MEDIUM CHAIN AND LONG CHAIN ALKANES HYDROXYLASE-PRODUCING WHOLE CELL BIOCATALYST FROM MARINE BACTERIA

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Abstract

Alkanes are major component of crude oil that could be hydrolyzed by the enzyme of alkane hydroxylase. The are three types of alkane hydroxylase based on the chain length of alkane such as short-chain length/SCL (C_2-C_4), medium-chain length/MCL (C_5-C_{17}), and long-chain length/LCL ($C>_{18}$). The aims of this study were to characterize and identify alkanes-degrading bacteria from these bacteria. The 30 strains from marine were grown on MCL (Pentane-C_5H_{12}, Decane-$C_{10}H_{22}$, and Pentadecane-$C_{15}H_{32}$) and LCL (n-Paraffin-$C_{12}H_{19}C_{17}$ and branch of Pristane-$C_{19}H_{40}$). The study showed twenty-nine isolates have the ability to degrade alkanes compounds, whereas 14 isolates have grown ability on MCL and LCL medium, 11 isolates have the ability to grow on MCL and n-LCL, 3 isolates have the ability only to grow on MCL medium and 1 isolate has the ability only grow on n-LCL medium. The growth test result indicated that 29 isolates have medium-chain alkane monooxygenase and long-chain alkane hydroxylase. Based on 16S rDNA gene analysis, we obtained twenty nine of oil- degrading bacteria, namely α-proteobacteria (57 %), γ-proteobacteria (30 %), Flavobacteria (7 %), Bacilli (3%) and Propionibacteriales (3 %). γ-Proteobacteria and α-proteobacteria which seems to play an important role in the alkane biodegradation.

Keywords: alkane, alkane hydroxylase, marine, bacteria

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Introduction

Crude oil constituents are classified into four fractions: saturates hydrocarbons (alkane), aromatics hydrocarbons, resins and asphaltenes (Harayama *et al.,* 1999). Alkanes constitute about 20-50% of crude oil, depending on the source of the oil (Head *et al.,* 2006). As alkanes are non-polar molecules with very low chemical activity, their utilization by microorganisms faces significant challenges, due to several factors, such as low water solubility, high degree accumulation in cell membranes, and higher activation energies (Labinger & Bercaw, 2002). At least sixty genera of aerobic bacteria, and five genera of anaerobic bacteria (Liu *et al.,* 2014), such as Pseudomonas (Zhang *et al.,* 2011), *Acinetobacter* (Maeng *et al.,* 1996), *Rhodococcus* (Van Hamme & Ward, 2001) and Dietzia (Wang *et al.,* 2011), have been reported capable to degrade aliphatic hydrocarbons.

n-alkanes hydrolysis could be initiated by enzymes that belong to different families, among others the microorganisms capable to degrade of short-chain alkanes (SCL) (C_2-C_4) have enzymes related to methane monooxygenase (van Beilen JB & Funhoff EG. 2005). The microorganisms degrading medium-chain length alkanes (MCL) (C_5-C_{17}) generally contain P450s and non-heme iron monooxygenase, such as AlkB (Rojo, 2009). Furthermore, the alkane hydroxylases of long-chain length (LCL) alkanes (>C_{18}) are unrelated to the above alkane hydroxylases as characterized recently. One such hydroxylase, AlmA, is an LC-alkane monooxygenase from *Acinetobacter*. A second hydroxylase is LadA, which is a thermophilic soluble LC-alkane monooxygenase from *Geobacillus* (Cerniglia, 1992; Throne-Holst *et al.,* 2006).

Isolation of alkane-degrading bacteria, the biochemical and genetic analyses of alkane degradation from sub-tropical marine have been reported (Cerniglia, 1992; Throne-Holst *et al.,* 2006), and there is few report of alkane degrading bacteria from tropical marine (Widada *et al.,* 2002; Harwati *et al.,* 2007). However, information of alkane hydroxylase from these isolates not yet been reported. Oil-degrading bacteria from Jakarta bay have been isolated (Yopi *et al.,* 2006), but their ability on degrading the alkane have not been analyzed. It is important to analyze the potency of those isolates and the existing of alkane hydroxylase gene.

The aims of this study were to characterize and identify alkanes-degrading bacteria from the marine area in Jakarta Bay, Indonesia. In this study, we report 29 bacterial strains which were capable of growing on medium with medium chain and long chain alkane that indicated they have various alkanes hydroxylase.

Materials and Methods

Bacteria strain and media

Thirteen bacterial isolates from marine used in this study are the collection of Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). The bacteria were grown in ONR7 medium (Dyksterhouse *et al.*, 1995) including alkane as substrate. The composition of ONR7 medium are 22.79 g NaCl, 11.18 g $MgCl_2 \cdot 6H_2O$, 3.98 g Na_2SO_4, 1.46 g $CaCl_2 \cdot 2H_2O$, 1.3 g 3-[N-tris (hydroxymethyl) methylamino]-2-hydroxypropane sulfonic acid (TAPSO), 0.72 g KCl, 0.27 g NH_4Cl, 89 mg $Na_2HPO_4 \cdot 7H_2O$, 83 mg NaBr, 31 mg $NaHCO_3$, 27 mg H_3BO_3, 24 mg $SrCl_2 \cdot 6H_2O$, 2.6 mg NaF, and 2.0 mg $FeCl_2 \cdot 4H_2O$ for 1 L solution.

Growth Test on Alkanes

The isolates were analyzed for their ability to utilize a variety of alkanes compound with an addition of 50 ppm of n-Pentane (C_5H_{12}), Decane ($C_{10}H_{22}$), Pentadecane ($C_{15}H_{32}$) as substrate-classified this medium into MCL. Paraffin and Pristane ($C_{19}H_{40}$) in the medium were used as a substrate for LCL. The cultures were incubated at 30°C, 1400 rpm for seven days at on Deepwell Maximizer (Bioshaker MBR-022UP) and triplicates for all treatments. The cell density of isolates was measured at λ=600 nm.

16S rRNA gene analysis

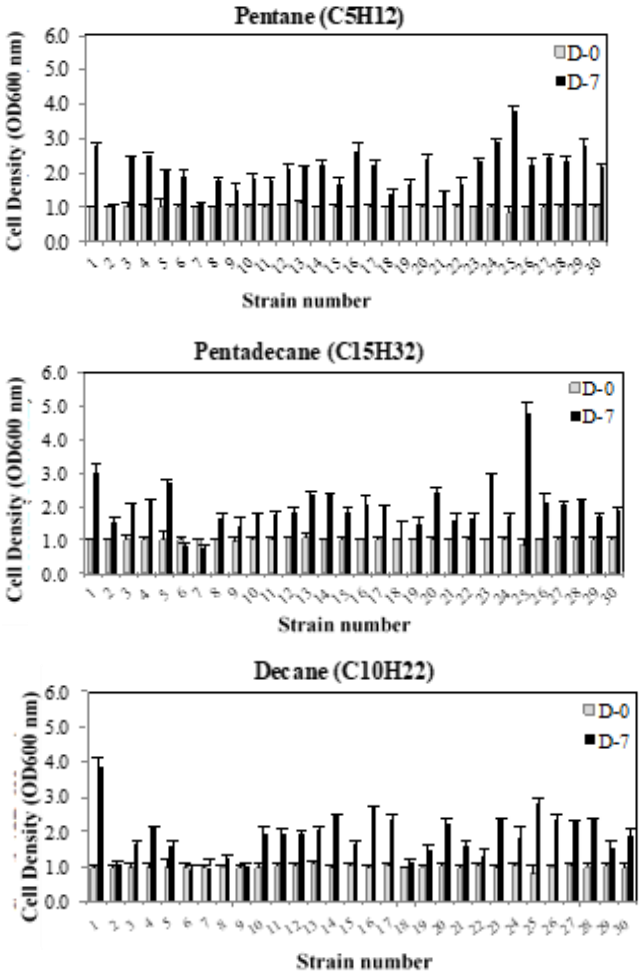
Genomic DNA was isolated using InstaGene (BioRad). DNA of the partial 16S rDNA gene was amplified by using 9F primer (5-AGRGTTTGATCMTGGCTCAG-3) and 1492R primer (5-TACGGYTACCTTGTTAYGACTT-3) (Cayalca *et al.*, 1999). PCR cycle condition are 95°C, 2 min (1 cycle); 95°C, 30 seconds, 65°C, 1 min, 72° C, 2 min (10 cycles); 95°C, 30 sec, 55°C, 1 min, 72°C, 2 min (30 cycles); and 72°C, 2 min (1 cycle). The PCR products were analyzed by electrophoresis on 0.8% agarose gel. The PCR product was also purified using *AGENCOURT® CLEANSEQ® Dye-Terminator Removal* (Beckman Coulter-USA) according to the manufacturer recommendations for sequence analysis. Purified DNA was sequenced using DNA sequencer ABI 310 (Pharmacia). The obtained nucleotide sequences were analyzed with BioEdit. Either BLSTX (Altschul *et al.*, 1997) was used for homology searching. Multiple alignments and phylogenetic tree of 16S rDNA gene were produced by CLUSTAL X (Higgins & Sharp, 1988) and the phylogenetic tree was visualized by the NJ plot program (Thompson *et al.*, 1994) and Mega 3.1 *ABI sequencer software* (Kumar *et al.*, 2004).

Results

Cell Growth in Alkanes

Biodegradation of n-alkanes compound was investigated in presence of medium- and long-chain n-alkanes. The growth test was performed in ONR7 medium in the presence of 50 ppm mixed alkanes (Pentane, Decane, Pentadecane, Paraffin, and Pristane), given as the only carbon and energy source and tested in 7 days. Pentane, decane, and pentadecane are representative of MCL alkane, while paraffin and pristane are LCL alkane. An increase of biomass accumulation was observed by spectrophotometric analysis at OD600.

The 30 strains were tested for their cell growth in ONR7 media containing MCL alkanes (C_5-C_{15}), i.e pentane, decane, and pentadecane. Based on the growth of cells, these isolates could use MCL alkane compounds, except isolates number 5 (Fig.1).



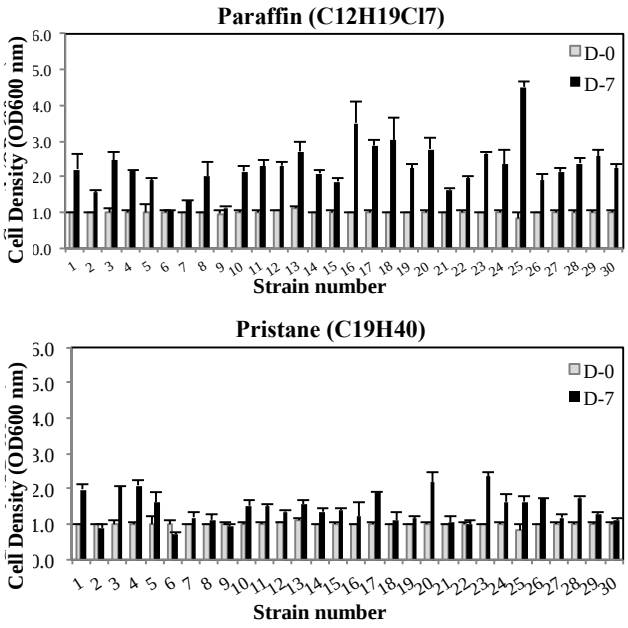
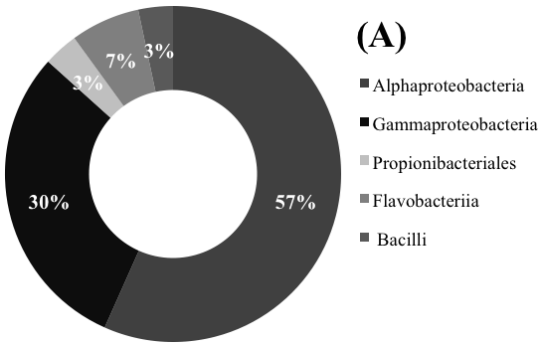


Fig 1. The cell growth of the isolates in ASW medium containing 50 mM alkanes at 30 ^{o}C during 7 days incubation. D-0: day 0 (□); D-7:day 7 (■).

Growth test of 30 strain bacteria were conducted in ONR7 media containing LCL alkanes (>C^{12}), ie paraffin and pristane. Paraffin represents a compound having a linear chain alkane. These compounds have a range of chain length C^{10}-C^{40}. It is expected to be easier to screen bacteria with the ability of the alkane degradation (Throne-Holst *et al.*, 2006). While pristane represent compounds which have branched-chain alkanes.

Alkane Hydroxylase Biocatalyst Diversity

n-alkanes hydrolysis could be initiated by enzymes that belong to different families. Based on a capability of alkanes consumption, 14 could grow on MCL and LCL medium, 11 could grow in MCL and n-LCL, 3 isolates could only grow MCL medium and 1 isolate could only grow in n-LCL medium (Fig. 3). The growth test result has indicated that 29 isolates produce medium-chain alkane monooxygenase and long-chain alkane hydroxylase.



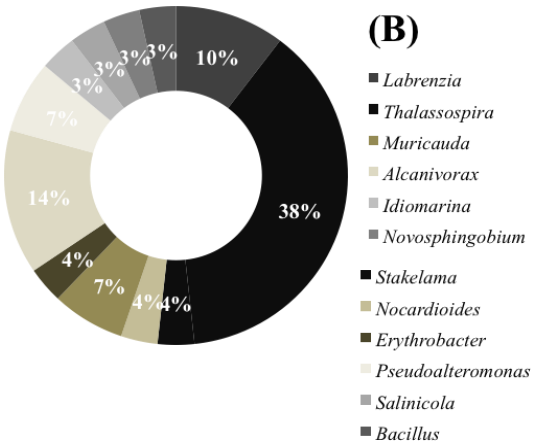


Fig. 2. Biodiversity of alkanes degrading bacteria based on (A) family and (B) species

Table 1. Bacteria identities were used in this study

No.	Private Culture Number	Most similar 16S rDNA sequence	Reference	%
1	LBF-1-0001	*Labrenzia aggregata* IAM 12614	NR115659	99
2	LBF-1-0002	*Stakelama pacifica* JLT832	NR116368	99
3	LBF-1-0003	*Thalassospira permensis* SMB34	NR116841	99
4	LBF-1-0004	*Nocardioides perillae* I10A-01402	NR109521	99
5	LBF-1-0005	*Muricauda aquimarina* SW-63	NR042909	99
6	LBF-1-0006	*Erythrobacter nanhaisediminis* T30	NR116764	99
7	LBF-1-0008	*Alcanivorax dieselolei* B5	NR074734	99
8	LBF-1-0009	*Muricauda aquimarina* SW-63	NR042909	100
9	LBF-1-0010	*Pseudoalteromonas shioyasakiensis* SE3	NR125458	99
10	LBF-1-0011	*Pseudoalteromonas shioyasakiensis* SE3	NR125458	99
11	LBF-1-0012	*Stakelama pacifica* JLT832	NR116368	99
12	LBF-1-0013	*Stakelama pacifica* JLT832	NR116368	99
13	LBF-1-0014	*Alcanivorax dieselolei* B5	NR074734	99
14	LBF-1-0015	*Labrenzia aggregata* IAM 12614	NR115659	99
15	LBF-1-0016	*Labrenzia aggregata* IAM 12614	NR115659	99
16	LBF-1-0017	*Alcanivorax xenomutans* JC109	NR133958	99
17	LBF-1-0018	*Stakelama pacifica* JLT832	NR116368	99
18	LBF-1-0019	*Stakelama pacifica* JLT832	NR116368	99
19	LBF-1-0020	*Idiomarina baltica* OS145	NR027560	99
20	LBF-1-0021	*Stakelama pacifica* JLT832	NR116368	99
21	LBF-1-0022	*Alcanivorax xenomutans* JC109	NR133958	99
22	LBF-1-0023	*Stakelama pacifica* JLT832	NR116368	99
23	LBF-1-0024	*Stakelama pacifica* JLT832	NR116368	99
24	LBF-1-0025	*Salinicola peritrichatus* DY22	NR109731	97
25	LBF-1-0026	*Stakelama pacifica* JLT832	NR116368	99
26	LBF-1-0027	*Stakelama pacifica* JLT832	NR116368	99
27	LBF-1-0028	*Novosphingobium resinovorum* NCIMB 8767	NR044045	99
28	LBF-1-0029	*Stakelama pacifica* JLT832	NR116368	99
29	LBF-1-0030	*Bacillus subtilis* subsp. subtilis OS-44.a	NR114997	99

Table 2. Strains producing medium chain alkane hydroxylase and long chain alkane hydroxylase

Type of alkane hydroxylase	Strain	Number of C	References
MCL	*Thauera butanivorans*	C_2-C_9	(Johnson & Harayama, 2006)
	Pseudomonas oleovorans GPo1	C_5-C_{12}	(Funhoff et al. 2006)
	Mycobacterium sp. HXN-1500	C_6-C_{11}	Maier et al. (2001)
	Acinetobacter sp. EB104	C_5	Zhou et al. (2011)
	Novosphingobium aromaticivorans DSM 12444	C_3-C_8	Kotani et al. (2003)
LCL	*Gordonia* sp. strain TY-5	C_{13}-C_{22}	Kotani et al. (2007); Li et al. (2008)
	Geobacillus thermodenitrificans NG80-2	C_{15}-C_{36}	Bihari et al. (2007)
	Acinetobacter haemolyti- cus strain AR-46	C_{16}-C_{35}	Lal & Khanna (1996)
	Acinetobacter sp. strain DSM 17874	C_{10}-C_{40}	Throne et al. (2006)
	Acinetobacter sp. DSM17874	C_{10}-C_{40}	Throne et al. (2006
	Alcaligenes odorans P20	$-C_{33}$	Schneiker et al. (2006)
	Alcanivorax borkumensis AP1	C_{10}-C_{20}	van Beilen & Funhoff (2005)
	Alcanivorax borkumensis SK2	C_8-C_{32}	Radwan et al. (1996)
	Arthrobacter nicotianae KCC B35	C_{10}-C_{40}	Chaerun et al. (2004)
	Bacillus thuringiensis/cereus A2	C_6-C_{28}	Yan (2006)
	Brachybacterium sp.	C_{10}-C_{20}	Wang et al. (2016)
	Burkholderia cepacia RR10	C_{12}-C_{34}	Yuste et al (2000)
	Desulfatibacillum aliphaticivorans CV2803	C_{13}-C_{18}	Cravo-Lauren et al. (2000)
	Dietzia cinnamea P4	C_{11}-C_{24}	von der Weid et al. (2006)
	Dietzia psychralcaliphila	C_{13}-C_{24}	Yumoto et al. (2002)
	Geobacillus thermodenitrificans NG80-2	C_{15}-C_{36}	Wang et al. (2006)
	Gordonia sp. TY-5	C_{3}, C_{13}-C_{22}	Kotani et al. (2003)
	Marinobacter hydrocarbonoclasticus 617	C_{16}-C_{30}	Doumennq et al. (2001)
	Marinobacter sp. BC36, BC38, and BC42	C_{18}	Bonin et al. (2004)
	Mycobacterium sp. HXN 600	C_6-C_{24}	van Beilen et al. (2002)
	Paracoccus sereniphilus/marcusii A7	C_6-C_{28}	Chaerun et al. (2004)
	Paracoccus sp. strains Ophe1 and Sphe1	C_{10}-C_{28}	Zhang et al. (2003)
	Planococcus alkanoclasticus MAE2	C_{11}-C_{33}	Enegelhardt et al. (2001)
	Pseudomonas aeruginosa PAO1	C_{12}-C_{24}	Smits et al. (2002)
	Pseudomonas aeruginosa strains A1, A3, A4, A5, A6	C_6-C_{28}	Chaerun et al. (2004)
	Pseudomonas fluorescens CHA0	C_{12}-C_{32}	Smits et al. (2002)
	Pseudomonas sp. PUP6	C_{12}-C_{28}	Naik & Sakthivel (2006)
	Rhodococcus sp. strains T12 and TMP2	C_9-C_{22}	Kunihiro et al. (2005)
	Thalassolituus oleivorans	C_7-C_{20}	Yakimov et al. (2004)
	Thermus sp. C2	C_9-C_{39}	Hao et al. (2004)
	Weeksella sp. RR7	C_{12}-C_{34}	Yuste et al. (2004)
	Xylella fastidiosa RR15	C_{14}-C_{34}	Yuste et al. (2004)

Discussion

In general, bacterial isolates tested were able to grow better in pentane than in the decane and pentadecane. This is understandable because the compound has a number of C atoms pentane less than others. So that these bacteria could utilize pentane as C source and energy better than the decane and pentadecane.

Meanwhile, isolate number 25 had the highest cell growth after incubated in ONR7 media containing pentadecane. This isolate has good cell growth in the all medium-chain alkane compounds. We hypothesized that isolate have an alkane hydroxylase which was capable to utilize the medium chain alkane compounds.

The highest bacterial growth in media containing decane is achieved by isolate number 25. These isolates identified as *Salinicola peritrichatus* (Table 1). *S. peritrichatus* is a Gram-negative bacteria, aerobic and rod-shaped bacterium. This strain was isolated from sediment in Pacific Ocean (Huo *et al.*, 2013) and the mangrove ecosystem in India (Raiu *et al.*, 2015). This genus has never been reported capable of utilizing pentane/alkanes for his growth and also for naphthalene-polycyclic aromatic hydrocarbons (Anan'ina *et al.*, 2007).

The highest bacterial growth in media containing decane is achieved by isolate number 1. This isolate was identified as *Labrenzia aggregata* (Table 1). *L. aggregata* is a Gram-negative bacteria, aerobic and motile (Wang *et al.*, 2016). This genus has been isolated from the hipersaline environment (Biebl *et al.*, 2007; Bibi *et al.*, 2014; Bacosa *et al.*, 2015). Genus of *Labrenzia* was able to grow in an oil contaminated environment as reported by several investigators (Overholt *et al.*, 2013; Bacosa *et al.*, 2015; Militon *et al.*, 2015).

Based on the growth of cells, in general, bacteria were grown better in paraffin than pristane (Fig. 1). This phenomenon occurs because pristane is branched-chain alkane which is more difficult to degrade. Thus the bacteria take longer to degrade than when grown in a paraffin which is a straight chain alkane.

One method for microbial identification is a molecular analysis by using some genes of bacteria. Currently, part of DNA that is often used for the taxonomy analysis of bacteria is 16S rDNA gene (Bottger, 1989; Pace *et al.*, 1997). This gene has unique properties in a biosynthesis system and also this gene is widely distributed in the cell. Technically, this gene is stored well on a broad ranges phylogeny data and the availability of complete data in the GenBank make PCR amplification of this gene become easier.

The composition of marine bacteria is not different from that of the composition of marine bacteria in the North Sea and Road Bay, Antartica (Harayama *et al.*, 1999; Ouatrini *et al.*, 2008). Beside these two classes, there are also families Actinobacteria, Bacillales, and Flavobacteria. The different results are reported from the diversity of oil-degrading bacteria in marine waters Semarang, Central Java, Indonesia. The reported α-proteobacteria was even more isolated from this area (Harwati *et al.*, 2007). These results indicate a wide range of oil-degrading bacteria in the regions of the waters of Indonesia, making it an attractive study for further exploration. Meanwhile, actinomycetes class dominate and hold the key bioremediation alkane compounds in the Mediterranean Sea (Chen *et al.*, 2010). Oil- degrading bacteria diversity in several different locations is thought to occur because of differences in location and

environmental conditions (O_2, electron receiver, temperature, redox potential, salinity, and pH) (Harayama *et al.,* 1999).

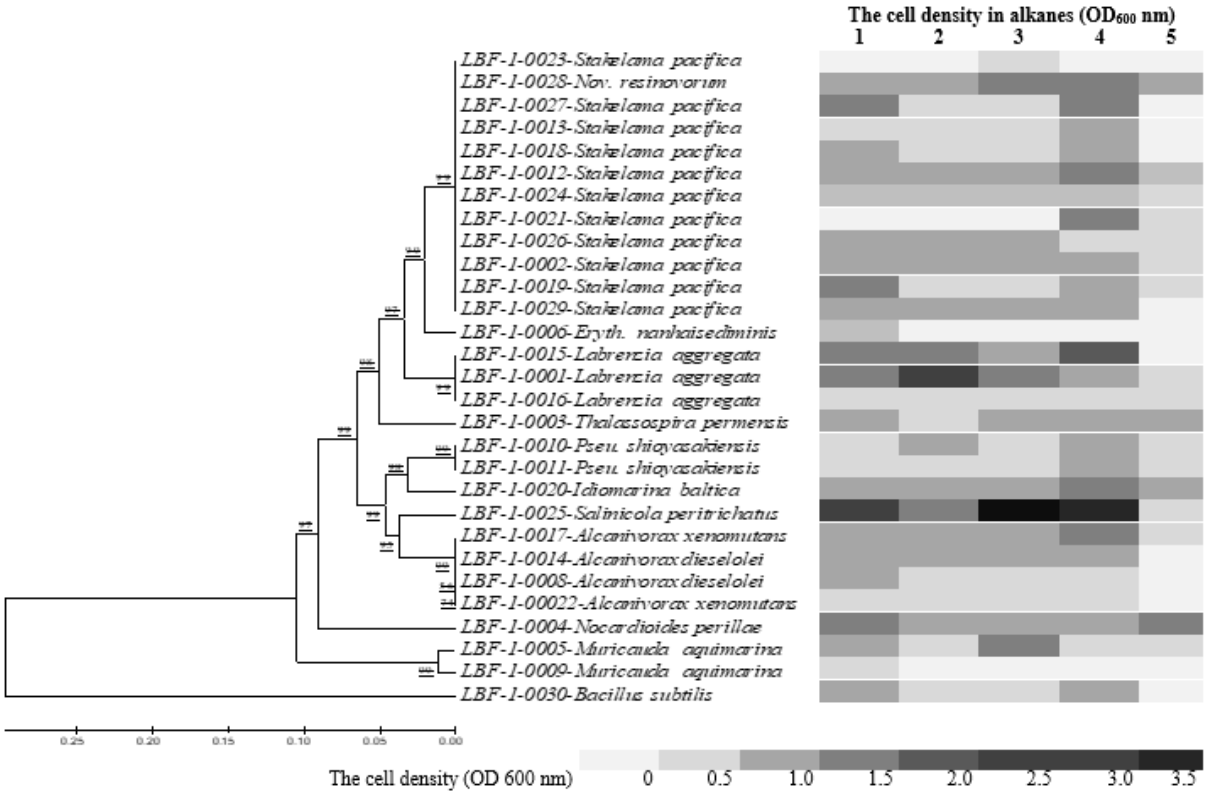


Fig 3. Biodiversity of alkane degrading bacteria and their phenotypic activity for alkanes degradation. 1: n-Pentane (C_5H_{12}), 2: Decane ($C_{10}H_{22}$), 3: Pentadecane ($C_{15}H_{32}$), 4: Paraffin, and 5: Pristane ($C_{19}H_{40}$).

Based on the genus information of alkanes degrader, we identified 12 species of bacteria. Genus of *Stakelama* dominates alkane-degrading bacteria. *Stakelama* is a Gram reaction-negative, weakly motile, non-spore-forming, rod-shaped, and aerobic bacterium (Thawng *et al.,* 2012). This genus was isolated at Pacific ocean (Thawng *et al.,* 2012), and tidal flat sediment (Kostka *et al.,* 2011). One paper reported that isolate isolated from Gulf Mexico seawater could be degrade oil (van Beilen & Funhoff, 2005).

One isolate of alkanes degrader has a difference of more than 3% of identity value or less than 97% with gene bank database, LBF-1-0025 isolate (Table 1). This isolate suspected to be new species (Yurui *et al.,* 2013). However, this proposal must be verified using several methods of identification of bacteria, such as morphology and biochemistry. Unfortunately, these tests were not done in this study. Bacterial identification is only evaluated by analyzing the 16S rDNA gene. We obtained bacteria which have MCL and LCL alkane hydroxylase from Indonesia sea water area. This result indicated that the biodiversity of alkane hydroxylase very diverse in Indonesia. As a comparison of the results was shown several types of microorganisms and their ability to produce MCL and LCL (Table 2). Genus of *Pseudomonas*, *Bacillus,* and *Alcanivorax* were reported to have MCL alkane hydroxylase (Fox *et al.,* 1992; van Beilen & Funhoff, 2005). Also for LCL alkane hydroxylase from *Geobacillus thermodenitrificans* NG80-2, utilizes a terminal oxidation pathway for the conversion of long-chain n-alkanes (from C_{15} to at least C_{36}) to corresponding primary alcohols (Halsey *et al.,* 2006). Further study of alkane hydroxylase from Indonesia marine bacteria will be carried out more detail.

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