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ISOLATION AND IDENTIFICATION OF HYDROCARBON-DEGRADING BACTERIA THAT TOLERANT TO SAPONIN OF *Sapindus rarak* PLANT

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Abstract. *A commercial saponin as biosurfactant can reduce the surface tension of water and increase of hydrocarbon degradation. However, this saponin can be toxic to some hydrocarbonoclastic bacteria. This study aimed to obtain bacterial isolates that were tolerant and incapable to degrade saponin, and to identify them based on 16S rDNA sequence. Bacteria were isolated from petroleum contaminated soil in Wonocolo Village, Bojonegoro Regency, East Java, Indonesia. The soil samples were acclimated using Bushnell-Haas (BH) broth with 0.5% crude oil at room temperature for 3 weeks. The culture was spread onto BH agar incubated at 30°C for 7 days. The first screened, isolates were grown in nutrient broth with addition of saponin 0%, 8%, and 12% (v/v) then incubated at 30°C for three days. The bacterial cell density was measured using a spectrophotometer. Second screened, the isolates were grown on BH broth with addition of 0.5% saponin as a sole carbon source, and their cell densities were measured. The selected isolates were identified based on 16S rDNA sequences. Among 34 bacterial isolates, nine isolates were tolerant to 12% saponin. Three bacterial isolates IHT1.3, IHT1.5, and IHT3.24 tolerant to high concentration of saponin and did not use this substance as growth nutrition. The IHT1.3, IHT1.5, and IHT3.24 isolates were identified as Ochrobactrum pseudogrignonense (99% similarity), Pseudomonas mendocina (99%), and Ochrobactrum pituitosum; (97%), respectively. Those three selected isolates are good candidates as hydrocarbon-degrading bacteria to bioremediation of soil contaminated crude oil. However, the combined activity of bacteria and saponin to degrade hydrocarbon needs further study.*

Keywords: *Ochrobactrum pituitosum, Ochrobactrum pseudogrignonense, sapindus rarak plant, saponin commercial, Pseudomonas mendocina*

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INTRODUCTION

Indonesia is one of the countries in the world that has a lot of petroleum reserves. Petroleum reserves, in 2016 were approximately 3.6 billion barrels and half of them are located in East Java Province (Kompas, 2016). Bojonegoro Regency, Wonocolo Village, as one of the petroleum producing areas in East Java Province is known to have

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several petroleum wells which cause pollution to the soil. Petroleum contaminated soil can be used as a carbon source for microorganism growth, such as hydrocarbonoclastic bacteria, fungi and yeast (Chikere & Azubuike, 2014). Application of hydrocarbonoclastic bacteria is cost-efficient and environmental friendly (Quatrini et al., 2008). Hydrocarbonoclastic bacteria can oxidize various types of hydrocarbon compounds by producing biosurfactant (Kostka et al., 2011).

Biosurfactants aren't only produced by bacteria but also produced by plants (Guo et al., 2008). *Sapindus rarak* can produce compounds in the form of saponins. Saponins can be obtained by extracting the plants themselves or available commercially. Commercial saponins are popular traditional detergent product among local people, used as batik washing material in Indonesia. There are many benefits from saponin application, especially in the field of biotechnology. Saponins can decrease water surface tension and it is less harmful (Kobayashi et al., 2012). In previous research, the interaction between *Sphingomonas* sp. and saponins 0.25%-5%, obtained from *Sapindus mukorossi* plants was able to increase degradation of pyrene and phenanthrene (aromatic hydrocarbons) (Kobayashi et al., 2012). Other studies also mentioned that combination treatment of 5% commercial saponins and bacteria increased hydrocarbon degradation (Soeder et al., 1996).

However, not the whole extract saponins from each plants species can synergize with the hydrocarbonoclastic bacteria. This has been proven by other studies, where saponin extract from some plants was toxic to bacterial cells from the rumen of sheep and ruminants. Some of the plants are *Sesbania sesban*, *Quillaja saponaria*, *Acacia auriculiformis*, *Sapindus saponaria* and *Enterolobium cyclocarpum* (Guo et al., 2008). So, the purpose of this study was to isolate bacteria based on the level of tolerance to saponins and don't have degradation activity of saponins.

MATERIALS AND METHODS

A Sampling of Hydrocarbon from Contaminated Soil

Soil samples were collected from traditional petroleum mining at Wonocolo Village, Bojonegoro Regency, East Java Province, Indonesia. Petroleum contaminated soil were taken from three-sites at 0-10 cm depth below the soil surface using a sterile shovel (Panda & Sarkar, 2012). Soil sample at each sampling site was taken randomly from five points and composite as one soil sample. Soil samples were collected into a labeled plastic bag and put into a cool box containing ice cubes. The soil samples were then taken to the laboratory within 18 hours.

Isolation and Enrichment of Hydrocarbonoclastic Bacteria

Soil samples as much as 2 g suspended in 100 mL BH broth in the Erlenmeyer flask 250 mL, 0.5 % crude oil at pH 6.8 were then added. The BH broth medium consist of 0.85 % NaCl, 0.02 g/L MgSO4, 0.002 g/L CaCl2, 0.1 g/L KNO3, 0.05 g/L FeCl3, 3.6 g/L K2HPO4, 1.2 g/L KH2PO4 and 1.0 liter of distillation water (Udgire et al., 2015). The cultures were incubated at room temperature on shaker incubator 120 rpm for 7 days. 5 mL of culture suspension was inoculated into BH broth medium and it conducted three times. The culture enrichment was done to obtain the most effective strains of hydrocarbon degrading bacteria (Udgire et al., 2015). The 0.1 mL of culture suspension at 10-7 dilution in 0.85 % sodium chloride solution was spread on BH agar with the addition of crude oil. The culture was incubated at 30°C for a week.

Screening of Hydrocarbonoclastic Bacteria

Pure culture of each isolate was screened in a two-step. The first step, bacteria inoculated into the nutrient broth with added by 0, 0.5, 8.0 and 12.0% of saponin (Soeder et al., 1996). The culture was then incubated at room temperature in shaker incubator 120 rpm for 20 hours. The optical density of each isolate was adjusted to get similar cell density. Each bacterial culture with similar cell density of approximately 3 mL was inoculated into 27 mL of nutrient broth containing saponins 0, 8 and 12%. The culture incubated at room temperature in shaker incubator 120 rpm for 3 days. The growth of the bacterial isolates was measured using a spectrophotometer at 600 nm wavelength (Al-Wasify & Hamed, 2014). The Isolates that were able to growth in nutrient broth with a high concentration of saponins to be used for the next screening. The second step, suspension of bacteria culture was inoculated into BH broth with the addition of 0.5% of saponin as a sole carbon source and it was incubated at room temperature on shaker incubator 120 rpm for 20 hours. The suspension of 3 mL bacterial culture with similar cell density was inoculated into 27 mL BH broth containing 0.5% saponin. The culture incubated at room temperature in shaker incubator 120 rpm for 3 days. The bacterial cell density was measured using a spectrophotometer at 600 nm wavelength (Al-Wasify & Hamed, 2014). The selected bacteria isolate was determined based on the highest ability to growth in BH broth with contain hydrocarbon and highest concentration of saponin and it could not able to use of saponin as a sole carbon source.

Identification of Bacteria Based on 16S rDNA Sequence Similarity

The chromosomal DNA of selected bacteria was isolated using i-genomic DNA Extraction Soil Mini Kit from gene aid Biotechnology, Inc. The 16S rDNA sequence was amplified using 16S rDNA universal primers, 27f (5'-GAG AGT TTG ATC CTG GCT CAG -3') and 1495r (5'- CTA CGG CTA CCT TGT TAC GA -3') (Gochhait et al., 2007). The amplification of 16S rDNA sequence using Polymerase Chain Reaction machine. The PCR procedure was started with initial denaturation at 94°C for 5 minutes, it continued with total 35 cycles of denaturation (94°C; 30 s), annealing (52°C for 30 s) and elongation (72°C; 1.5h) and final elongation at 72°C for 7 minutes (Promega, 2016). The amplicon of 16S rDNA sequence was visualized using 1.5% agarose gel electrophoresis.

Statistical Analysis

All of the data results were statistically analyzed using One Way Analysis of Variance (ANOVA) by SPSS 16.0 software for windows with significant level p-value <0.05.

1st-BASE of amplicons sequencing at PT. Genetika Science, Jakarta. Afterward, the sequences were used to identify the bacteria using BLAST nucleotides from NCBI. The identified isolates were compared to the reference sequences from the GenBank database to construct the phylogeny tree using MEGA 5 software for windows based on the Neighbor-Joining algorithm and Tamura-Nei model.

RESULTS AND DISCUSSION

Isolates of Hydrocarbonoclastic Bacteria from Petroleum-Polluted Soil

The total of 34 bacterial isolates were found from Petroleum-Polluted Soil that could growth on BH agar containing crude oil. Those isolates consist of 6 isolates of Gram-positive and 28 isolated of Gram-negative and cell-shape consist of 6 isolates of coccus and 28 isolates of rod (Table 1). The whole of isolates

have morphology of colony spherical shaped, entire margin and 18 isolates flat-elevation, while 16 isolates have an elevation-convex.

A lot of isolates that were isolated from petroleum contaminated soil samples it was due to the enrichment process that carried out 3 times.

Table 1. Macros and micro characteristics of bacteria isolated from petroleum contaminated soil in Wonocolo Village, Bojonegoro Regency

	Colony characteristic					Cell characteristic	
Isolate	Shape	Elevation	Margin	Pigmentation	Diameter	Shape	Gram
IHT1.1	circular	flat	entire	white	0.52	coccus	+
IHT1.2	circular	flat	entire	white	0.52	rod	-
IHT1.3	circular	convex	entire	tan	0.63	rod	-
IHT1.4	circular	convex	entire	cream	0.54	coccus	-
IHT1.5	circular	convex	entire	cream	0.47	rod	-
IHT1.6	circular	flat	entire	white	0.42	rod	+
IHT1.7	circular	flat	entire	cream	1.27	rod	+
IHT2.1	circular	convex	entire	white	0.26	rod	-
IHT2.2	circular	flat	entire	white	1.13	rod	-
IHT2.3	circular	convex	entire	cream	1.20	coccus	-
IHT3.1	circular	flat	entire	white	0.77	rod	-
IHT3.2	circular	flat	entire	white	0.78	rod	-
IHT3.3	circular	convex	entire	white	1.12	rod	-
IHT3.4	circular	convex	entire	tan	0.39	coccus	-
IHT3.5	circular	flat	entire	tan	1.43	rod	-
IHT3.6	circular	convex	entire	white	0.75	rod	-
IHT3.7	circular	flat	entire	white	1.97	rod	-
IHT3.8	circular	convex	entire	cream	0.46	rod	-
IHT3.9	circular	flat	entire	cream	1.13	rod	-
IHT3.10	circular	flat	entire	white	1.30	rod	-
IHT3.11	circular	convex	entire	white	0.55	rod	-
IHT3.12	circular	flat	entire	white	1.24	rod	+
IHT3.13	circular	flat	entire	cream	1.18	rod	+
IHT3.14	circular	convex	entire	white	2.16	rod	-
IHT3.15	circular	flat	entire	white	1.44	coccus	+
IHT3.16	circular	convex	entire	cream	0.32	coccus	-
IHT3.17	circular	flat	entire	tan	2.28	rod	-
IHT3.18	circular	flat	entire	white	2.09	rod	-
IHT3.19	circular	convex	entire	tan	1.12	rod	-
IHT3.20	circular	convex	entire	cream	0.33	rod	-
IHT3.21	circular	flat	entire	cream	1.76	rod	-
IHT3.22	circular	convex	entire	white	0.75	rod	-
IHT3.23	circular	flat	entire	white	1.94	rod	-
IHT3.24	circular	convex	entire	tan	0.46	rod	-

The Selection of Hydrocarbonoclastic Bacteria

Hydrocarbonoclastic bacteria were selected based on ability to growth on nutrient broth medium with the addition of saponin. At the first screening, 9 isolates namely IHT1.3, IHT1.5, IHT3.8, IHT3.14, IHT3.17, IHT3.19, IHT3.20, IHT 3.23 and IHT3.24 growth better than other isolates (Figure 1a, b, c). Futhermore, those 9 isolates were screened for second stage.

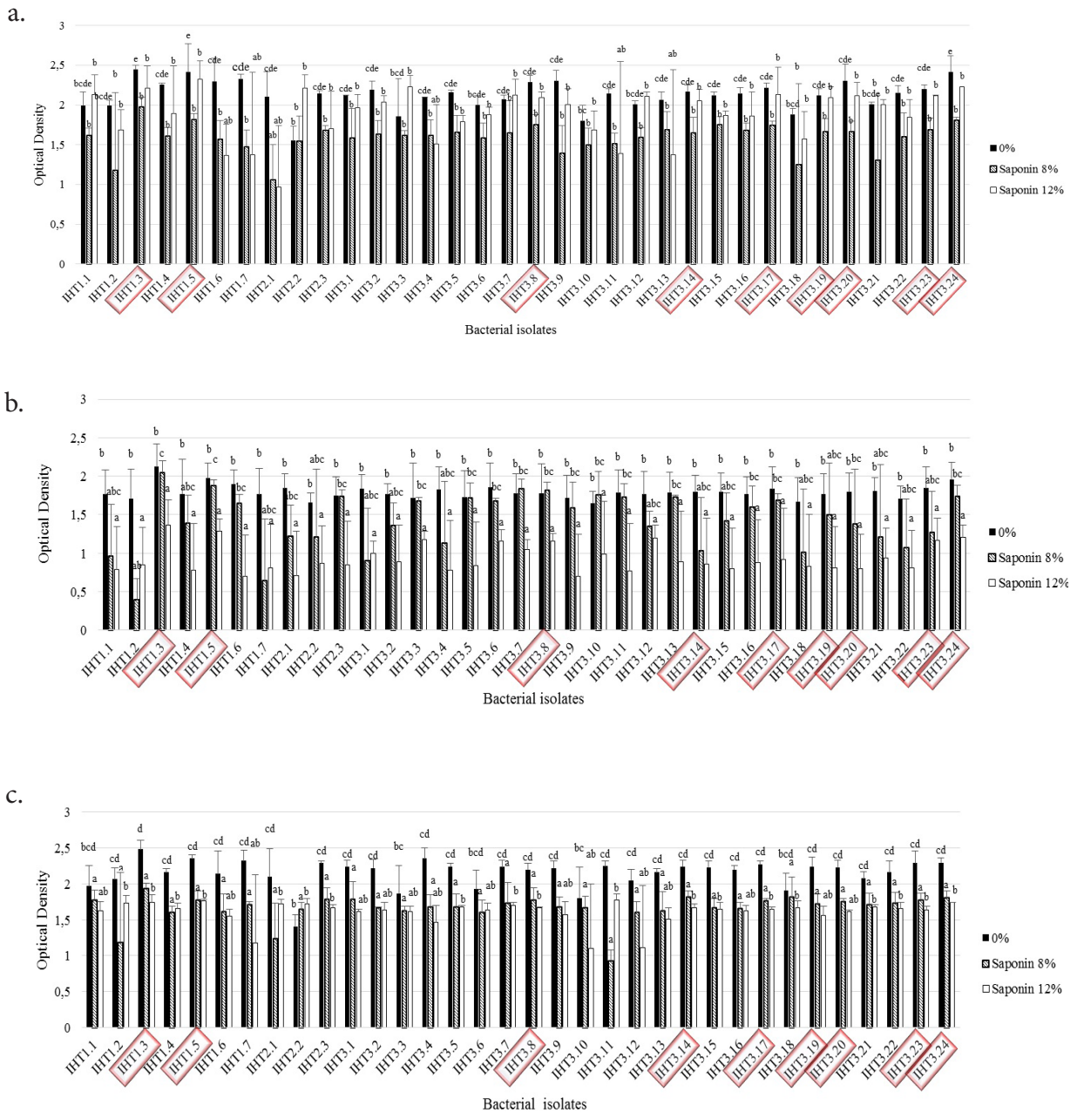

Figure 1. Cell density of Hydrocarbonoclastic bacteria on nutrient broth with the addition of saponin first day (a), second day (b), and third day (c) incubation time

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The hydrocarbonoclastic bacteria was growth significantly at the first day on the nutrient broth containing saponin. Whereas, cell density of those hydrocarbonoclastic bacteria did not differ significantly at second and third day of incubation, it caused by the bacteria culture was at a stationary growth phase. This means that the number of new cells is equal to the number of cells that die or its growth completely stopped and generate stationary growth (Marecik et al., 2012). This is due to lack of nutrients and decreasing of medium pH, so the bacterial cell change physiologically. This change aims to let cells survive on a longer period of time or in unfavorable condition (Carrero-Colón et al., 2006). This stationary growth phase was evidenced by the absence of significant growth (increase cell density significantly) at the second and third days of incubation time (Figure 1b & 1c). The presence of saponin at some concentrations, didn't have a negative impact on bacterial cell growth. Saponin from various plants caused a wide range of cell toxicity mechanism from molecular to cellular level (Guo et al., 2008). However, the 9 selected isolates were able to grow well in this condition, it showed that the bacteria have resistance to high concentrations of saponin. Whereas from other studies it was explained that saponins with a concentration of 10% can have a toxic effect on bacterial cells (Kobayashi et al., 2012). This experiment showed that saponins with high concentrations of up to 12% did not negatively affect to the Hydrocarbonoclastic bacteria. So, when combined with bacteria to degrade hydrocarbons, these saponins don't damage bacterial cells rather accelerate the rate of degradation. Those selected of Hydrocarbonoclastic bacteria were assayed for the growth activity on BH broth medium with saponin as a sole carbon source (Figure 2).

The second stage, the 9 bacterial isolates were inoculated into BH broth containing 0.5% concentration of saponin (Al-Wasify & Hamed, 2014). The result showed that 3 isolates (IHT1.3, IHT1.5 and IHT3.24) of hydrocarbonoclastic bacteria were unable to use saponin as sole carbon source for growth nutrition. Those isolates have cell density similarly over three day incubation time, the optical density less than 0.1 (Figure 2). While six isolates of hydrocarbonoclastic bacteria were growth significantly at first day of incubation and it cell density decrease significantly at the second and third of incubation time. Those six isolates showed able to used saponin as a sole carbon source for growth nutrition. So those six isolates were not selected as a candidate of hydrocarbonoclastic bacteria. While the other three isolates of hydrocarbonoclastic bacteria were selected based on ability to growth on BH broth medium contain saponin at high concentration and cannot use saponin as a sole carbon source as growth nutrition.

The Species of Selected Hydrocarbonoclastic Bacteria Based on Phylogeny Identification

The three isolated of hydrocarbonoclastic bacteria Namely IHT1.3, IHT1.5, and IHT3.24 were identified phylogenetically base on 16S rDNA sequence similarity. The figure showed that IHT1.3 and IHT3.24 isolate have higher concentration of 16S rDNA sequence than IHT1.5 (Figure 3) (Lorenz, 2012). The IHT1.3 and IHT 3.24 were identified as Ochrobactrum (Table 1, Figure 4a & 4 c) and IHT1.5 identified as Pseudomonas (Table 1, Figure 4b). Those IHT1.3 and IHT 3.24 isolate was identified as *Ochrobactrum pseudogrignonense* (99% similarity) and *Ochrobactrum pituitosum* (97%), while IHT 1.5 isolate as *Pseudomonas mendocina* (99% similarity).

In the process biodegradation of oil, bacteria must have a mobile genetic elements

(MGEs) such as plasmids. In the plasmid of hydrocarboclastic bacteria, it contain operon that coding some enzymes to degrade of hydrocarbon. Catabolic plasmids that responsible for degradation of hydrocarbon namely incP-9 or Incompatibilities (inc) Plasmid21. Plasmid incP-9 plays to start the process of degradation of oil. Other research explain that the IncP-9 plasmids detected in the Genus of Ochrobactrum and Pseudomonas (Dealtry et al., 2018). From the results of this research obtained three isolates belonging to the Genus of Ochrobactrum and Pseudomonas. These species can be used to biodegrade oil in the environment.

Among 34 isolates of hydrocarbonoclastic bacteria, 3 isolates IHT1.3, IHT1.5, and IHT3.24 were crude oil and saponin tolerant and did not utilize saponin as a carbon source. Those isolates were identified as *Ochrobactrum pseudogrignonense*, *Pseudomonas mendocina* and *Ochrobactrum pituitosum*. The saponin of *Sapindus rarak* can be used as biosurfactants for supporting those selected bacteria to degrade of hydrocarbon and it can be used as a candidate of bioremediation agent of petroleum-polluted environment.

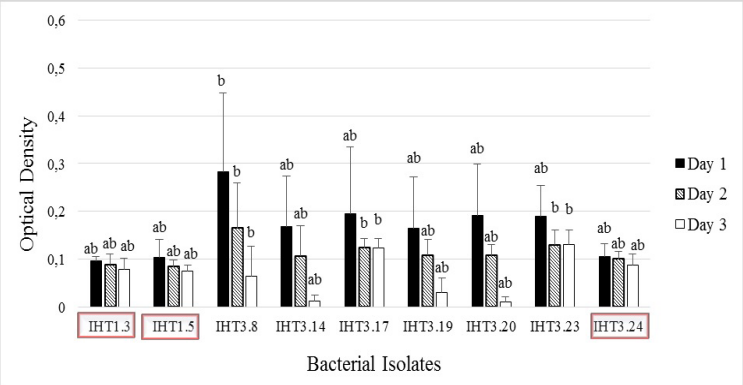


Figure 2. Cell density of Hydrocarbonoclastic bacteria on BH broth with 0.5% concentration of saponin

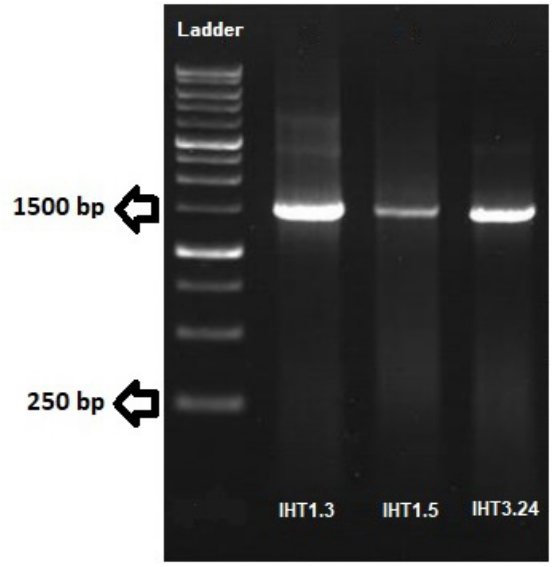
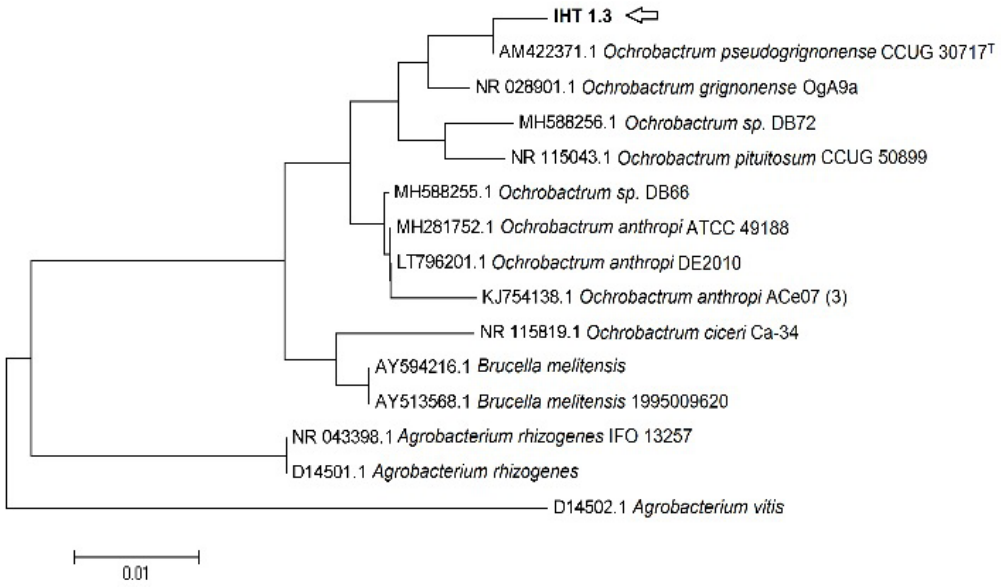
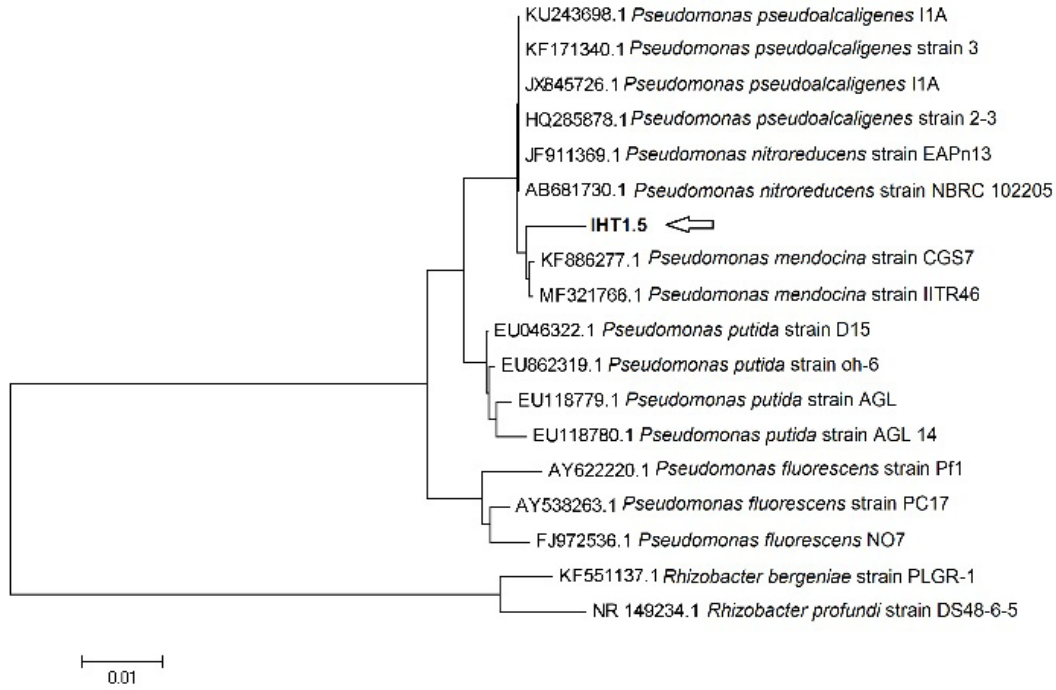


Figure 3. Amplicon of 16S rDNA


IHT 1.3
AM422371.1 Ochrobactrum pseudogrignonense CCUG 30717T
NR 028901.1 Ochrobactrum grignonense OgA9a
MH588256.1 Ochrobactrum sp. DB72
NR 115043.1 Ochrobactrum pituitosum CCUG 50899
MH588255.1 Ochrobactrum sp. DB66
MH281752.1 Ochrobactrum anthropi ATCC 49188
LT796201.1 Ochrobactrum anthropi DE2010
KJ754138.1 Ochrobactrum anthropi ACe07 (3)
NR 115819.1 Ochrobactrum ciceri Ca-34
AY594216.1 Brucella melitensis
AY513568.1 Brucella melitensis 1995009620
NR 043398.1 Agrobacterium rhizogenes IFO 13257
D14501.1 Agrobacterium rhizogenes
D14502.1 Agrobacterium vitis
0.01


KU243698.1 Pseudomonas pseudoalcaligenes I1A
KF171340.1 Pseudomonas pseudoalcaligenes strain 3
JX845726.1 Pseudomonas pseudoalcaligenes I1A
HQ285878.1 Pseudomonas pseudoalcaligenes strain 2-3
JF911369.1 Pseudomonas nitroreducens strain EAPn13
AB681730.1 Pseudomonas nitroreducens strain NBRC 102205
IHT1.5
KF886277.1 Pseudomonas mendocina strain CGS7
MF321766.1 Pseudomonas mendocina strain IITR46
EU046322.1 Pseudomonas putida strain D15
EU862319.1 Pseudomonas putida strain oh-6
EU118779.1 Pseudomonas putida strain AGL
EU118780.1 Pseudomonas putida strain AGL 14
AY622220.1 Pseudomonas fluorescens strain Pf1
AY538263.1 Pseudomonas fluorescens strain PC17
FJ972536.1 Pseudomonas fluorescens NO7
KF551137.1 Rhizobacter bergeniae strain PLGR-1
NR 149234.1 Rhizobacter profundi strain DS48-6-5
0.01

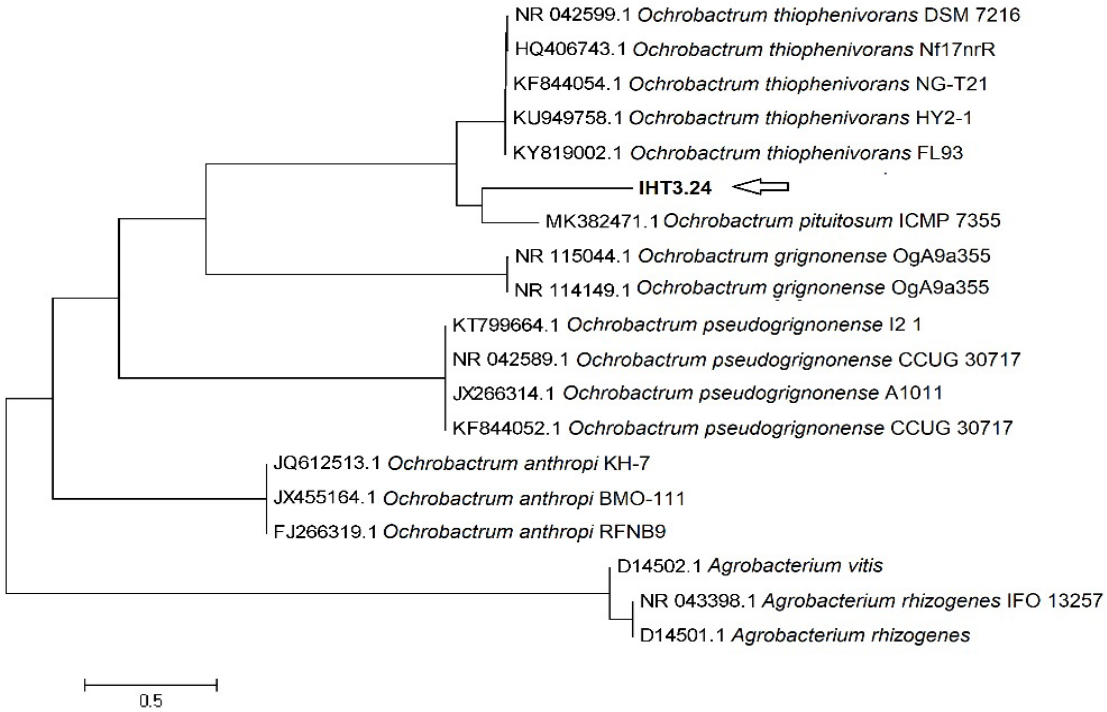


Figure 4. The phylogenetic tree of selected hydrocarboclastic bacteria with reference strains base on 16S rDNA sequence similarity according Neighbor-Joining algorithm and Tamura-Nei model *Ochrobactrum pseudogrignonense* (a), *Pseudomonas mendocina* (b) and *Ochrobactrum pituitosum* (c)

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