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The Initial Dioxigenase Gene Squences Analysis of Marine Bacteria Strain M128

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Biodegradation of polyaromatic hydrocarbons (PAHs) are catalyzed by multicomponent enzymes from microbe. The initial dioxygenase was used as a key enzyme for attacking the aromatic ring structure of PAHs, furthermore its initial dioxygenase gene was used to select PAHs degrading bacteria. Marine bacteria M128 strain could grow on medium contained PAHs. Detection of its cellular initial deoxygenase gene was done by *nahAc* gene amplification. The *nahAc* gene commonly used as biomarkers of PAH degradation, and as a result, *nahAc* gene sequence analysis of marine bacteria M128 strain was similar to naphthalene dioxygenase of Pseudomonas genera with 99 % homology.

Keywords: Biodegradation, Dioxygenase, Gene sequence, Analysis, PAHs

INTRODUCTION

Consumption of petroleum product as an energy source has increased together with increasing of community live style as an impact of rapid consume growth. Petroleum product exploration activities caused waste pollution to environment, thus creating a seriously imbalance in the biotic and a-biotic regimes of the ecosystem (Okoh and Trejo-Hernandez, 2006). Oil and gas exploitation produced significant amount of petroleum sludge. Nowadays domestical petroleum production produced around 2 Kton oil sludge per day (Helmy *et al*, 2010).

Characterization of petroleum sludge from Pertamina Refinery Unit V, Balikpapan, Indonesia show that around 13.24 % oil sludge contains PAHs. PAHs are one of the most widespread organic pollutants and potentially hazard to human health (Arun *et al*, 2011).

Natural characteristic of PAHs are carcinogenic, mutagenic, and teratogenic which expose to the environment, human health, and aquatic ecosystems, and become severe hazard effect, therefore PAHs remediation for reducing its hazard effect become one of the government concern. One of the most effective and efficient way to remove this contamination

is bioremediation by using selected microorganism locally isolated from Indonesian territorial that have high capability to degrade PAHs.

Bioremediation is commonly used for recovery PAHs contaminated environment. The usage of potential fitted microbia will increase the efficiency of environment recovery processing. Guo *et al* (2010) reported the high ability of microbia to degrade PAHs influenced by the precense of dioxygenase gene. Sho *et al* (2004) and Zhou *et al* (2006) also reported that the initial dioxgenase was became biomarker for PAHs degradation microbia.

Genetic sequence analysis of PAHs degradation ability from domestically bacteria that was isolated from Indonesian marine was done and also potential to increase biological remediation of oil sludge. Detection of the dioxigenase gene (*nahAc* gene) from M128 strains was done in order to revealed the potential ability of the strain for PAHs degradation and as result, genetical sequence of this dioxogenase gene was also reported.

MATERIALS AND METHODS

Microorganism

Bacterial strain M128 is a collection strain from Research Center for Biotechnology, Indonesian Institute of Science and isolated from Indonesian marine which then was selected after cultivation in agar plate contained 20 mg/l pyrene and phenanthrene.

Culture media

Genomic DNA of M128 strain was extracted by using Miobio-laboratories kit after growth on LB medium for 18 hours (Prágai and Harwoord, 2000).

Detection of PAH-degrading dioxygenase M128 strain (Zhou *et al*, 2006)

The presence of the initial dioxygenase gene was detected base on PCR amplification. The primers for *nahAc* gene amplification were listed in Table 1.

Table 1. PCR Primer for the detection *nahAc* gene (Zhou *et al*, 2006)

Primer	Sequence
nahAc – first PCR	Forward primer: Nah-for TGCMVNTAYCAYGGYTGG
	Reverse primer : Nah-rev 1 CCCGGTARWANCCDCKRTA
nahAc- nested PCR	Forward primer *:* Nah-for TGCMVNTAYCAYGGYTGG
	Reverse primer: Nah-rev 2 CRGGTGYCTTCCAGTTG

PCR solution contain 5 µL buffer, 4 µL dNTPs, 1 µL forward primer, 1 µL reverse primer, 1 µL DNA template, 0.25 µL Ex Taq DNA polymerase and sterile distilled water to a final volume 50 µL. The PCR cycle conditions were listed in Table 2.

PCR amplification of *nahAc* gene is performed using genomic DNA of M128 strain as template. The first round PCR products is used as template for second PCR. The first and second round PCR products is analysed by electrophoresis in 2 % agarose gel.

Ligation and transformation of *nahAc* gene (Prágai *et al*, 1997)

The second round PCR product is separated by electrophoresis in a 2 % (w/v)

Table 2. PCR condition for *nahAc gene* amplification (Zhou *et al*, 2006)

First round PCR	- Initiation 94^{0}C for 3 min - Amplification 30 cycles (94 ^{0}C 45 s, 55 ^{0}C 45 s, and 72 ^{0}C 45 second) - Final extension : 72 ^{0}C for 5 min
Second round PCR (nested PCR)	- Initiation 94^{0}C for 3 min - Amplification 40 cycles (94 ^{0}C 45 s, 55 ^{0}C 45 s, and 72 ^{0}C 45 second) - Final extension : 72 ^{0}C for 5 min

agarose gel and ethidium bromide staining. The target bands were cut off on UV Trans-illuminator then recovered and purified by gene clean using glass-milk. The purified DNA fragment was linked to pMD20 vector by using ligation mixture at 16 ^{o}C for 15 min, and then this recombinant vector is transformed into competence cells *Escherichia coli* DH5α. The blue-white selection and PCR screening method are carried out to identify positive recombinant clone.

Plasmid Extraction

Plasmid extraction is done after the competent cell *Escherichia coli* DH5α growing on LB ampicillin medium for 18 hours. This extraction is done by using alkali-SDS method (Prágai and Harwoord, 2000; Prágai *et al*, 1997).

Sequence analysis of nahAc gene strain M128 (Herrick *et al*, 1993)

Polymerase chain reaction (PCR) is performed for *nahAc gene* strains M128 sequence analysis. Composition of PCR solution for *nahAc gene* sequence analysis is listed in Table 3.

The PCR condition of *nahAc gene* sequence analysis consist of initial denaturation 96 ^{o}C for 5 min, than 25 cycles (96 ^{o}C for 1 min, 50 ^{o}C for 30 seconds, 60 ^{o}C for 1 min). PCR product is purified using BigDye®XTerminator™Purification Kit. Purified PCR product is sequenced using BigDye®TerminatorV3.1 Kit Applied

Table 3. PCR solution component for sequencing *nahAc gene*

Reverse sequence	100-250 ng purified plasmid (ds DNA template)
	2 µl Reverse Primer (M13-Rv primer)
	1 µl BigDyeV3.1 Primer
	2 µl sequence buffer
	dH_2O up to 10 µl
forward sequence	100-250 ng purified plasmid (ds DNA template)
	2 µl forward primer (M13M4 Primer)
	1 µl BigDyeV3.1 Primer
	2 µl sequence buffer
	dH_2O up to 10 µl

Biosystems. Furthermore, this genetical sequence is submitted to GenBank data base to search for similarity with other dioxygenase gene sequences by using Blast alignment tool on The National Center of Biotechnology Information (NCBI) website.

RESULTS

Detection of *nahAc gene* M128 strain

For investigation of dioxygenase gene that was encoded initial polyaromatics hydrocarbons dioxygenase, M128 strain was analyzed by PCR amplification to detect the presence of *nahAc gene*. The *nahAc gene* encoded naphthalene dioxygenase was commonly used as biomarkers of PAH degradation (Zhou *et al*, 2006).

PCR amplification of *nahAc gene* used genomic DNA of M128 strains as template. Product of first and second round of PCR product were analyzed by electrophoresis in 2% agarose gel. Figure 1 shows first round PCR product for about 900 bp, and Figure 2 shows second round PCR product that have molecular weight for about 400 bp.

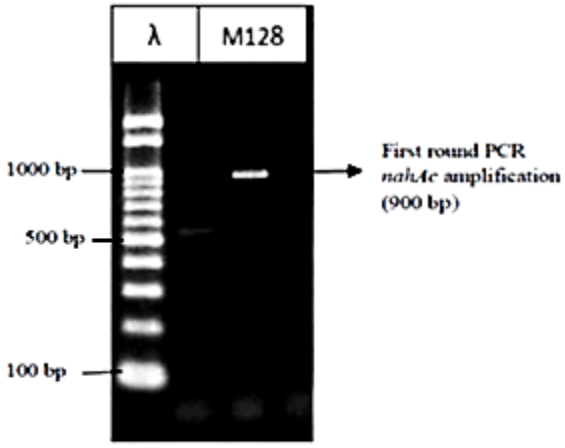

Fig. 1: First round PCR product of amplified *nahAc* gene fragmen separated by 2% agarose gel

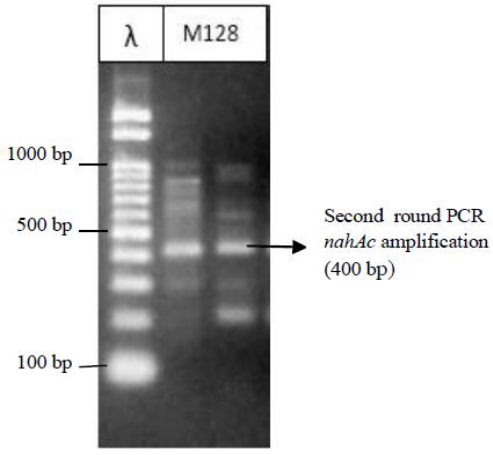

Fig. 2: Second round PCR product of amplified *nahAc* gene fragment separated by 2% agarose gel.

Ligation and Transformation of *nahAc gene*

Positive recombinant clone were identified by blue-white selection and PCR screening method. Figure 3 shows PCR screening result of *E. coli* DH5α that was inserted by *nahAc* gene from first round PCR product strain M128.

Plasmid Extraction

Figure 4 shows bands of plasmid after cutting by both *BamHI* and *XbaI* restriction enzyme. This plasmid have been cut completely by this restriction enzyme, therefore there are 2 bands, for about 900 bp comes from *nahAc* fragment gene and ±2800 bp from pmD20 vector band.

Sequence Analysis of dioxygenase gene M128 strain

Sequence analysis of *nahAc gene* M128 strain by using Blast alignment tool revealed that *nahAc* gene M128 strain has similarity with *nah* gene from *Pseudomonas* genera with 99 % homology. Table 4 show comparison result of similarity of *nahAc*

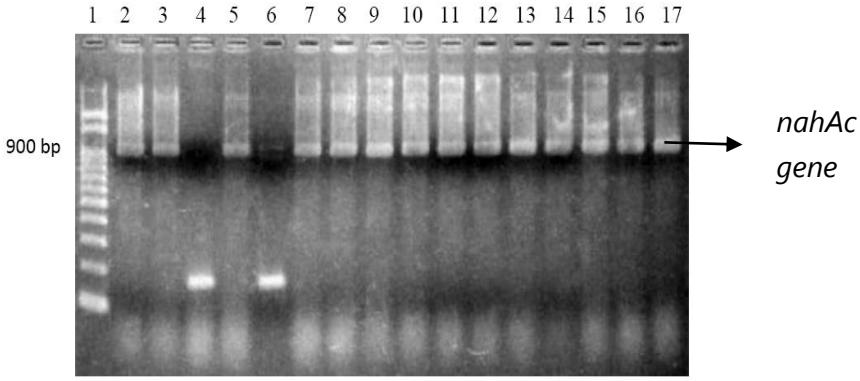

Fig. 3: Selection of competence cells *E. coli* DH5α transformed with plasmid contain *nahAc* gene strain M128. (**1**.Standard, **2.** M128-1, **3.** M128-2, **4.** M128-3, **5.** M128-4, **6.** M128-5, **7.** M128-6, **8.** M128-7, **9.** M128-8, **10.** M128-9, **11.** M128-10, **12.** M128-11, **13.** M128 12, **14.** M128-13, **15.** M128-14, **16.** M128-15, **17.** M128-16)

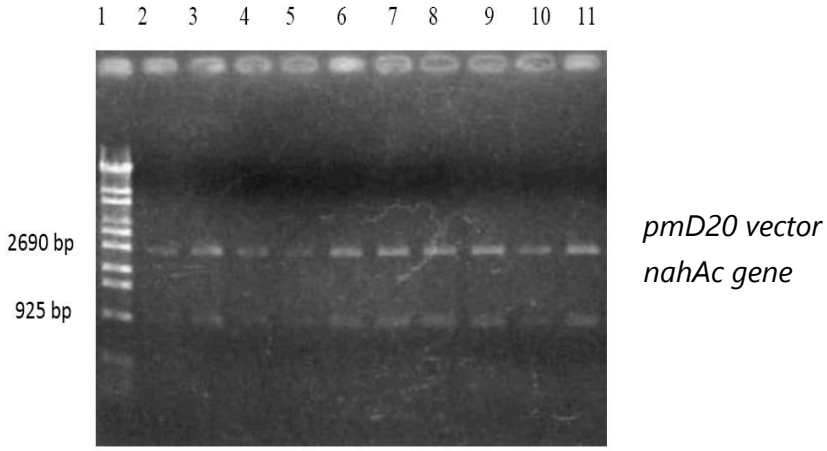

Fig. 4: Plasmid contains *nahAc* gene was cut by *BamH-I* and *XbaI* restriction enzymes (**1**.Standard, **2.** M128-7, **3.** M128-8, **4.** M128-9, **5.** M128-10, **6.** M128-11, **7.** M128-12, **8.** M128-13, **9.** M128-14, **10.** M128-15, **11.** M128-16)

Table 4. Compare result of the protein similarity of *nahAc* gene M128 strain with other bacterial gene cluster

Accession No.	Microbia	Similarity
dbj|D84146.1| PSEORF1	**Pseudomonas aeruginosa PAH** genes for 12 ORFs (components of naphthalene dioxygenase, dehydrogenase, hydratase-aldolase, isomerase), Length = 12808	Score = 1696 bits (918), Expect = 0.0 Identities = 944/957 (99%), Gaps = 0/957 (0%)
gb|HM368649.1	**Pseudomonas sp. N1** naphthalene dioxygenase gene cluster, complete sequence, Length = 3461	Score = 1694 bits (917), Expect = 0.0 Identities = 943/956 (99%), Gaps = 0/956 (0%)
gb|AF039533.1|AF039533	**Pseudomonas stutzeri** naphthalene degradation upper-pathway gene cluster (*nahAa*, *nahAb*, *nahAc*, *nahAd*, *nahB*, *nahF*, *nahC*, *nahE*, and *nahD*) and transposon-like protein (*tnpA1*) gene, complete cds, Length = 11514	Score = 1677 bits (908), Expect = 0.0 Identities = 941/957 (98%), Gaps = 2/957 (0%)

gene M128 strain with other bacterial gene which was downloaded from GenBank database.

Phylogenetic analysis of M128 strain's *nahAc* gene found that this *nahAc* gene homolog with *Pseudomonas* genera's *nahAc* gene which was known have ability to degrade PAHs (Grimm and Harwood (1997), Figure 5).

DISCUSSION

PAHs biodegradation process was initiated by dioxygenase of aromatic rings. Kim *et al* (2007) was reported pyrene biodegration by *Mycobacterium vanbaalenii* strain was initiated by the dioxigenase of pyrene compounds formed *cis-4,5- pyrene dihydrodiol* and *4,5-dihydroxy pyrene*. Iwabuchi and Harayama (1997) and Stingley *et al* (2004) reported that phenanthrene biodegradation was also initiated by dioxygenase of phenanthrene compounds for producing *phenanthrene 3,4-dihydrodiol*. Soe *et al* (2009) revealed that bacterial PAHs biodegradation was also initiated by dioxgenase reaction. Therefore the dioxygenase gene became important prevalent for microbial ability in PAHs degradation.

Zhou *et al* (2006) found that presence of dioxygenase gene for selecting the potential PAHs degradation isolates. This selection was done by amplification of *nidA, phdA, narAa, pdoA, nahAc, nagAc, phnAc,* dan *arc gene*. Furthermore *Guo et al* (2010) also selected potential bacteria by using *nahAc*, *nidA*, and *pdoB* gene amplification. Guo's result concluded that high biodegradation of phenanthrene, fluoranthene, and pyrene were done by bacterias that have dioxygenase gene. Therefore M128 strain that has dioxygenase gene (*nahAc gene*) was also have high potential ability for PAHs

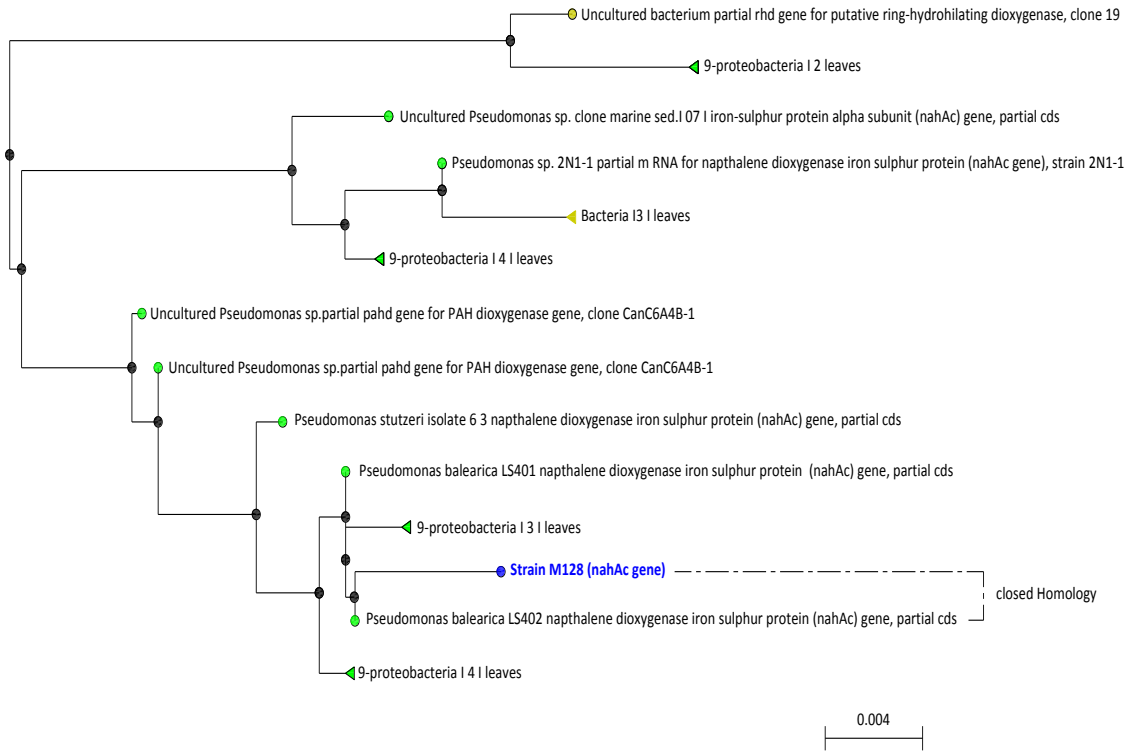

Fig. 5: Phylogenetics analysis of *nahAc* gene M128 strain.

degradation.

Sequence analysis of dioxygenase gene from M128 strain revealed that dioxigenase gene from this strain was similar with naphthalene dioxygenase (*nahAc*) gene from *Pseudomonas* genera with 99 % homology. Grimm and Harwood (1997) reported biodegradation of PAHs naphtalene by *nah* gene from *Pseudomonas sp*. and revealed that napthalene was degraded and formed salicilate, catechol and then continue by meta cleavage pathway to formed acetyl-CoA as shown in Figure 6. As a result, the *nah* gene is specify for napthalene degradation to be pyruvate and acetyl-coenzyme-A (CoA) via meta cleavage. From dioxygenase gene of M128 strain sequence analysis result, it was known that this strain could completely degrade PAHs to form pyruvate and acetyl-coenzyme A (CoA) via meta cleavage, and continued to tricarboxylic acid (TCA) cycle for completely degradation.

For next step research activity, based on PAH degradation pathway which was concluded in this experimental result, it is necessary to make more in – depth study on reaction kinetic of PAH bioremediation using this *Bacillus* strain as a complementary for large – scale oil sludge waste treatment purpose.

CONCLUSION

Domestical marine bacteria strain M128 which could grow on medium contained PAHs, has *nahAc* gene which is responsible for initial dioxygenase of PAHs degradation. This *nahAc* gene has similarity

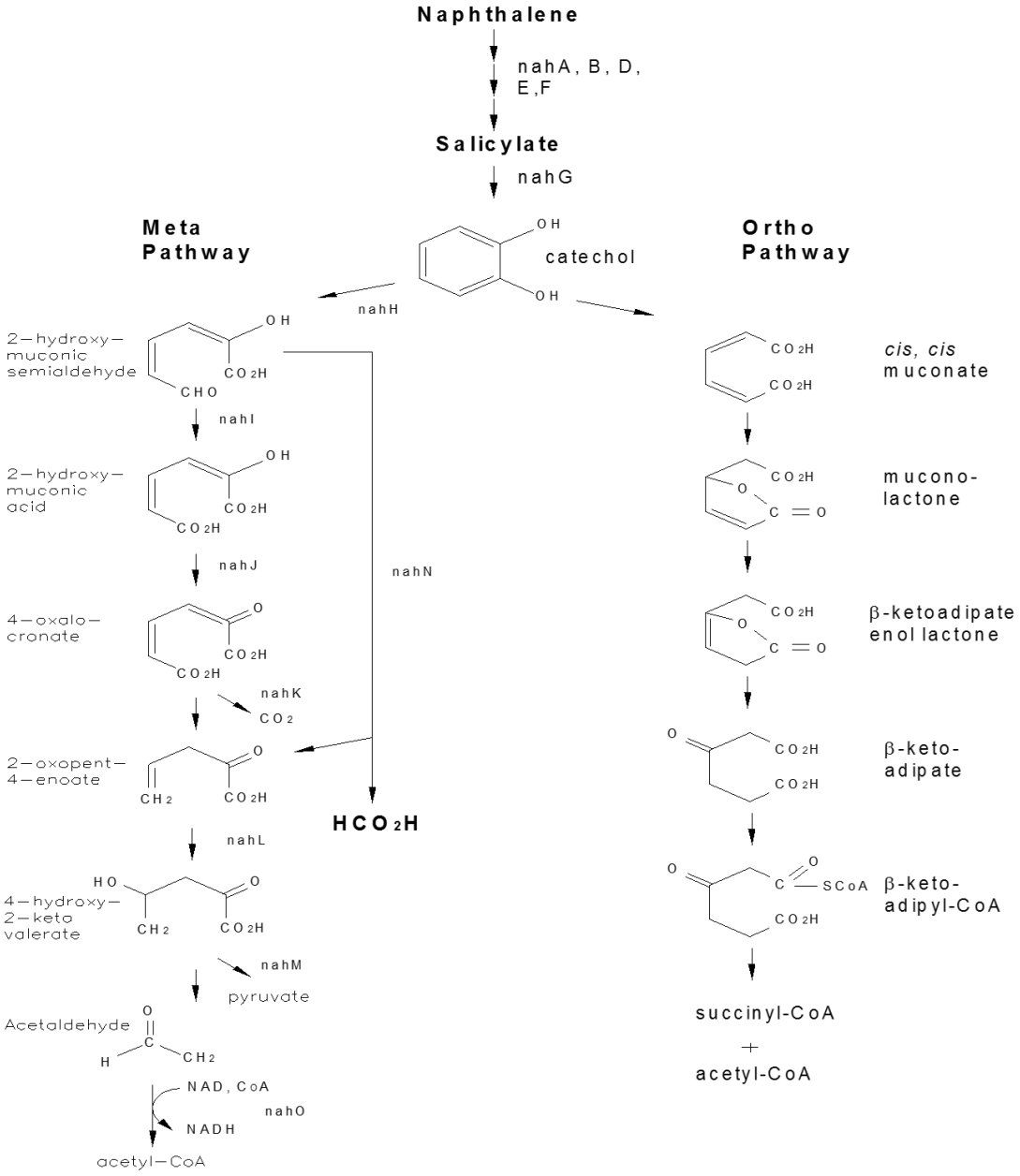

Fig. 6: Naphthalene degradation pathways, the *nah* gene specify the degradation of naphthalene to pyruvate and acetyl-CoA.

with *nah* gene belong to *Pseudomonas* genera with 99 % *homology* which was specify for napthalene degradation to be pyruvate and acetyl-coenzyme-A (CoA) via meta cleavage.

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