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Crude Oil Biodegradation Potential using *Acinetobacter baumannii* CYA20 and *Bacillus subtilis* CYA27 from the Bekasi Coast, Indonesia

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ABSTRACT

The pollution of coastal areas caused by oil spills is an environmental issue that needs further attention. Crude oil contains persistent organic pollutants (POPs) that are difficult to degrade. This study aimed to isolate bacterial strains capable of degrading crude oil from the Bekasi coast through bacterial isolate characterization and crude oil biodegradation tests using a crude oil-enriched microcosm model. Two strains with higher TPH degradation values were selected among the isolated bacteria, namely CYA20 and CYA27, which specifically showed values of 46% and 66%, respectively. These strains were tolerant to NaCl concentrations of up to 4% and 12%, respectively, and they were also tolerant to pH values ranging from 5 to 9 and temperatures between 10°C and 50°C. Both strains were shown to utilize POPs, such as phenanthrene and fluorene, produce biosurfactants, and exhibited an emulsification activity in paraffin ranging from 0.373 to 0.533. Phylogenetic analyses identified these bacterial strains as *Acinetobacter baumannii* CYA20 and *Bacillus subtilis* CYA27, respectively. The results of this study indicate that these isolates could be developed as biodegradation agents for the bioremediation of crude oil-contaminated environments.

1. Introduction

Indonesia is the world's largest archipelagic country, consisting of 13,558 islands (Andrefouet *et al.* 2022) with a coastline of 99,093 km and a marine area of about 6.32 million km^2 (KKP 2018). Indonesian marine waters are among the most vulnerable to oil pollution because they represent the main route through which oil is transported worldwide. Oil spills, which originate from crude oil processing, production, distribution, and utilization, can cause marine pollution due to the release of crude oil components into seawater (Kim *et al.* 2019).

Due to the increasing occurrence of oil spills, crude oil pollution in coastal areas has become an environmental issue that needs more attention. Several oil spill incidents have occurred in Indonesian seawaters, such as in the Malacca Strait, Riau Island, East Borneo, and North of Java Sea. In 2019, an oil spill event at Karawang Beach caused massive pollution extending for about 100 km along the northern coast of West Java Island. Crude oil pollution in the oceans has a far-reaching impact (Yang *et al.* 2020), and it affects also the surrounding environment, including the marine coastal populations, ultimately modifying the ecological system. Hydrocarbon compounds, such as polyaromatic hydrocarbons (PAHs), are among the most difficult crude oil pollutants to degrade. PAHs are persistent organic pollutants (POPs) found in all environmental compartments, including the atmosphere, water, sediment, and soil. They are semi-volatile and highly toxic compounds to living organisms and the environment that can accumulate in adipose tissue (Sheriff *et al.* 2021). As they contaminate surfaces, these compounds can evaporate, be washed away by rainwater, or enter the soil, where they exert their toxic effects. Therefore, further processing is required to separate crude oil from surfaces (Hu *et al.* 2013).

The activity of bacteria in crude oil degradation has attracted increasing attention due to non-

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carcinogenic, non-combustible, low operational cost, and eco-friendly materials compared to other conventional methods. Biodegradation was shown to remove the crude oil hydrocarbons from the marine environment and restores the oil-contaminated ecosystem (Fu *et al.* 2021; Sakthipriya *et al.* 2015). Indigenous and pre-adapted bacteria are critical factors in the degradation of crude oil derived from terrestrial oil spills as crude oil-degrading bacteria are abundant in oil-rich environments and considered efficient oil-degrading agents (Moghimi *et al.* 2017; Uribe *et al.* 2021). These bacteria can utilize hydrocarbon compounds as a carbon and energy source for growth and they can survive in a crude oil-polluted environment. In addition, they can degrade the crude oil components and oxidize hydrocarbons to CO_2 and H_2O, which are harmless to the environment (Fu *et al.* 2020), and they can also use hydrocarbons as electron donors and produce biosurfactants (Yalaoui-Guellal *et al.* 2021). This condition will reduce or eliminate crude oil pollutants (Yang *et al.* 2020). The most rapid and complete degradation is achieved under aerobic conditions. The initial intracellular enzyme that attacks organic pollutants is an oxidative process. The activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases to degrade the oil (Das and Chandran 2011).

The main purpose of this study was to isolate and characterize bacterial strains capable of degrading crude oil from oil-polluted beaches and conducting crude oil biodegradation tests using a crude oil-enriched microcosm model.

2. Materials and Methods

2.1. Sampling Site

Sample were collected from the oil spill located in the northern coast of Bekasi, Indonesia (5°56'18.5''S 107°05'53.6''E) in December 2020. This area was contaminated by crude oil in middle of 2019 due to an oil spill incident by an oil company. The TPH value of the contaminated coastal sand samples was 2.1%. Sampling was conducted using a randomized sampling method. The samples, i.e. beach water (5°57'03.3''S 107°05'45.8''E), pond water (5°57'03.0''S 107°05'44.2''E), and mangrove water (5°56'09.3''S 107°05'10.4''E) were collected at a depth of 50 cm from the surface and were placed into sterile sample bottles. The surface samples of mangrove (5°56'10.2''S 107°05'09.0''E) and pond grass rhizosphere (5°57'03.3''S 107°05'44.8''E) were collected at depth between 5 and 20 cm. Then the samples were sealed into plastic bags and labeled with detailed information based on the sampling location and period. All samples were packed in an icebox and stored in cold storage before isolation for two days.

2.2. Enrichment of Crude Oil-Degrading Bacteria

The isolation of the crude oil-degrading bacterial consortium was carried out using the modified enrichment culture method Perdigao *et al.* (2021). Bacteria were extracted by mixing a 5 g sample (for surface samples of mangrove and pond grass rhizosphere) and a 5 ml sample (for beach water, pond water, and mangrove water) into the flask with 45 ml of oil medium (Ijah *et al.* 2008). The composition of oil broth medium was: KH_2PO_4 (1.2 g/L), K_2HPO_4 (1.8 g/L), NH_4Cl (4 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), NaCl (0.1 g/L), and $FeSO_4 \cdot 7H_2O$ (0.01 g/L). The crude oil (2% v/v) was injected into the oil medium as a carbon source. The flask was shaken for two weeks on an orbital shaker at room temperature at 150 rpm. Subsequently, the grown culture was transferred to the fresh oil broth medium enriched with crude oil (2% v/v). The consortium bacteria were shaken for two weeks. The cell growth was calculated by monitoring the cell turbidity, which was measured an optical density of 600 nm ($O.D._{600\ nm}$) (Cai *et al.* 2021; Loureiro *et al.* 2020) using a UV-VIS Shimadzu UV 1,280 at observation times of 0, 1, 3, 5, 7, 9, 11, 13, and 14 days, and using the total plate count method at observation times of 0, 7, and 14 days during the incubation period. The total petroleum hydrocarbons (TPHs) were measured on day 14 of the culture period using hexane extraction as a gravimetric method (Behera *et al.* 2021).

2.3. Bacterial Isolation and Screening

After obtaining the enriched bacterial consortium, 1 ml of bacterial suspension was serially diluted to 10^{-6} and inoculated onto the 10% nutrient agar plates. All the isolated bacteria were screened in terms of their morphological properties, including shape, margin, elevation, pigmentation, and optical properties. The bacterial colonies that exhibited a different morphology during growth were taken at one loop and were streaked on the nutrient agar plate for purification using the quadrant-streaking method. A

pure culture was one isolate of crude oil-degrading bacteria coded for initial naming.

2.4. Pathogenicity Test

A hypersensitivity response (HR) test was carried out to evaluate the bacterial pathogenicity in plants, allowing it can be released into the environment. Bacteria with a density of 10^7 CFU/ml in nutrient broth were injected into the lower surface of tobacco leaves (*Nicotiana tabacum* L.) using a syringe and were observed for one week (Umesha *et al.* 2008), necrosis was identified by the presence of brown-colored leaves. A hemolysis test was performed by streaking the bacterial colonies on a blood agar plate for a pathogenicity test in humans and animals (Darmawati *et al.* 2021). Bacterial strains were incubated for 18–24 hours at room temperature to observe colony growth. The formation of a clear zone around the colony denoted that bacteria were pathogenic to humans and animals

2.5. Metabolic Characterization

The metabolic ability of the representative crude oil-degrading bacteria was ascertained by performing different biochemical and physiological tests and through further identification with 16S rRNA. Gram staining is the most common technique used to differentiate Gram-positive (Gram+) and Gram-negative (Gram-) bacteria *in vitro* (Becerra *et al.* 2016). The tolerance of bacterial strain towards various physiological stresses was assessed by growing the isolates under different NaCl concentrations (0–12% w/v), pH (3–10), and temperature (-18–50°C). To determine the catalase activity of each strain, the pure grown colony was streaked onto the object-glass and was dropped in a 3% (v/v) hydrogen peroxide (H_2O_2) solution (Sarkar *et al.* 2017; Upadhyay *et al.* 2015). The Oxidase test was performed by streaking the bacterial colony on the oxidase strip (Microbact™), where the bacteria that produced oxidase were marked in blue. A sugar fermentation test was also conducted for several substrates: glucose, lactose, and sucrose.

The bacterial strains to be used for the potential degradation of crude oil were selected based on their continuous growth and were subjected to 16S rRNA sequencing (PT. Genetika Science Indonesia) for identification. The obtained sequences were submitted in GenBank and by using the Basic Local Alignment Search Tool (BLAST), and the homology of the sequences was matched with the known 16S rRNA. The phylogenetic tree was constructed to establish the relationship among the strains using MEGA 7.0 software with a maximum likelihood method.

2.6. Microcosm Biodegradation Experiment

To ensure the exponential log-phase growth of the bacterial culture, a pre-culture was prepared from the pure colony selected for inoculation. First, one loop of the colony was aseptically transferred into a flask of oil broth medium + 0.001% yeast extract. The flask was shaken for three days on an orbital shaker at room temperature at 150 rpm. After gaining density of 10^7 CFU/ml, 5 ml of bacterial suspension was inoculated into a flask containing oil medium with crude oil (2% v/v). The total volume of the solution was 50 ml, with the amount of medium 45 ml, and the crude oil added 2% is 1 ml. The control used was a medium containing crude oil but without bacterial isolates. The flask was shaken for two weeks on an orbital shaker at 150 rpm at room temperature. Cell growth was calculated by monitoring cell turbidity, which was measured at an optical density of 600 nm ($O.D._{600\ nm}$) (Cai *et al.* 2021; Loureiro *et al.* 2020) with a UV-VIS Shimadzu UV 1,280. (TPHs) were measured on day 14 using hexane extraction as a gravimetry method (Behera *et al.* 2021).

2.7. Hydrocarbon Substrate Utilization Test and Biosurfactant Production

Crude oil-degrading bacteria were grown in an oil broth medium containing 500 mg/L phenanthrene (Phe) and fluorene (Flr) as carbon and energy sources (Acer 2021; Oyehan and Al-Thukair 2017). The bacterial cultures were incubated on a 120-rpm shaker for seven days at room temperature. After gaining a density of approximately 10^7 CFU/ml, 1 ml of bacterial culture was transferred to a new oil broth medium containing 500 mg/L Phe and Flr, and the solution was incubated under the same conditions for another period of seven days. This procedure was performed to ensure that the bacteria could withstand the applied pressure. After incubation, the bacterial cultures (10^7 CFU/ml) were grown on oil agar media coated with 50 µl of Phe and Flr.

The biosurfactant-producing bacteria were screened through the techniques of blood agar lysis (Ibrahim 2018), oil-spreading (Techaoei *et al.* 2007, 2011), and emulsification with solid paraffin (Johnson *et al.* 1992). The blood agar lysis test was

carried out based on the modified method described in Ibrahim (2018) method by dipping the sterile disc paper into the supernatant and placing it on a blood agar medium. A clear zone on blood agar indicated that the bacteria could produce biosurfactants. The oil spreading test was carried out on a petri dish containing 50 ml distilled water, added with 20 µl oil, which formed a thin layer on the surface. The 10 µl supernatant was carefully inserted into the middle of the oil layer for each bacterial strain. A clear zone was formed when the supernatant contained biosurfactants. The emulsification test was carried out with 4.5 ml supernatant mixed with 0.5 ml of solid paraffin. Then, this mixture was vortexed for one minute and was incubated for two hours to measure the emulsion stability at 610 nm OD.

2.8. Total Petroleum Hydrocarbon Analysis

The (TPH) analysis was carried out by modifying methods from Behera *et al.* (2021), Li *et al.* (2021), and Mostafa *et al.* (2021). The 50 ml culture broth in the flask extracted the residual TPH by mixing the broth with 50 ml *n*-hexane (1:1), and then adding an anhydrous Na_2SO_4 to remove the water content. The mixture was shaken for 10 minutes, and the upper organic layer containing TPH was separated using a separating-funnel. Then, a silica gel was added to remove the polar compounds before the filtration process, after which the extract obtained was transferred into a boiling flask and was concentrated with a rotary evaporator until entirely separated from the solvent. The extract was placed in a weighed porcelain dish and was dried in an oven at 70°C for 45 minutes then, it was cooled in a desiccator and weighed.

3. Results

3.1. Isolation and Identification of Crude Oil-Degrading Bacteria

Based on the pathogenicity test results, two bacterial strains were identified as potentially non-pathogenic, namely CYA20 and CYA27, which both appeared morphological different. The identification and characterization details, including colony shape, margins, elevation, pigmentation, and optical shape, are shown in Table 1.

Based on the phylogenetic analysis, the CYA20 and CYA27 strains showed a 100% sequence similarity (Figure 1) to *Acinetobacter baumannii* (accession KF498713.1) and *Bacillus subtilis* (accession AY162130.1).

Table 1. Characteristics of strains

Characteristics	Strains code	
	CYA20	CYA27
Location	Pond grass rhizosphere	Mangrove surface
Identification 16S rRNA	*Acinetobacter baumannii*	*Bacillus subtilis*
Colony shape	Irregular	Circular
Colony margins	Undulate	Entire
Colony elevation	Convex	Flat
Colony pigmentation	Nonpigmented	Nonpigmented
Colony optical properties	Opaque	Opaque
Gram stain	Gram-	Gram +
Cell shape	Cocci	Bacilli

3.2. Pathogenicity Test Response

On the sixth day, the results showed no necrosis symptoms, followed by no color change in the leaf bones. This indicates that none of the bacterial strains were pathogenic to plants. At 24-hour incubation, none of the strains showed clear zones and color changes.

3.3. Metabolic Characterization of Bacterial Strains

The physiological and biochemical characteristics of bacterial strains were determined in relation to relevant properties associated with the survival under crude oil-enriched conditions (Figure 2). It was found that bacterial strains could perform catalase and oxidase activities, grow under aerobic conditions, be motile, and ferment several sugars, including glucose, lactose, and sucrose.

3.4. Enumeration of Total Bacterial Population

TPH degradation depends on the adaptability and viability of the bacteria after inoculation. Bacterial strains that could adapt and survive using crude oil as the only carbon and energy source could increase their biomass after incubation. The total bacterial population counted using two methods and calculations were repeated three times (Figure 3).

3.5. Microcosm Biodegradation Experiment

The TPH degradation analysis of the CYA20 and CYA27 bacterial strains is shown in (Figure 4). The

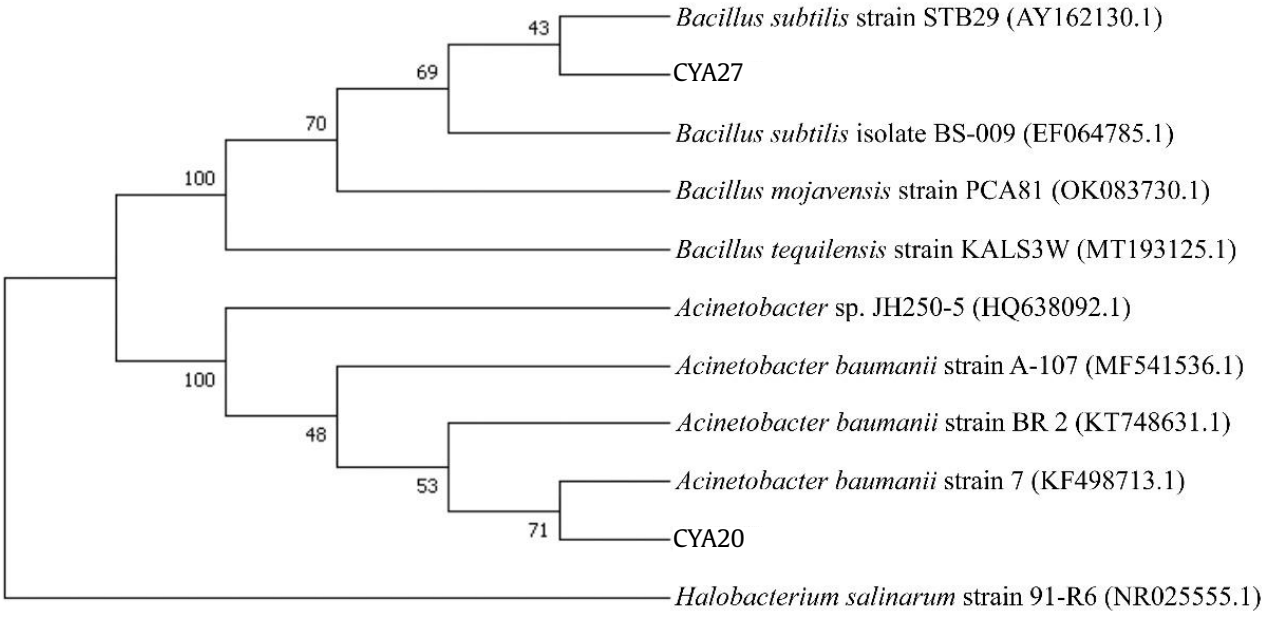

Figure 1. Phylogenetic tree of isolated CYA20 and CYA27 strains using the maximum likelihood with a kimura 2-parameter model, the bootstrap percentage level is presented by the number located at the nodes following the maximum likelihood estimation with 500 replications

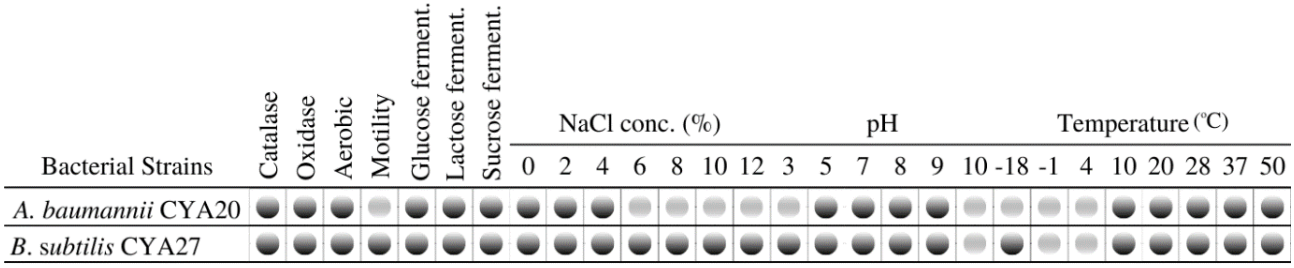

Figure 2. Metabolic properties of crude oil-degrading bacteria. Metabolic properties are presented in terms of positive (black circles) and negative (white circles) responses for each tested character

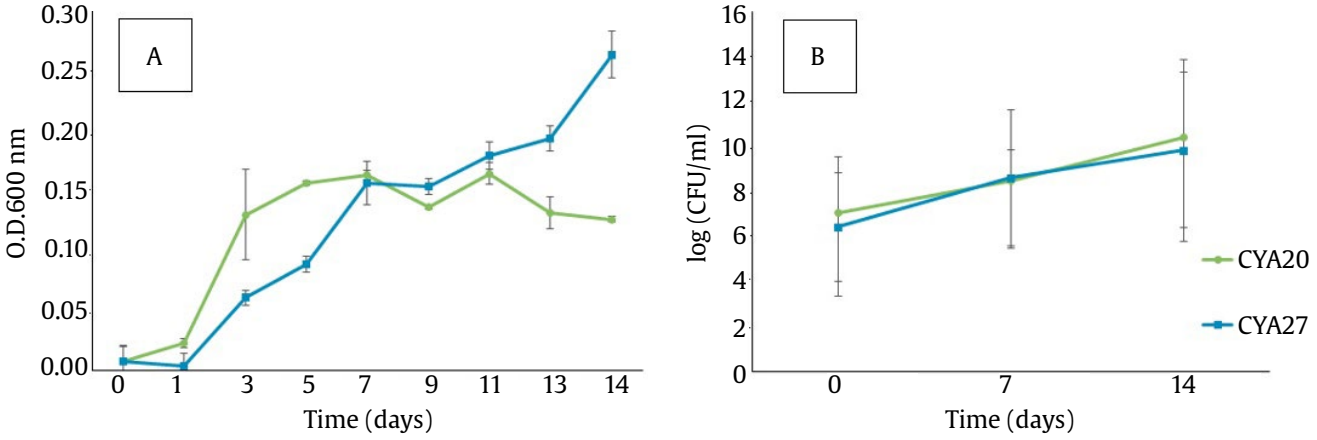

Figure 3. The bacterial growth curve in an oil broth medium containing 2% crude oil using the (A) optical density method and (B) total plate count method

analysis was carried out for 14 days with 2% or 20,000 mg/L crude oil repeated three times. After 14 days of incubation, a decreased TPH value was detected along with a decreased efficiency in CYA20 and CYA27, measured at 45.67% and 66.33%, respectively, and in the control at 0.7%. The concentration of crude oil was reduced from 20,000 mg/L to 10866.67 mg/L in CYA20, 6733.67 mg/L in CYA27, and 19860 mg/L in the control.

At the beginning of the incubation period (Figure 5A), crude oil was spread over the medium surface and remained clearly separated from the medium layers. During seven day incubation period (Figure 5B), the crude oil dispersion was observed. Several tiny crude oil droplets were formed, dispersed, or emulsified in the aqueous phase. Some crude oil was aggregated in tiny droplets (biofloccula), which were observed at the water surface and aqueous phase. After 14 days of the incubation (Figure 5C), the crude oil was reduced. This phenomenon shows that both strains produced biosurfactants, or secreted a surface exopolymer that acted as a surfactant, to facilitate the crude oil biodegradation process.

3.6. Utilization of Hydrocarbon Substrates and Production of Biosurfactants

The CYA20 and CYA27 strains were cultured separately in an oil broth medium containing phenanthrene and fluorene at a concentration of 500 mg/L, respectively, were grown over a period of 48 hours (Table 2).

The results of the blood agar lysis, oil spreading, and emulsification tests showed that the CYA20 and CYA27 strains produced biosurfactants (Table 2). The supernatant of the two strains showed hemolytic activity as a β-hemolysis pattern, indicating a complete red blood cell lysis (Figure 6A and B). This hemolytic activity is related to the strains ability to produce biosurfactants. Positive oil-spreading test results were marked as a clear zone when the strain supernatant was dropped (Figure 6C and D).

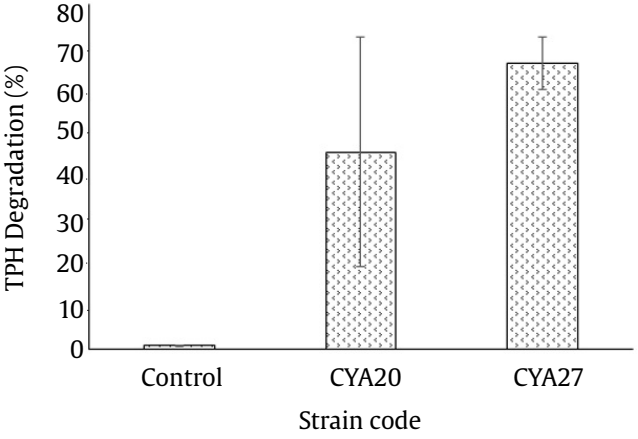

Figure 4. Percentage of TPH removal

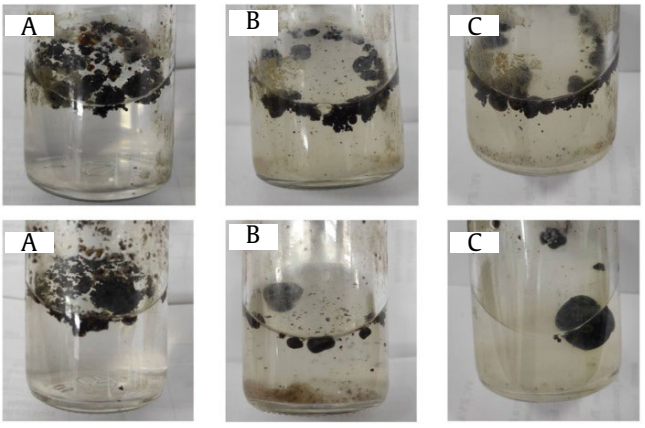

Figure 5. The visual of the microcosm flasks for the two bacterial strains at the start of the experiment period (A), after 7 days (B), and after 14 days (end of the experiment period) (C). The top picture is the CYA20 strain, and the bottom is the CYA27 strain

Table 2. Results of tests on hydrocarbon substrate utilization and production of biosurfactants

Strain code	PAHs compounds		Biosurfactant-producing		
	Phe	Flr	Blood agar lysis	Oil spreading	Emulsification activity (O.D.610 nm)
CYA20	+	+	+	+	0.533
CYA27	+	+	+	+	0.373
Control	-	-	-	-	0.068

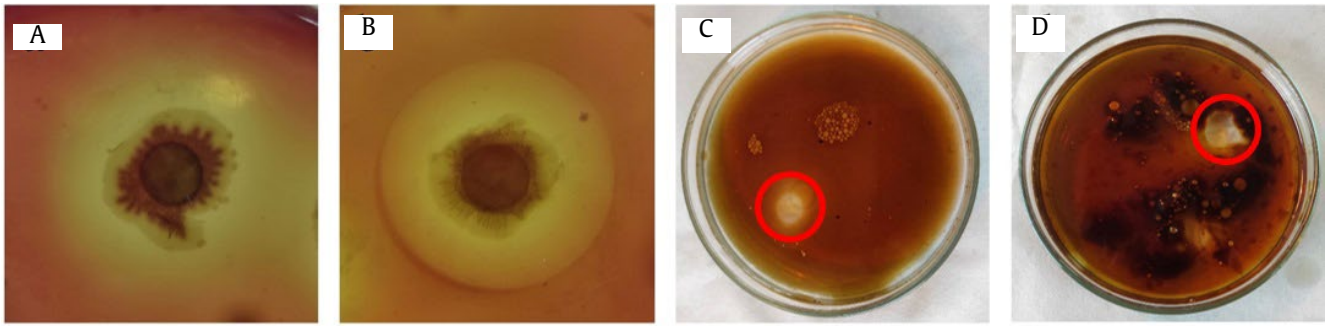

Figure 6. Identification of clear zones for strains CYA20 and CYA27 using the blood agar lysis method, (A) and (B), respectively; and the oil spreading method, (C) and (D), respectively. Biosurfactant production is presented as positive (+) and negative (-) responses for each test

4. Discussion

4.1. Isolation, Characterization, and Identification of Crude Oil-Degrading Bacteria

Based on the samples collected in six locations along the northern coast of Bekasi, two isolates of single culture were identified from an enrichment medium containing crude oil as a carbon source, namely the CYA20 and CYA27 strains, which were tolerant to NaCl concentrations of up to 4% and 12%, respectively. These strains were also tolerant to the pH values ranging between 5 and 9, and temperatures between 10°C and 50°C (Figure 2). Kim *et al.* (2019) showed that bacteria that were tolerant to high-salinity(12%) had degradation efficiency under extreme conditions, which could specifically possible to degrade crude oil in soils and sediments in coastal or deep-sea areas. Various crude oil-degrading bacteria have been isolated from the environment, such as *A. baumannii* from the pond water (Tan *et al.* 2018), *Acinetobacter* from coastal seawater (Chen *et al.* 2020), *B. subtilis* from the polymer dump site (Sakthipriya *et al.* 2015) and *Bacillus* from an oil well in the oil field (Li *et al.* 2020).

All the bacterial strains identified in the present study are non-pathogenic. Bacteria could not grow on tobacco leaves because their cells were incompatible with these plant components. Necrosis only occurred in tobacco leaves due to the compatibility with leaf cells. Both the CYA20 and CYA27 strains were marked as non-pathogenic plants, and neither of them showed a clear zone based on the hemolysis test on blood agar medium. This means that none of the bacterial strains could lyse blood in the medium or were pathogenic to humans and animals. Based on Maheshwari *et al.* (2019), the condition where no blood lysis occurrs in the blood agar medium is referred to as gamma (γ) hemolysis.

The phylogenetic analysis revealed that the CYA20 and CYA27 strains had a 100% sequence similarity (Figure 1) to *Acinetobacter baumannii* (accession KF498713.1) and *Bacillus subtilis* (accession AY162130.1). These bacteria commonly correspond to a bacterial group known for crude oil hydrocarbons degradation capability in the sea and mangroves, such as *Bacillus, Acinetobacter, Pseudomonas*, and *Rhodococcus* (Chen *et al.* 2020; Kim *et al.* 2019; Syakti *et al.* 2013; Salamat *et al.* 2018). Tan *et al.* (2018) and Sakthipriya *et al.* (2015) reported that *A. baumannii* and *B. subtilis* were marine bacteria capable of degrading crude oil pollutants.

4.2. Enumeration of Total Bacterial Population

The increased OD value observed in each strain denoted presence of bacterial growth in the medium. Specifically, a more turbid medium indicates that the bacterial strain could degrade crude oil by using it as a carbon source. The OD values in CYA20 and CYA27 strains increased after 14 days of incubation (Figure 3A). The log unit (CFU/ml) represented the increased bacterial biomass obtained during the treatments. The initial bacterial population or log-phase was about log 6–7, and during this log-phase, bacteria adapted to efficiently utilize oil hydrocarbons as the sole carbon source, which led to their increased growth. During the first seven days of incubation, bacterial growth enters an exponential phase marked by an increase in the bacterial population (Figure 3A), where on day seven, the population of oil-degrading bacteria was about log 7–9 and continued to increase to log 9–10 at the end of the incubation period (Figure 3B). The degradation of crude oil pollutants by indigenous bacteria occurred more quickly because these species could adapt to the conditions of the oil source. Behera *et al.* (2021), have noted that it is essential to maintain bacterial activity during the degradation phase to obtain a better degradation level.

4.3. Microcosm Biodegradation Experiment

Crude oil could be used as a carbon and energy source by both strains during incubation period. Generally, at the beginning of the degradation process,

Table 3. PAH degraders isolated from different environments worldwide

Sites	Country	Isolates	Substrates	References
Pond grass rhizosphere from the North Coast of Bekasi	Indonesia	*Acinetobacter baumannii*	Crude oil, phenanthrene, fluorene	This study
Mangrove surface sediment from the North Coast of Bekasi	Indonesia	*Bacillus subtilis*	Crude oil, phenanthrene, fluorene	This study
Rhizosphere soil of rice paddies	China	*Acidobacteria, Actiobacteria, Chloroflexi, Pseudomonas, Sphingomonas, Comamonadaceae,*	Naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(α) anthracene, chrysene, benzo(b) fluoranthene, benzo(k) fluoranthene, benzo(α)pyrene, dibenzo(α,h) anthracene, benzo(g,h,i) perylene, indeno(1,2,3-cd) pyreneRice	Lu *et al.* 2021
Soil contaminated by heavy oil waste (HOW)	Indonesia	*Bacillus altitudinis, Salipiger* sp. PR55-4, *Ochrobactrum anthropi*	PAHs, phenanthrene, dibenzothiophene, and fluorene	Yani *et al.* 2020
Beach sediment, Taean	Korea	*Planococcus halocryophilus, Vibrio alginolyticus, Bacillus algicola, Planomicrobium alkanoclastiucm, Zobellella* sp., *Isoptericola chiayiensis, Cobetia marina, Rhodococcus soli, Pseudoalteromonas agarivorans,*	Phenanthrene, pyrene	Lee *et al.* 2018
Sediment of oil-polluted coastal, Jubail	Saudi Arabia	*Pseudomonas aeruginosa, Ochrobactrum intermedium, Cupriavidus taiwanensis, P. citronellolis*	PAHs, pyrene, anthracene, phenanthrene	Oyehan and Thukair 2017

Table 3. Continued

Sites	Country	Isolates	Substrates	References
Mangrove, Cilacap	Indonesia	*Bacillus aquimaris, B. megaterium, B. pumilus, Flexibacteraceae bacterium, Halobacilus trueperi, Rhodobacteraceae bacterium*	Complex hydrocarbons	Syakti *et al.* 2013
PAH-contaminated soil, Guiyu	South China	Mycorrhiza, *Acinetobacter* sp.	Phenanthrene, pyrene	Yu *et al.* 2011

oil is dispersed and forms a thin layer. Then, crude oil-degrading bacteria secrete surface exopolymers that allow them to bind to the oil and begin the emulsion formation in an aqueous environment (Tan *et al.* 2018). The degree of oil dispersion determines the oil surface area that bacteria can utilize. Bacteria are active at the oil-water interface, and the availability of this layer can increase their biodegradation activity (Cai *et al.* 2021). A higher interface area can easily trigger bacterial activity and initiate the degradation process, which lead a reduced to viscosity and biofloccule formation (Tan *et al.* 2018), ultimately decreasing the TPH value.

4.4. Utilization of Hydrocarbon Substrates and Production of Biosurfactants

An oil medium is a mineral-minimum medium. After the medium's nutrient sources are used for growth, bacteria begin to utilize aromatic carbon compounds, namely phenanthrene and fluorene, as carbon and energy sources (Table 2). Several bacteria can utilize hydrocarbon substrates in the form of these two compounds for their growth (Yani *et al.* 2020). Many researchers are interested in contributing to discovering PAH decomposers from various sources to treat PAH pollutants (Table 3).

One of the bacterial mechanisms of oil degradation is the production of biosurfactants, which can reduce the oil and water surface tension and mobilize the oil trapped in soil particles by increasing the capillary numbers. The oil layer is emulsified and formed micelles which spread over the surface. Micelles are created due to hydrophobic and hydrophilic fusion and surface tension reduction (Techaoei *et al.* 2011). Base on the spectrophotometry results at wavelength of 610 nm, the CYA20 and CYA27 strains produced biosurfactants that could emulsify solid paraffin and showed OD values of 0.533 and 0.373 for CYA20 and CYA27, respectively, and 0.068 for the control. Various biosurfactant-producing strains have been isolated from the marine environment, such as *A. baumannii* OCB1 from the petroleum refining industry waste (Goveas and Sajankila 2020), *A. baumannii* BJ5 from petroleum-contaminated soil (Gupta *et al.* 2020), *B. subtilis* B30 from crude oil-contaminated soil (Al-Wahaibi *et al.* 2014), *B. subtilis* RI4914 from oilfield production water formation (Fernandes *et al.* 2016), and *B. subtilis* MG495086 from oil reservoir water formation (Datta *et al.* 2018). Biosurfactants can function as emulsifiers by decreasing the surface tension and forming micelles. The microdroplets encapsulated in the hydrophobic microbial cell surface are assimilated and degraded (Das and Chandran 2011).

In conclusions, both strains the *Acinetobacter baumannii* CYA20 and *Bacillus subtilis* CYA27 were shown to survive at high salinity levels, pH values between 5 and 9, and temperatures between 10°C and 50°C, where they were isolated from a crude-oil contaminated environment in the northern coast Bekasi, Indonesia. They could maintain the growth levels ranging from log 6 to log 10 on day 14 of the incubation period, and were also able to disperse crude oil in tiny droplets (biofloccula) with a TPH reduction efficiency of 45.67% in *A. baumannii* CYA20 and 66.33% in *B. subtilis* CYA27. Both strains could biosurfactants production and emulsify paraffin to degrade the crude oil and POP compounds (phenanthrene and fluorene) as the main carbon and energy sources.

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