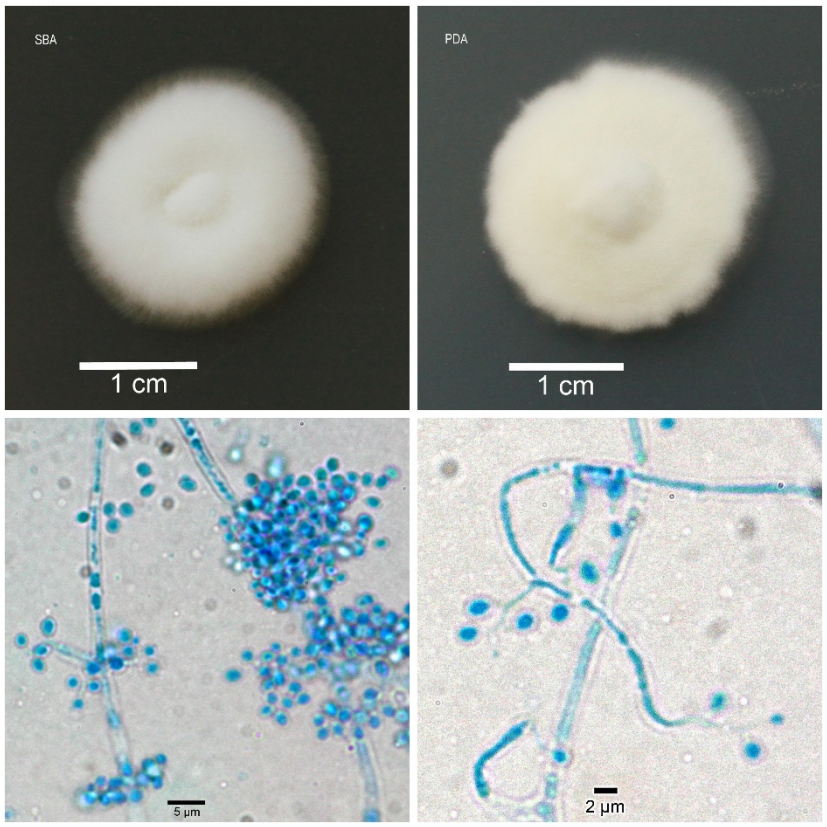
Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
19	AH-1.1	- Color and tint in colony surface: white and light grey-green - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, homogeneous - Colony margin: smooth, spreading - Pattern: flowery - Pigment exuded: many colorless droplets on inner area and unchanged medium color	- Size: 3.8-4.0 cm after 7 days incubation - Color: N9 in outer and 10GY 6/1 in inner - Shape: hyphae with septate	- Color and tint in colony surface: white and light grey-green - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, homogeneous - Colony margin: smooth, spreading - Pattern: flowery - Pigment exuded: many colorless droplets on inner area and unchanged medium color	- Size: 3.1 cm after 7 days incubation - Color: 5GY 6/1 - Shape: hyphae with septate	*Penicillium*
20	DT-2.5.3	- Color and tint in colony surface: white in outer and light white-grey in inner area - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth - Pattern: zonate to 2 concentric circles, white in outer and light white-grey in inner, - Pigment exuded: no	- Size: 3.8 cm after 7 days incubation - Color: N9 for outer and 10YR 8/1 for inner area - Shape: hyphae with septate	- Color and tint in colony surface: white in outer and grey-green in inner area - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, - Pattern: zonate to 2 concentric circles, white in outer and grey-green in inner - Pigment exuded: no	- Size: 2.5-2.7 cm after 7 days incubation - Color: N9 for outer and 5G 6/1 for inner area - Shape: hyphae with septate	*Penicillium*
21	AH-3.3/2	- Color and tint in colony surface: white - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, velvety, powdery - Colony margin: smooth, restricted - Pattern: fan-shaped colony with many furrow-drains from center - Pigment exuded: no	- Size: 1.8 cm after 7 days incubation - Color: N9 - Shape: hyphae with septate	- Color and tint in colony surface: white - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, velvety, powdery - Colony margin: smooth, restricted - Pattern: indistinctly fan-shaped colony with some furrow-drains from center - Pigment exuded: no	- Size: 1.8 cm after 7 days incubation - Color: N9 - Shape: hyphae with septate	*Penicillium*

Characteristic of fungi						Genus
No.	Strain	SBA Medium		PDA Medium		
		Cultural characteristics	Morphology and physiological characteristics	Cultural characteristics	Morphology and physiological characteristics	
22	AH-3.3/1	- Color and tint in colony surface: green - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, white in outer and green in inner, and fan-shaped colony with some furrow-drains from center - Pigment exuded: many colorless droplets on inner area but unchanged medium color	- Size: 2.7-2.8 cm after 7 days incubation - Color: 5G 8/1 - Shape: hyphae with septate	- Color and tint in colony surface: green - Color and tint in colony reverse: white - Aerial hyphae: yes - Colony surface texture: cottony, shrunken, embedded - Colony margin: smooth, wavy, restricted - Pattern: zonate to 2 concentric circles, white in outer and green in inner, and fan-shaped colony with some furrow-drains from center - Pigment exuded: many colorless droplets on inner area but unchanged medium color	- Size: 2.1 cm after 7 days incubation - Color: 5G 6/2 - Shape: hyphae with septate	*Penicillium*
23	DT-3.3	- Color and tint in colony surface: light yellow - Color and tint in colony reverse: yellow - Aerial hyphae: yes - Colony surface texture: cottony, sparse, homogeneous - Colony margin: smooth, spreading - Pattern: no - Pigment exuded: change medium color to yellow	- Size: 8.0 cm after 3 days incubation - Color: 5GY 9/3 - Shape: hyphae with septate	- Color and tint in colony surface: white with patchy green - Color and tint in colony reverse: light yellow - Aerial hyphae: yes - Colony surface texture: cottony, powdery, heterogeneous - Colony margin: smooth, spreading - Pattern: zonate with white and some small patchy green outer - Pigment exuded: change medium color to yellow	- Size: 8.0 cm after 3 days incubation - Color: N9 and 5GY 8/6 - Shape: hyphae with septate	*Trichoderma*

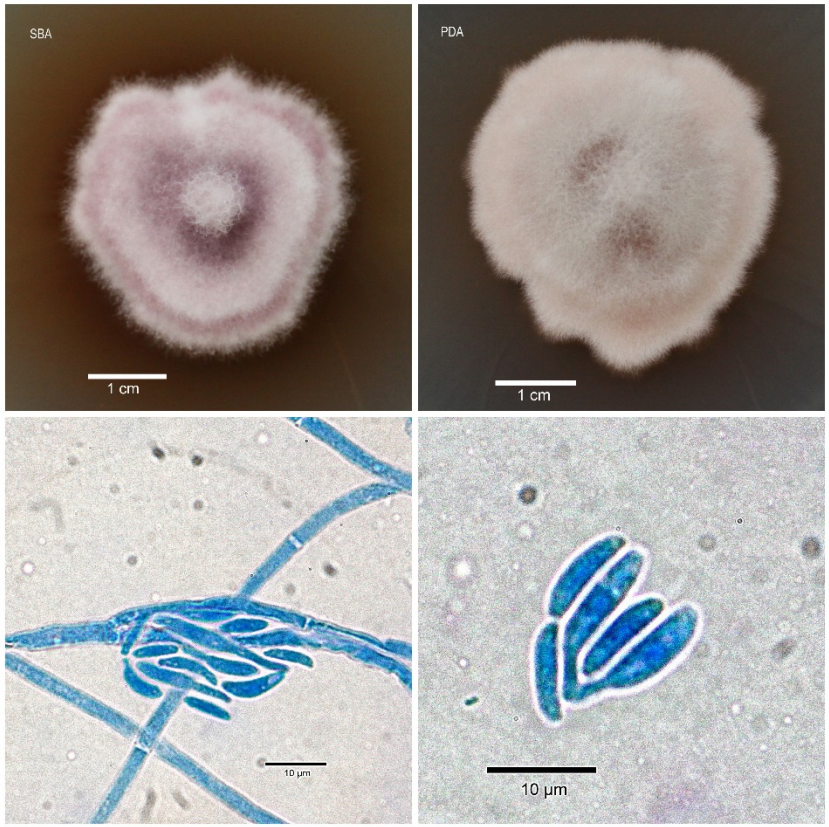
*: *refer to the standard color charts JIS Z 8721*

Appendix 3 Morphological pictures of oil-degrading fungi

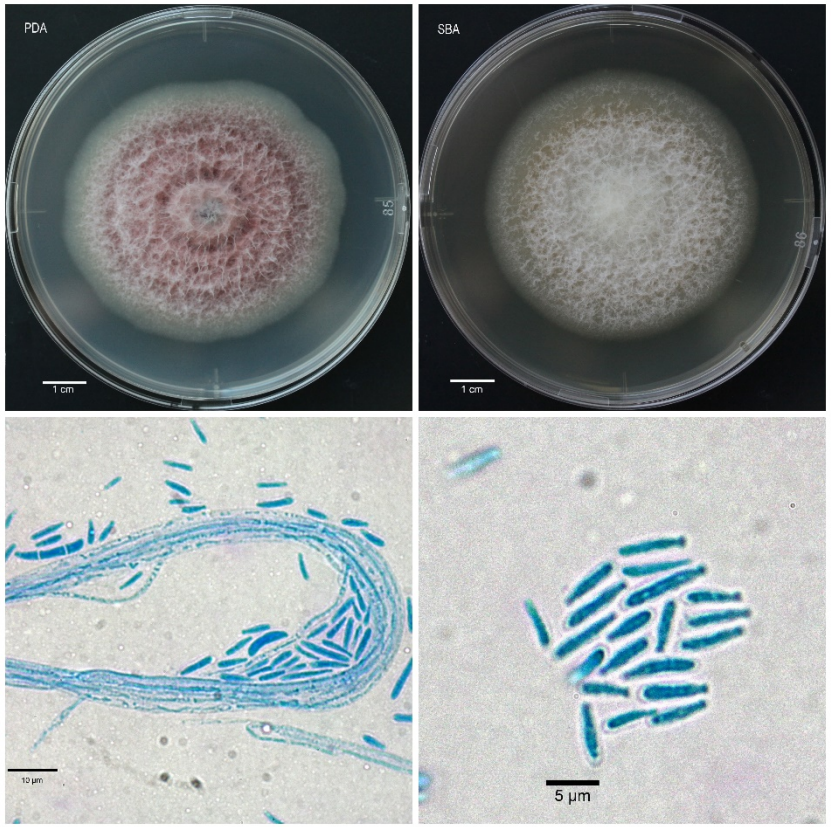
Iriomote



1. Fu-1.12



2. Fu-1.1.1



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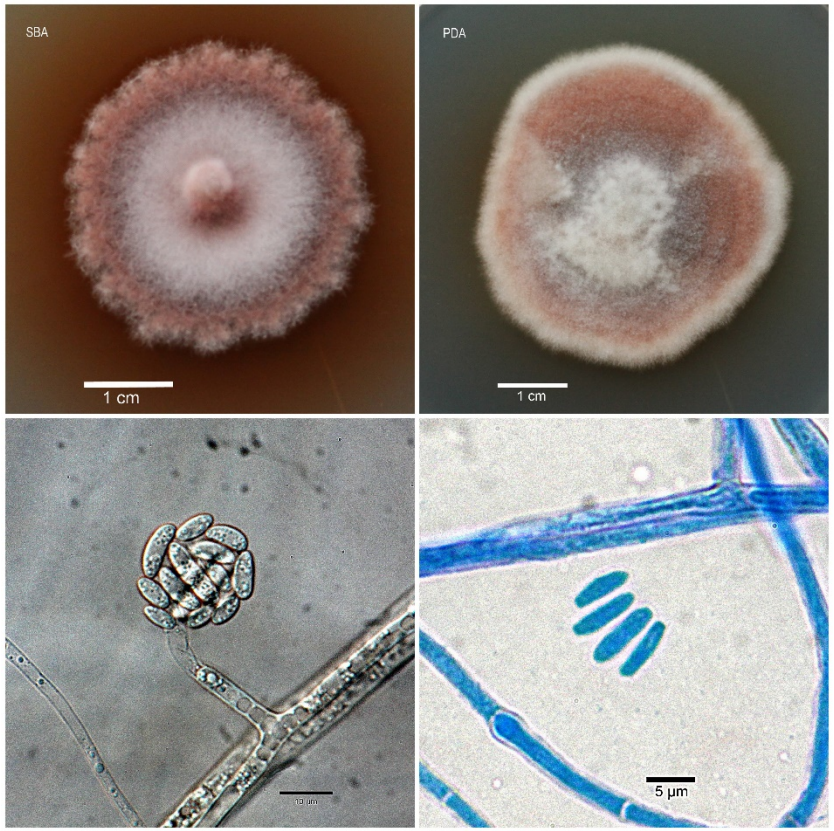

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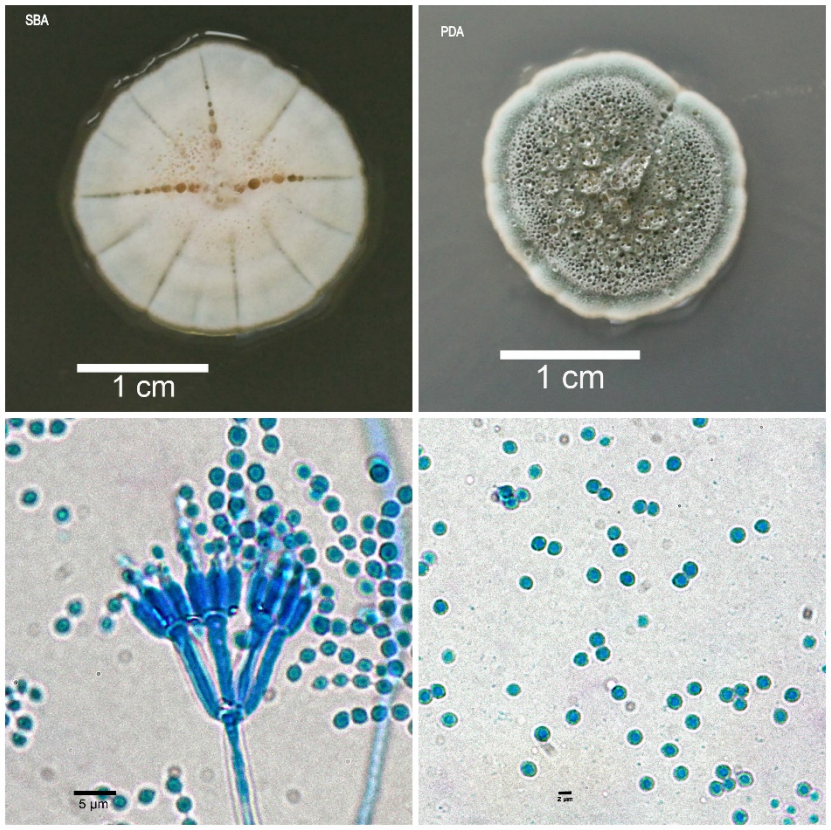
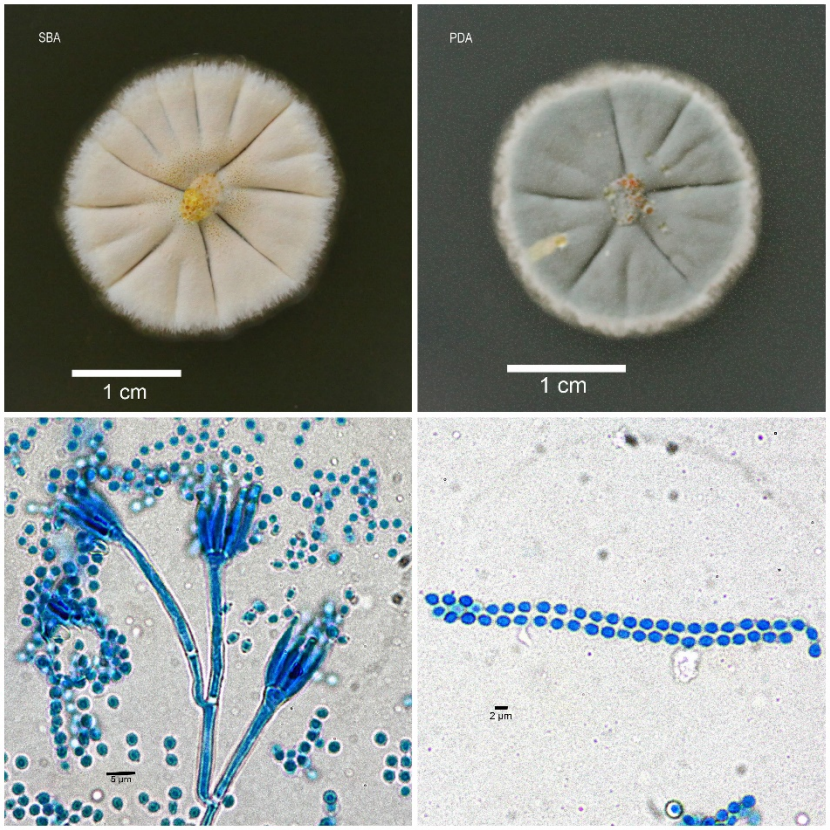
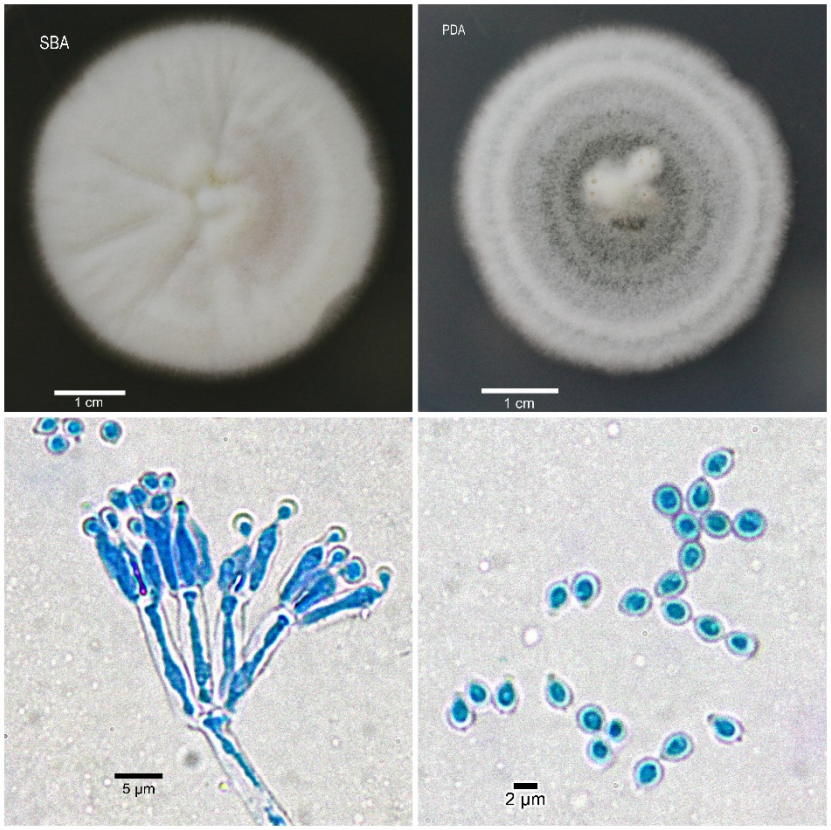


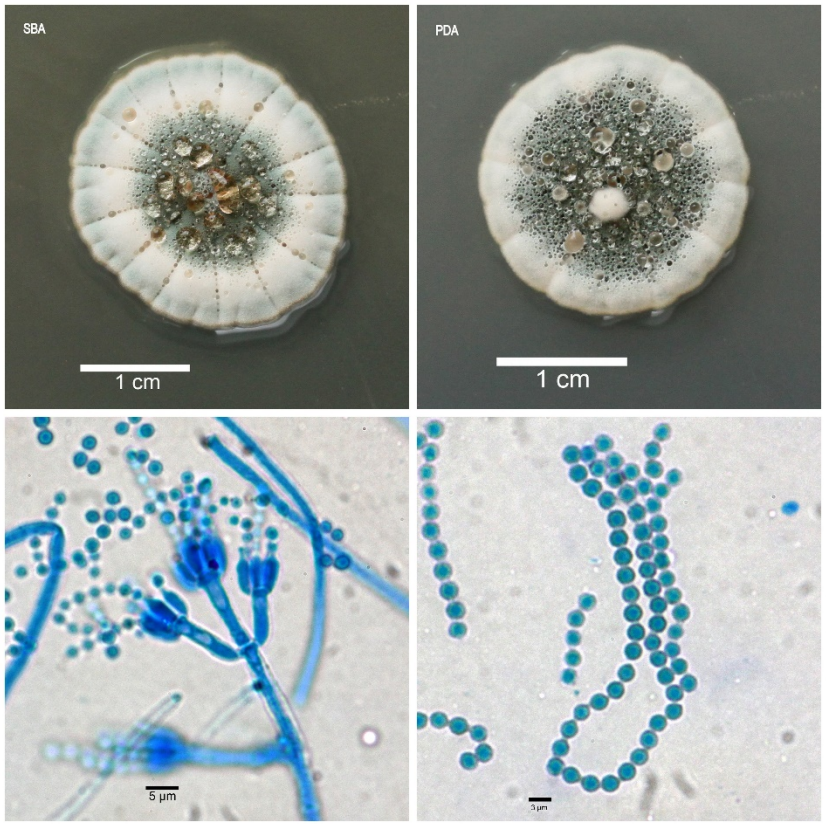
5. Fu 1.14



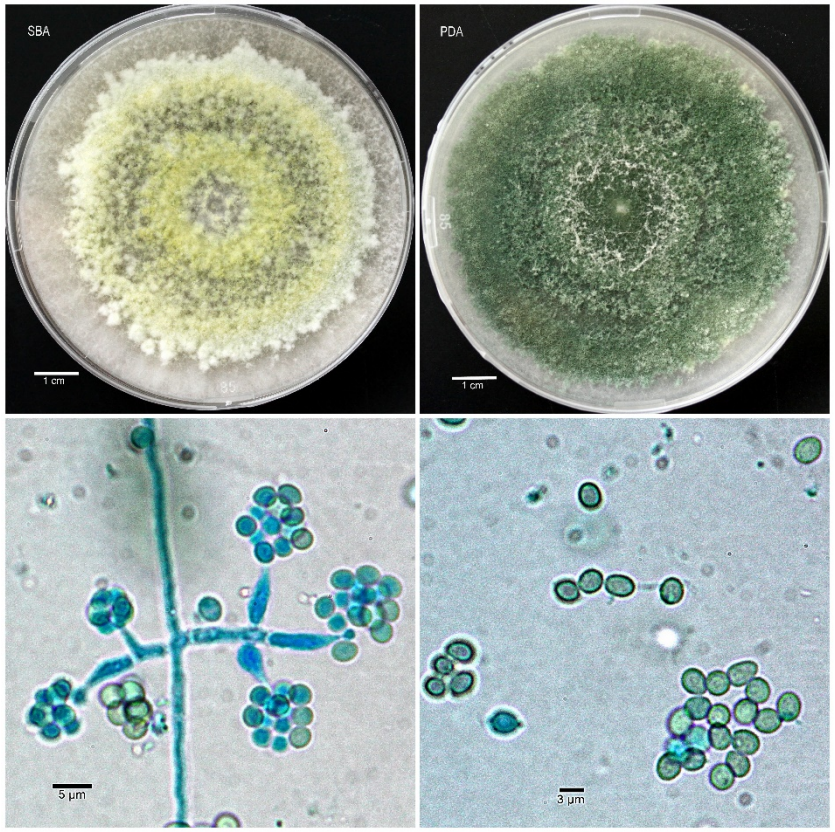
6. Oh-1.7



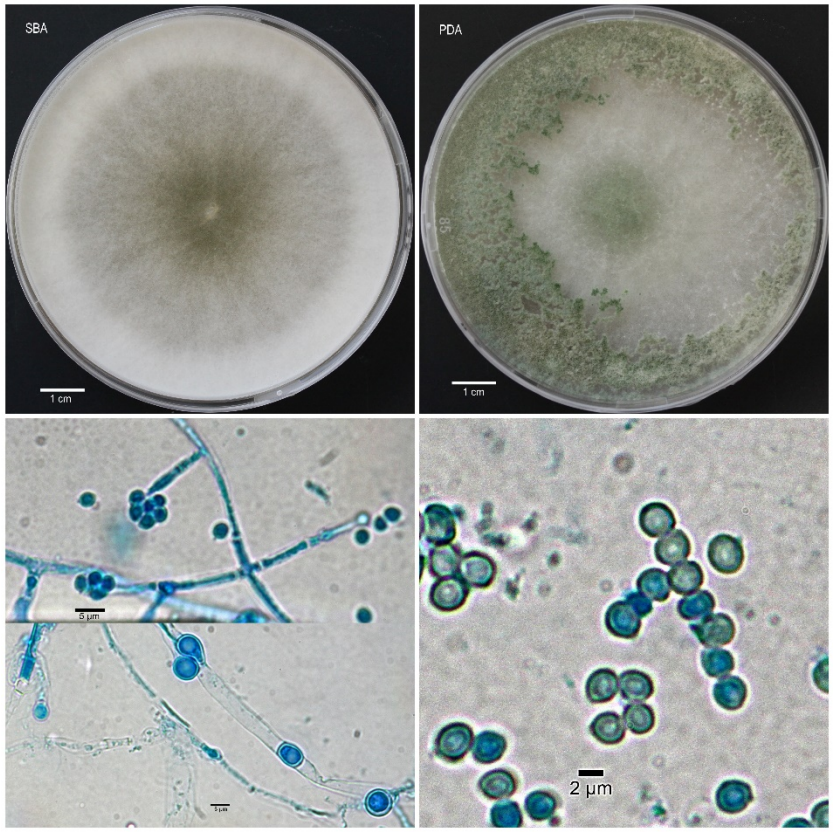
7. Oh-1.6.1



8. Fu-1.13



9. Oh-2.1



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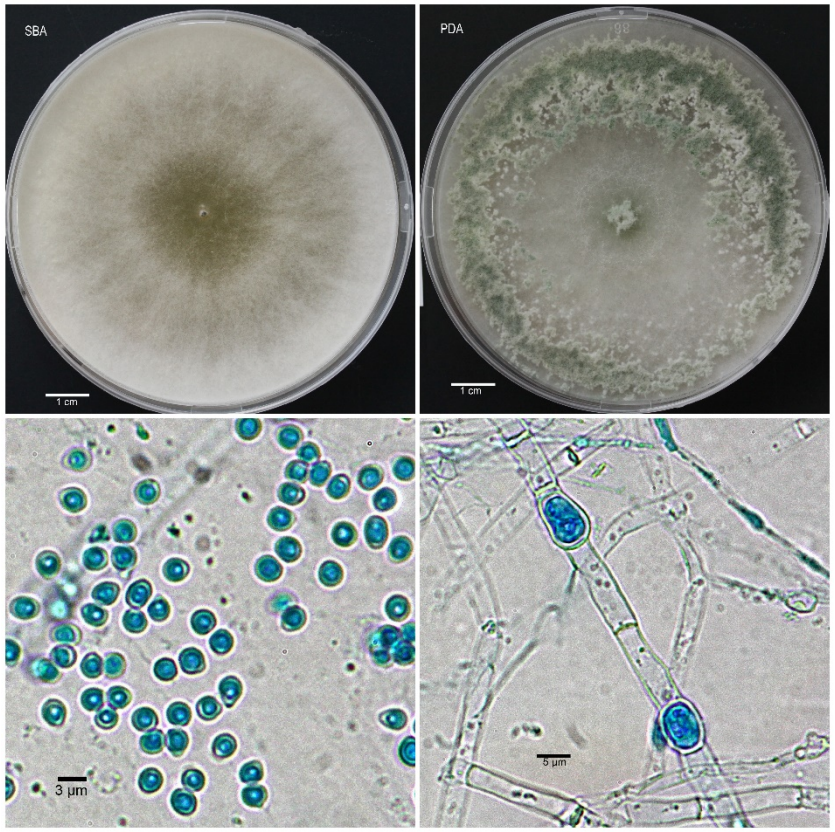
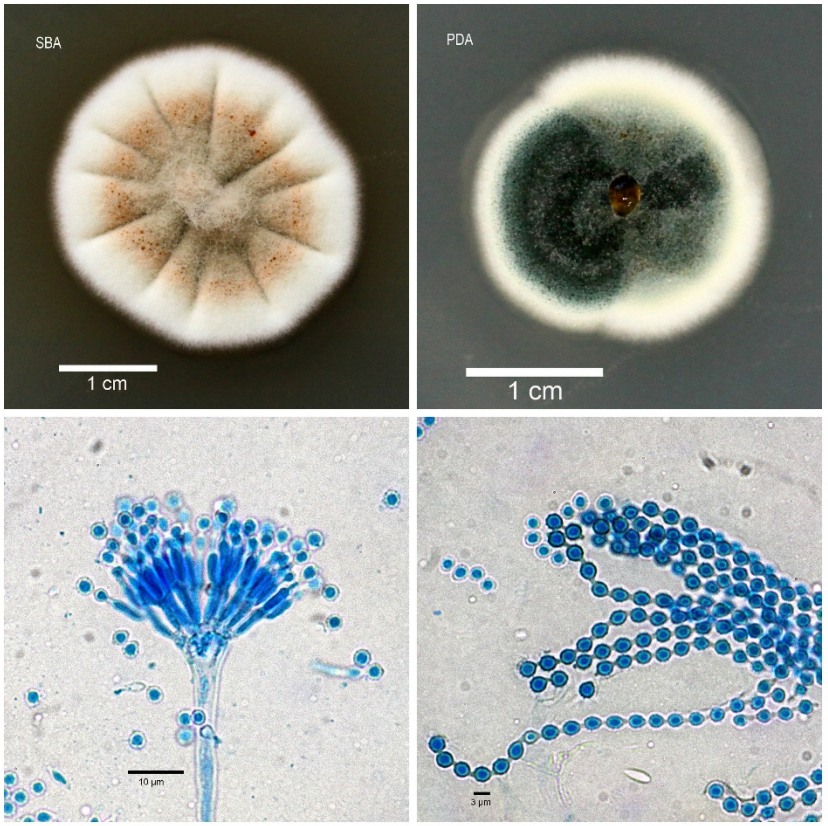
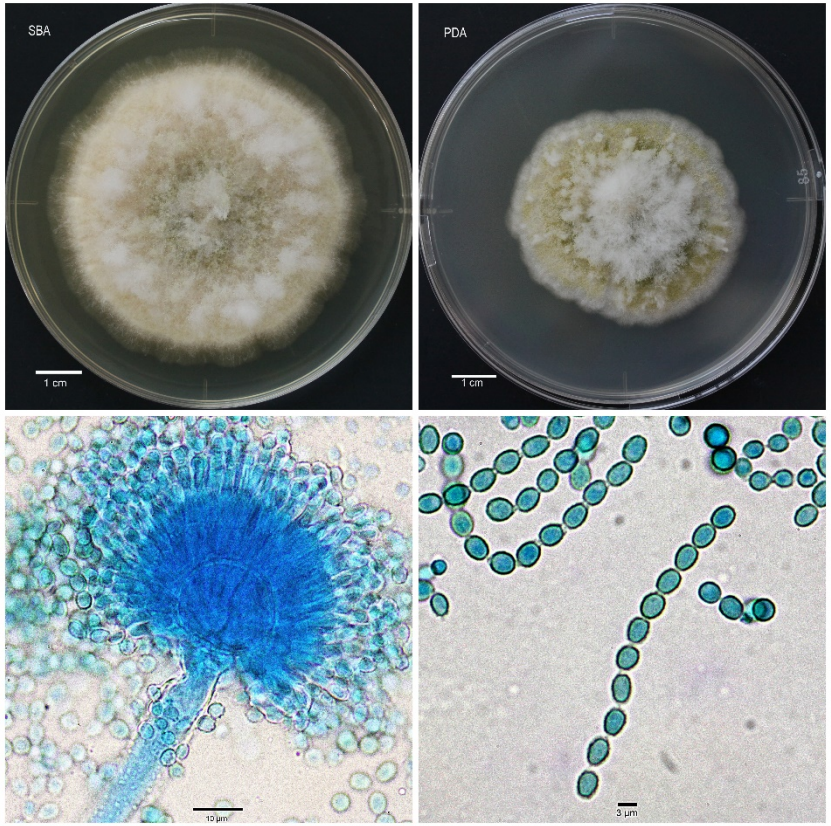


11. Oh-1.5

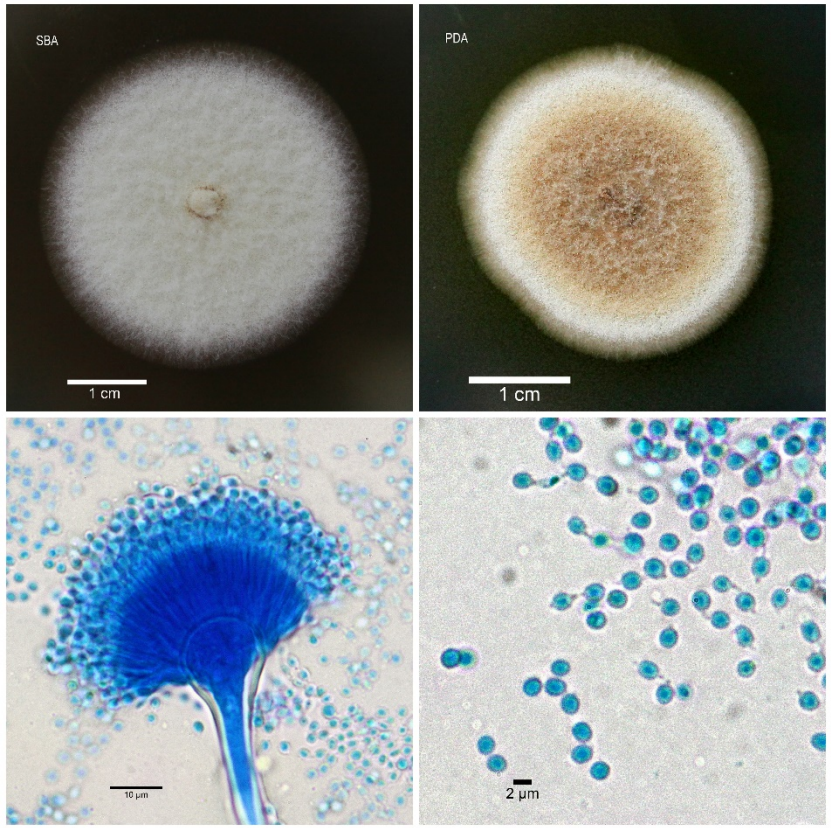
Con Dao



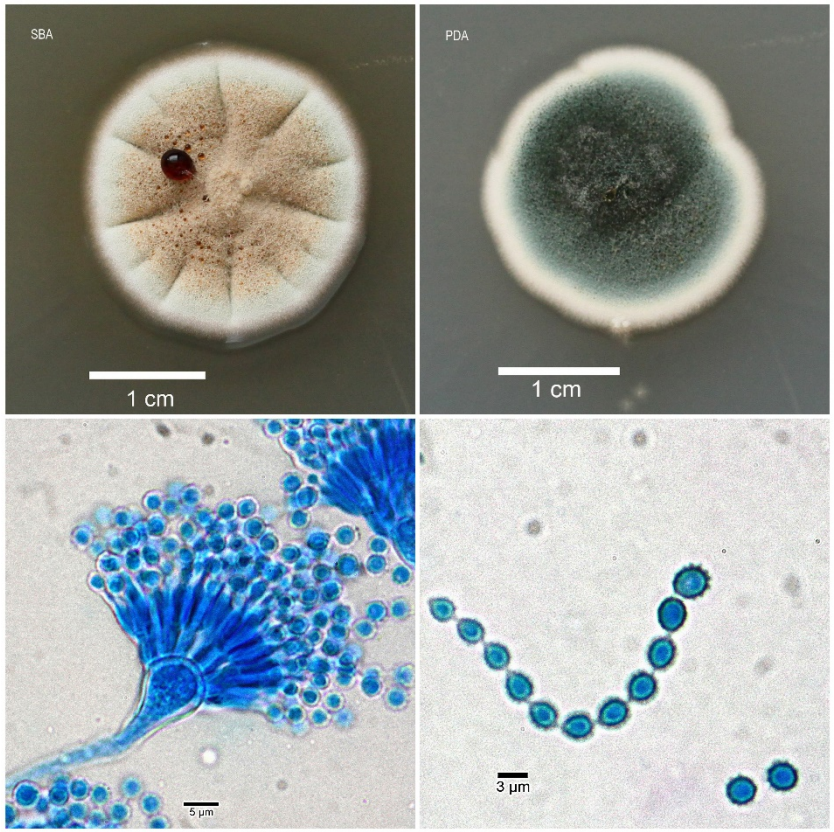
12. BD-3.4.2



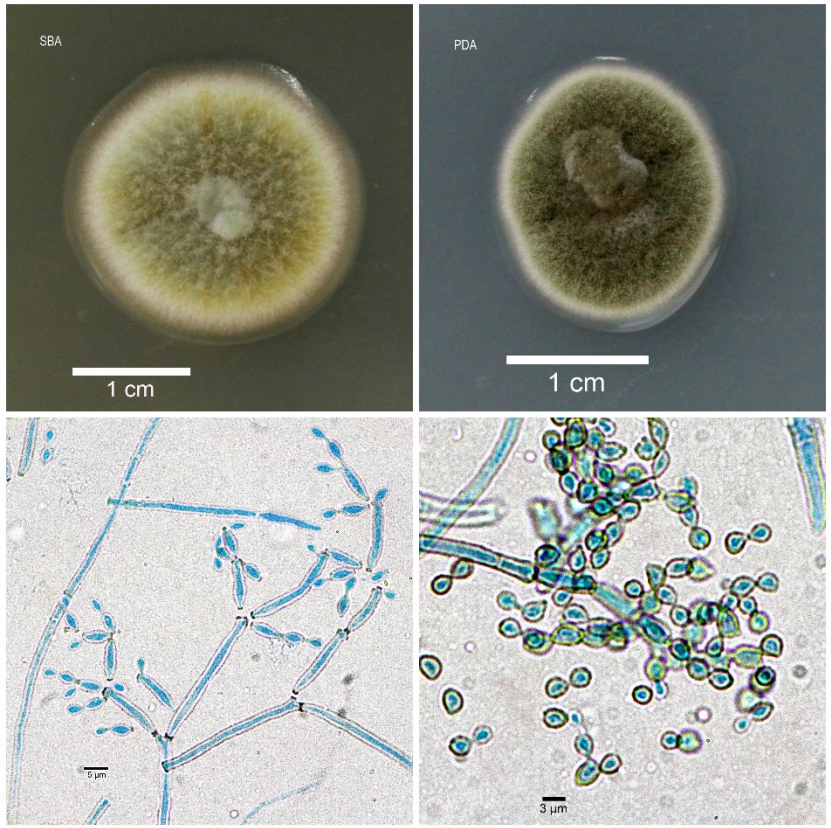
13. DT-2.5.2



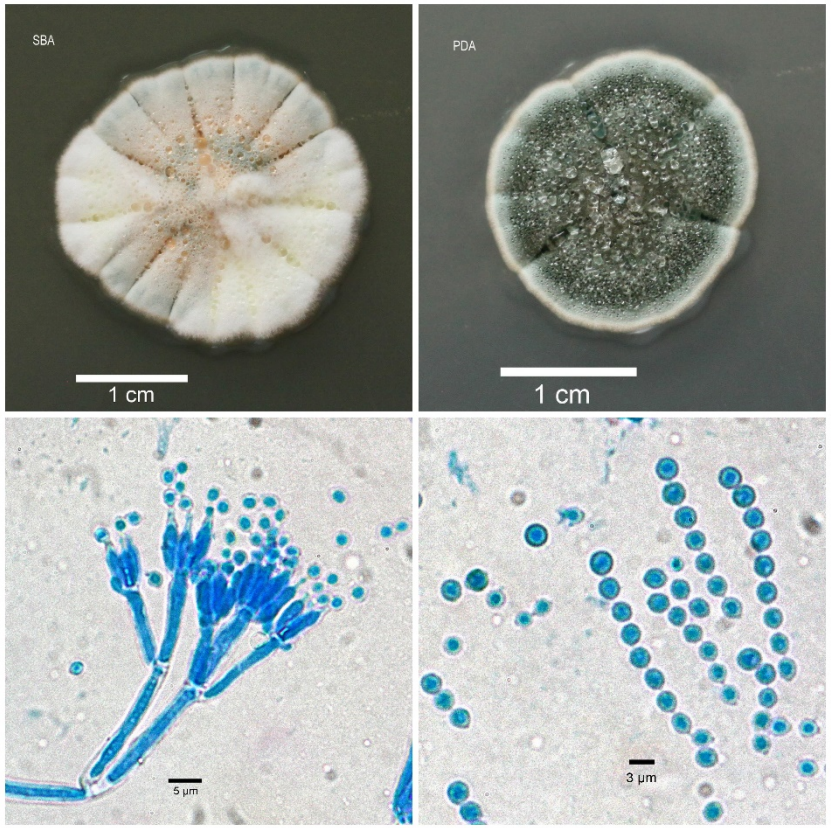
14. DT-3.4



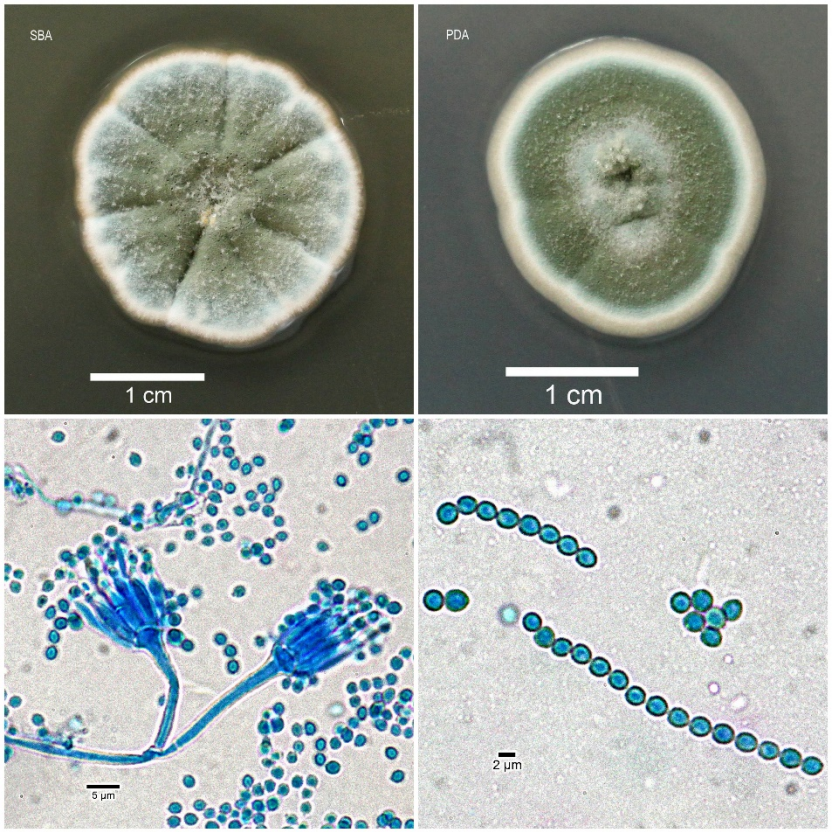
15. DT-2.5.1.2



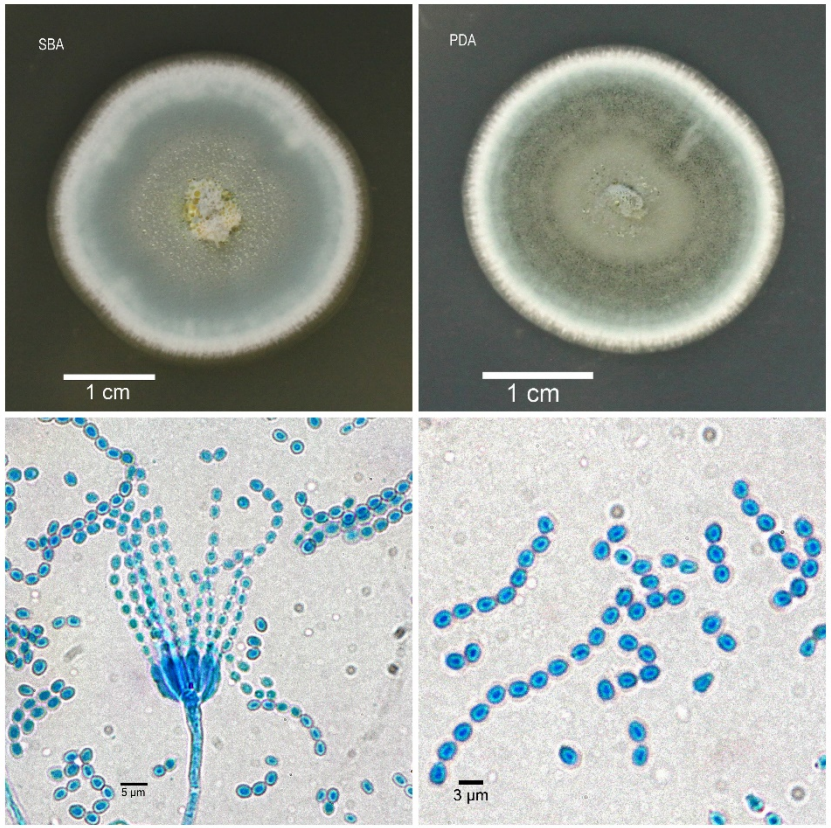
16. DT-2.3



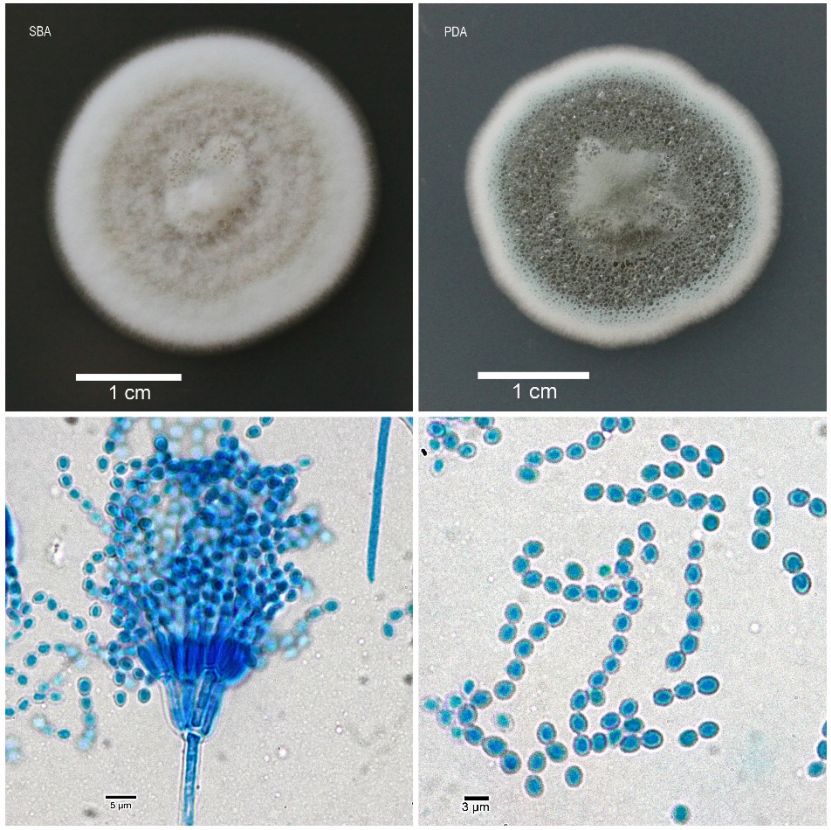
17. VB-3.5



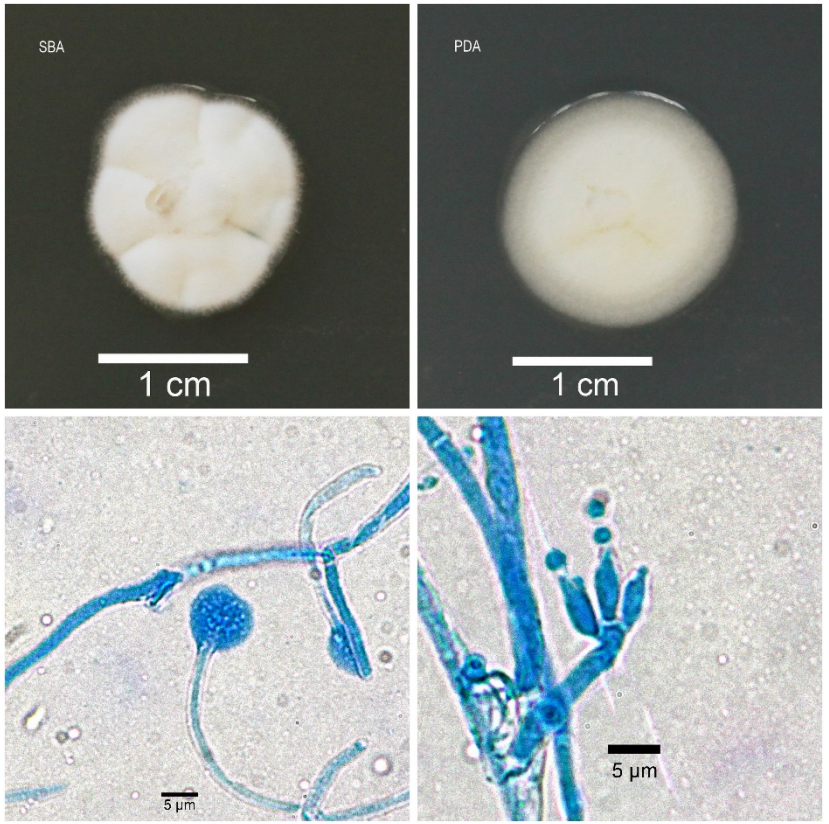
18. DT-3.5



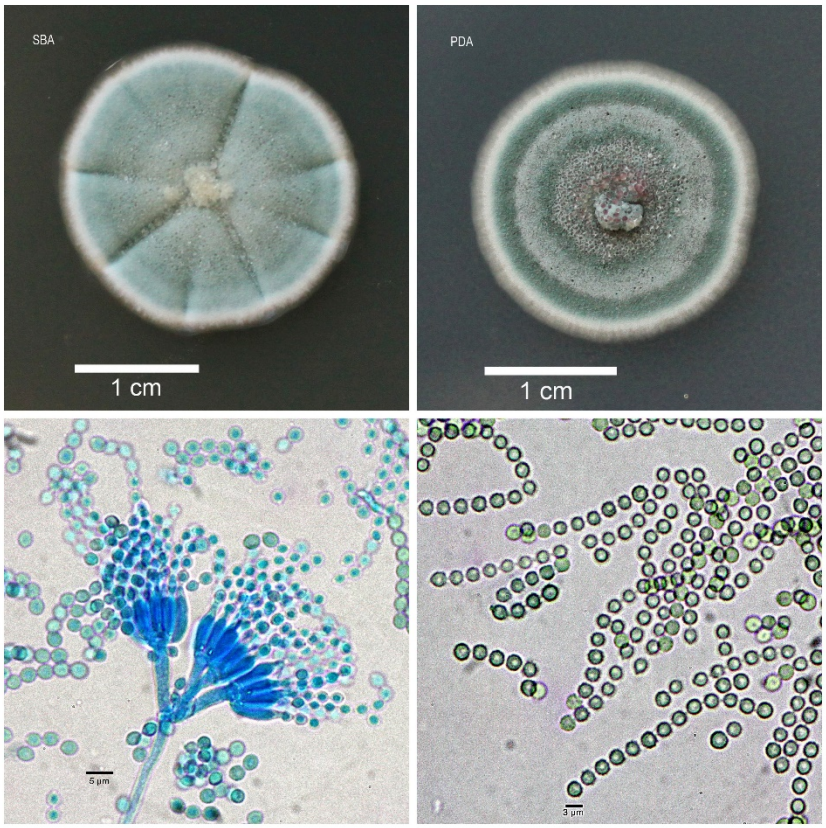
19. AH-1.1



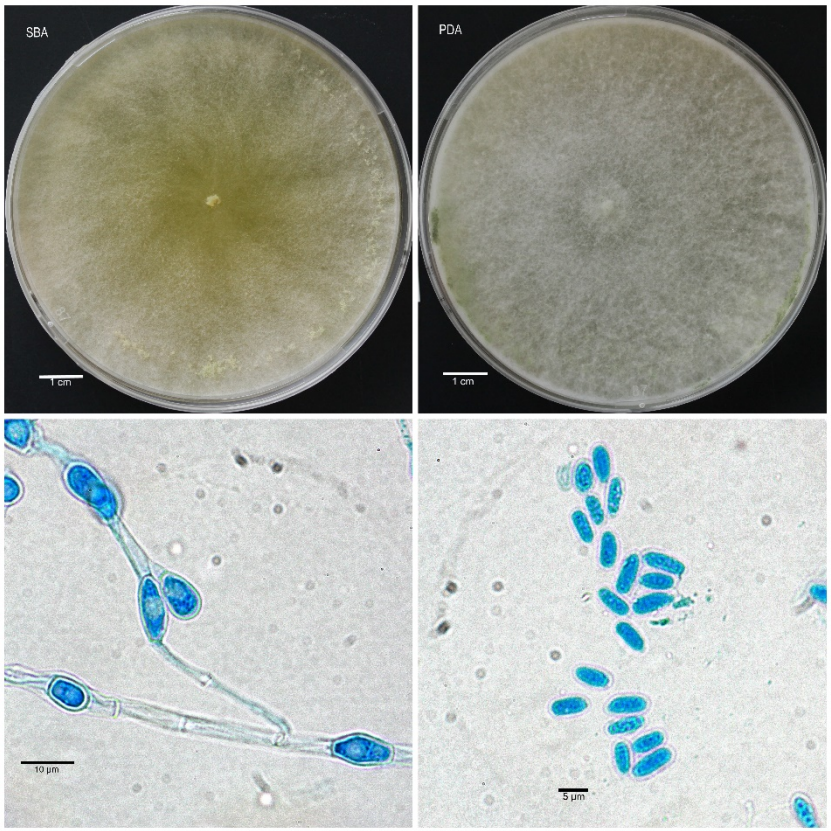
20. DT-2.5.3



21. AH-3.3.2



22. AH-3.3.1



23. DT-3.3