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per litre. e culture medium was adjusted to a nal pH of 7.00 by using 0.5 mol/L of sodium hydroxide solution. e MM-pyrene plates were prepared with MM and 1.5% of powder agar, followed by the spraying of 2000 g of pyrene on the surface of the solid medium. A portion of 0.5 g soil samples were added into the 200 mL-Erlenmeyer ask containing 50 mL of MM and 100 mg/L of pyrene. It was shaken in an orbital shaker at a speed of 180 rpm at 30°C in the dark. e enrichments were incubated under aerobic conditions for four weeks. One mL aliquots of enrichment cultures were transferred into another sterile Erlenmeyer ask containing fresh MM and pyrene. One mL of aliquots was also serially diluted subsequently, and 0.1 mL of culture broth was spread onto the MM-Pyrene plates. ey were incubated at 30°C and routinely checked for colony growth. A er about two weeks, the bacterial colonies were growing and showing zones of pyrene clearing. e colonies were picked and serially diluted, and then streaked onto new MM-Pyrene agar plates. A single colony was picked and streaked again on other new MM-Pyrene plates.

Polymerase chain reaction (PCR)

irteen isolates were examined for ampli cation of the target genes. All PCR ampli cations were carried out using the TAKARA TP600 thermal cycler (Takara Bio, Inc, Kyoto, Japan). e Go-Taq Master Mix (Promega, Madison, Wisconsin) was used with 49 µL of total volume. e PCR reagent mix consisted of colony picked up as a template, 2 µL of forward and reverse primer, of which the concentration was 25 µM each, 25 µL of Go-Taq Green, and 20 µL of nuclease-free water. Sequences of used primer sets were summarized in Table 1. A er preheating at 95°C for 5 minutes, 30 cycles of heating at 95°C for 30 s, annealing, and extension at 72°C for 2 minutes were performed. Annealing temperatures were 62°C for 30 s, 63°C for 45 s, 54°C for 45 s, 54°C for 45 s, and 62°C for 60 s applied with primer sets including nidA 508F/508R, CD34F/CD34R, pdoA2F/ pdoA2R, nidA3F/ nidA3R, and

analysis of the partial 16S rRNA gene sequence. As presented in Table 2, the isolated bacteria clasi ed four classes, namely -Proteobacteria, -Proteobacteria, -Proteobacteria and Actinobacteria with 98-99% of similarity.

Pyrene degradation ability of isolates

Biodegradation tests of pyrene with thirteen identi ed bacteria within 16 days of incubation were carried out and their degradation abilities were shown in Figure 1. e percentage of pyrene degradation was from 26.3%-31.5% for 10 isolates. However, isolate no. 1 (close to Burkholderia fungorum), isolate no. 12 (close to Mycobacterium vanbaleeni) and isolate no. 13 (close to Mycobacterium gilvum) showed high degradation percentage such as 96.2%, 82.2% and 100%, respectively.

e PCR ampli cations targeting nidA, nidA3, pdoA2, and pcaH genes were carried out for all isolates. Only three isolates, namely isolate no. 1, isolate no. 12 and isolate no.13 which showed high pyrene degradation ability had all four genes. Isolate no. 9, which was close to Pseudomonas vancouverensis, had nidA and pcaH genes. Other 9 isolates had only nidA gene. It seemed that the whole pathway was necessary for the complete degradation of pyrene.

e nidA, nidA3, pdoA2, and pcaH genes were identi ed for the degradation of PAHs in other reports [7,9,11,13]. e naphthalene inducible pyrene dioxygenase gene (nidA), with the -subunit pyrene dioxygenase, has a critical role in the initial hydroxylation of PAHs, which was identi ed to be a transmembrane protein; PAHs that are hydrophobic tend to split into the cell membranes [22]. Furthermore, particularly for pyrene, M. vanbaalenii PYR-1 yielded pyrene cis-4,5-dihydrodiol using this enzyme during the initial degradation pathway [12]. e presence of the pcaH gene-encoding enzyme of protocatechuate 3,4-dioxygenase has an important part in the -ketoadipate pathway [9]. e existence of the nidA3 and pdoA2 genes suggested that these genes had key roles for the degradation of pyrene into a more hydrosoluble compound in the 16 days of cultivation. e pdoA2 gene encoding of the -subunit of phenanthrene ring-hydroxylating oxygenase was involved in the initial PAH-degradation pathway [12]. is gene has a role in the detoxi cation of PAH catechols into more hydrosoluble compounds by delivering less reactive methoxy-derivatives compounds. Furthermore, the nidA3 gene encoding of the -subunit of uoranthene/pyrene ring-hydroxylating oxygenase (RHO) functions as a terminal oxygenase of RHO for degradation (nidA3) [illegible]

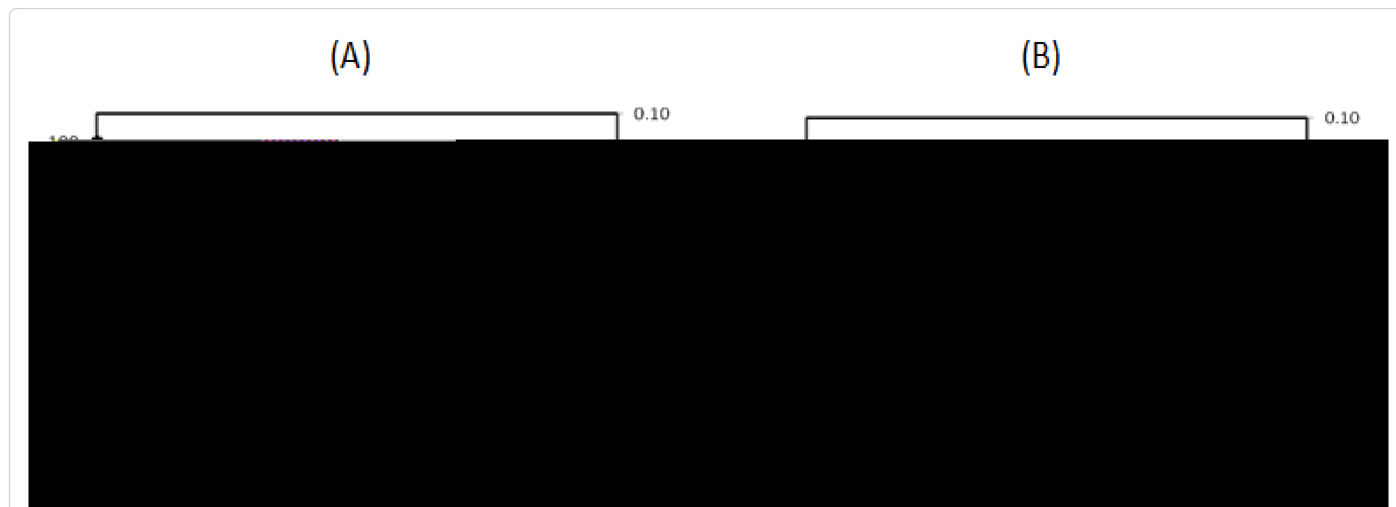

Figure 2: 'HJUDGDWLRQ FXUYHV RI $ S\UHQH DQG B. fungorum % ÀXRUDQWKHQH ZLWK

Previous studies have reported that there are a very limited number of bacteria taxonomic groups, including the dominant PAH degraders, namely Sphingomonas, Pseudomonas, Burkholderia, and Mycobacterium [24-26]. According to some other reports [27-29], bacteria belonging to genus Burkholderia and Mycobacterium could degrade PAHs signi cantly. Burkholderia sp. was isolated to utilize several PAHs, such as naphtalene, penantherene, and pyrene [27,30]. e rst report on Mycobacterium sp. showed that the strain could degrade 94.9% in 0.5 mg/L, with pyrene as the sole energy source in a minimal basal salt medium a er 4 days of incubation [29,30]. Mycobacterium sp. could be the major member genus of the indigenous PAH-degrading bacteria, wherein the genus had been isolated from many polluted environments [25,31]. In this study, B. fungorum isolate no. 1 and M. gilvum isolate no. 13 were isolated from Tanoura Bay, Yatsushiro Sea, as promising bacteria to degrade pyrene and uoranthene for future bioremediation. ese strains could degrade both pyrene and uoranthene properly into more hydrosoluble compounds. During the PAH-degrading experiment, the medium coloring was observed. ere was no switch in color on pyrene degradation. In contrast, in the uoranthene degradation experiment, both isolates showed the color change of medium to a reddish hue a er 10-days of incubation. e medium color probably came from metabolite accumulation [13].

e PAHs' concentration of abiotic control was decreased from 100% to 94.19%, and from 100% to 88.09% for pyrene and uoranthene, respectively. e decreasing concentration of abiotic controls was a possibility due to volatilization [23]. e growth controls increased to 0.009 OD and 0.014 OD for B. fungorum isolate no. 1 and M. gilvum isolate no. 13, respectively. e OD of growth controls was lower than with pyrene, in which the OD containing pyrene increased to 0.035 and 0.068 for B. fungorum isolate no. 1 and M. gilvum isolate no. 13, respectively. e higher OD containing pyrene compared with those of growth control in the experiments indicate that both B. fungorum

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of pyrene and uoranthene degradation of Mycobacterium 1B can be attained completely a er 6 day and 20 day, respectively, although it was supplemented with higher initial concentration of pyrene 250 mg/L. e PAH degradation of Mycobacterium 1B was better than M. gilvum isolate no. 13 utilizing MM medium, where 77.7% of pyrene and 70.9% of uoranthene can be consumed for 8 and 24 day of incubation period, respectively. e results indicate that both BSM and BSMY mediums could increase the degradation ability of PAHs. e BSM medium added with di erent additives, such as peptone, glucose, sucrose, ethanol, methanol and yeast have been investigated where the study indicating that the supplement of yeast extract could increase speci c growth and phenantherene degradation rate signi cantly [27].

e research results have shown that all of these genera can utilize PAHs, particularly pyrene and uoranthene, though they used di erent media. Four genes detected in these bacteria had important role in PAH degradation by encoding terminal oxygenase of the RHO enzyme. Nevertheless, this study showed that the both isolated bacteria, B. fungorum isolate no. 1 and M. gilvum isolate no. 13 can degrade the PAHs well, despite the use of a limited media, namely minimum medium (MM). e recent study suggests that minimum medium can be used as an alternate medium in PAHs degradation experiments.

Conclusion

is study has shown that PAH-degrading bacteria can be isolated from a polluted coastal environment and they have a good ability for PAHs degradation. ere is a positive correlation between the four genes considered and the ability for HMW PAH degradation. B. fungorum isolate no. 1 and M. gilvum isolate no. 13 can degrade pyrene and uoranthene almost completely, although the experiment used a nite medium, namely minimum medium (MM). e isolates might be applied as candidate bacteria for future bioremediation in polluted coastal sites. ese results may be applicable to hydrosoluble compounds as intermediate metabolite products a er PAHs degradation. Further studies need to be carried out in order to elucidate the degradation products.

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