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DIOXYGENASE GENE OF PAHs DEGRADING A *BACILLUS* STRAIN ISOLATED FROM MARINE INDONESIAN ENVIRONMENT AND ITS BIOSURFACTANT PRODUCTION ABILITY

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ABSTRACT

Bioemulsifier production, the biodegradation abilities and initial dioxygenase gene of a *Bacillus* strain isolated from marine Indonesia was investigated. The 16S rDNA sequence revealed that the strain was *Bacillus subtilis*. The *B. subtilis* possessed *nahAc* and *nidA* genes encoding the initial dioxygenase required for pyrene degradation. The *B. subtilis* also could completely degrade a polycyclic aromatic hydrocarbon (PAH) of pyrene in 30 days. We suggest that the bioemulsifier that was also produced by the *B. subtilis*, may assist the bacteria to attach the pyrene for degradation.

KEYWORDS: Bioemulsifier, *Bacillus subtilis*, pyrene, biodegradation, dioxygenase

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INTRODUCTION

Pyrene represent one of the most abundant high-molecular weight polyaromatic hydrocarbons (HMW-PAHs), possessing mutagenic and carcinogenic potential. Biodegradation of HMW-PAHs compounds with four or more rings, such as pyrene, are more difficult than low-molecular weight PAHs (LMW-PAHs) compound. The problem for pyrene degradation is low bioavailability of HMW-PAHs which are hydrophobic. (Das and Mukherjee, 2006) Bioremediation of waste materials, which contain hydrocarbon and their derivates, was based on the ability of microorganisms to increase their growing biomass on these substrates and degrading them to non-toxic products (Toledo *et al*, 2005).

The presence of emulsifier can increase pyrene solubility and bioavailability. The syntheticsurfactants in high concentration caused environment problems so that many researchers investigated the application of biosurfactant in culture medium to enhance the solubility and biodegradation of hydrophobic substrate, such as pyrene (Miller and Zhang, 1997; Das and Mukherjee, 2006; Millioli *et al*, 2009). Bacterial species that have high potential for hydrocarbon biodegradation and biosurfactant production are identified as *Bacillus subtilis*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Pseudomonas pseudomallei*, and *Bacillus polymyxa*, (Kumar *et al*, 2006; Bayoumy, 2009; Bayoumi and El-Nagar, 2009). Although several reports are available on the pyrene degradation by the actinomycetes group of bacteria (Heitkamp *et al*, 1998; Sho *et al*, 2004; Kim *et al*, 2006) pyrene degradation pathways by non-actinomycetes groups of bacteria need to be revealed. Kim *et al* (2006) have analyzed complete and integrated pyrene degradation pathway in Mycobacterium vanbaalenii PYR-1 based on system biology, and identified 27 enzymes necessary for constructing a complete pathway for pyrene degradation. Their analysis indicate that *Mycobacterium vanbaalenii* PYR-1 degrade pyrene to central intermediates through o-phthalate and β-ketoadiapate pathway. Sho *et al* (2004) have identified *nidA* and *pdoA* gene encoding pyrene degradation in *Mycobanterium sp*. S65 which utilized pyrene, phenanthrene, and fluoranthene as sole carbon and energy sources.

Capability of non-actinimycetes bacteria, *Pseudomonas* and *Bacillus*, for pyrene degradation and biosurfactant production was analyzed (Kumar *et al*, 2006; Das and Mukherjee, 2007). Biosurfactant was enhancing the solubility of pyrene in aqueous media and influence the cell surface hydrophobicity for higher pyrene's uptake. (Das and Mukherjee, 2007). PCR and DNA hybridization studies in *Pseudomonas putida* IR1 showed that enzymes involved in PAH metabolism were related to the naphthalene dioxygenase pathway (Kumar *et al*, 2006). In the present study, we analyze the capability of C19 strain to degrade pyrene and produce biosurfactant. The dioxygenase gene, *nidA* and *nahAc* gene, as well as the bacteria identification by sequence analysis of gene encoding 16S rDNA are also reported.

MATERIALS AND METHODS

(i) *Microorganisms:*

Microorganism used in this study was isolated from Indonesian marine environment, as microbe's strain collection of research center for biotechnology, Indonesian Institute of Science.

(ii) *Culture Media:*

Growth of C19 strain on pyrene was determined in liquid medium with the composition (L^{-1}) 10 g yeast extract, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g NaCl, 1 ml $MgSO_4$ 1 M, and 2.5 ml micro-element that contained ((L^{-1}) 53 mg $MnCl_4$ H_2O, 31 mg H_3BO_3, 36 mg $CoCl_2$ $6H_2O$, 10 mg $CuCl_2$ $2H_2O$, 30 mg Na_2MoO_4 $2H_2O$ and 50 mg $ZnCl_2$. The pH of this medium was adjusted to 7.0 and the solution was sterilized in high pressure sterilizer for 20 min at 121 ^oC. The

ethanol solution of 200 and 1000 ppm pyrene was added to sterilize liquid medium, and this experimental solution could be used after the ethanol had been completely volatilized.

(iii) *Identification of pyrene-degrading strain C19:* In order to identify C19 strain, 16S rDNA gene sequence analysis was carried out. Genomic DNA extraction was done by using Miobio-laboratories kit. The 16S rDNA gene fragment was amplified by PCR using the set of primer: 16S-F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 16S-R (5'-CGGCTACCTTGTTACGACTTC-3'). The PCR conditions (35 cycles of 30 s at 94^{o}C, 30 s at 50^{o}C, and 90 s at 72 ^{o}C) were performed with PCR system 9700 from Applied Bio-system. The PCR products were then purified and linked to the pMD20 vector. The DNA sequence of the positive clone with 16S rDNA gene fragment was compared with the available database (Genbank) using the BLAST program which is freely downloaded from the National Center for Biotechnology Information (NCBI) website.

(iv)*Detection of PAH-degrading dioxygenase:* Bacterial DNA was extracted by the bacterial DNA extraction (Miobio-laboratories kit). The presence of the initial dioxygenase gene was detected based on PCR amplification. The primer for detection of *nahAc* and *nidA* in C19 strain were listed in table 1.

Table 1
PCR Primer for the detection of initial PAH-degrading dioxygenese gene.

Primer	Sequence
nidA – first PCR	Forward primer: Nid-for. TCCRMTGCCCDTACCACGG
	Reverse primer: Nid-rev1 GAASGAYARRTTSGGGAACA
nidA – nested PCR	Forward primer: Nid-for. TCCRMTGCCCDTACCACGG
	Reverse primer: Nid-rev2 GCGSCKRKCTTCCAGTTCG
*nahA*c – 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

Components for the PCR were 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 condition were 94 ^{o}C for 3 min, then 40 cycles (first PCR) or 30 cycles (nested PCR) of 94 ^{o}C 45 s, 55 ^{o}C 45 s, and 72 ^{o}C 45 s, and final extension at 72 ^{o}C for 5 min. PCR amplification of *nidA* and *nahAc* gene was performed using genomic DNA of C19 strain as template. The first round PCR products were used as templates for second PCR. The first and second round PCR products were analyzed by electrophoresis in 2 % agarose gel (Zhou *et al*, 2006).

(v) *Biodegradation experiment:*
Bacterium was grown in batch culture in a 250 ml flask containing 100 ml of 1 % yeast extract supplemented by 200 and 1000 ppm pyrene as sole carbon source. The experimental flasks were inoculated with 2 % (v/v) inoculum which was incubated at 30 ^{o}C on a rotary shaker (150 rpm). The residual pyrene from culture flask

was analyzed by spectrophotometer UV-Visible. (Heitkamp *et al*, 1998; Das and Mukherjee, 2006).

(vi) *Emulsification assays and biosurfactant activity test:*

The emulsifying activity of the bioemulsifier synthesized by C19 strain grown in liquid medium was detected by a modified version of method which is previously described by Helmy *et al* (2010). The ability of C19 strain for bioemulsifier production was analyzed within 7 days incubation. To determine the emulsification activity, C19 isolate was incubated at 30 ^{o}C for 7 days. Centrifugation at 13000 rpm and after that separation of supernatant culture containing bioemulsifier from microorganism cell. A mixture of 1:1 between supernatant culture and oil test is agitated for about 2 minutes and then stabilized for 24 hours. Emulsification activity was determined by comparing with the blank sample.

RESULT

1. Identification of pyrene-degrading bacteria strain C19

Identification of C19 strain was done by 16S rDNA analysis, Genome extraction from C19 isolate has molecular weight for about 19329 bp (Fig. 1a). PCR amplification of 16S rDNA gene was performed using genomic DNA of strain C19 as template. The PCR products, about 1500 bp, were separated by electrophoresis in a 1% agarose gel. The length of the DNA fragment was in accordance with primer designing region size (Fig. 1b).

In order to verify whether ligation of 16S rDNA from strain C19 to pMD20 vector had been done correctly, white-blue selection method and PCR screening method was carried out. Furthermore, to identify whether the correct gene was inserted in the plasmid, cutting plasmid was done by using *AvaI* restriction enzyme for one recognize restriction site, and using both *AvaI* and *HindIII* were used for two recognize restriction sites (Fig. 2).

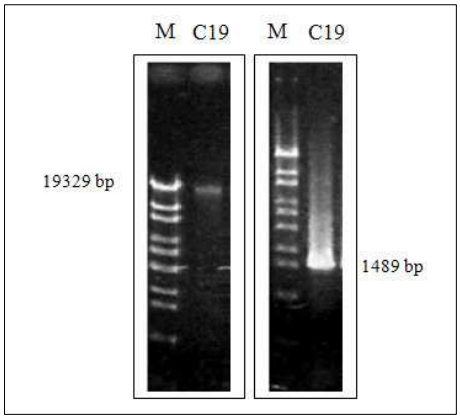

Figure 1

[A] Genome of C19 Strain (19329 bp) and λ marker (M - lane); [B] 16S rDNA gene of C19 Strain (1489 bp) and λ marker (M - lane).

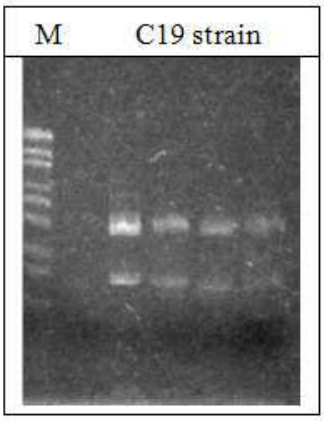

Figure 2.
Plasmid after cutting using Ava I and Hind III restriction enzymes, and λ marker (M - lane).

Analysis of the 16S rDNA strain C19 sequence revealed 100 % sequence similarity to *B. subtilis* K21 (JN58751-.1), *B. subtilis* NB-01 (HM214542.1), and *B. subtilis* L4 (GQ421472.1).

2. Detection of dioxygenase gene in strain C19

The *nidA* and *nahAc* genes are prevalent in the PAH-degrading bacteria and they are useful for determining the presence of PAH-dioxygenase genes. During the analysis of the PAHs-dioxygenase gene using nested PCR, second round PCR served as a confirmation step to check if the first round PCR was a PAH-dioxygenase gene. The *nidA* primer amplified DNA fragment of 600 bp and 300 bp in nested PCR. Fig. 3 shows the amplified DNA fragments by *nahAc* primer for about 900 bp and 400 bp in nested PCR. This result is appropriate with *nidA* and *nahAc* design primer for ring-hydroxilating alpha domain DNA amplification as described at Fig. 4. Both forward primers for the *nidA* and *nahAc* genes were located at the conserved region – C-X-X-H-X – of the [2Fe-2S] binding site. The internal reverse primers used in nested PCR were also located in similar regions, recognizing the conserved – N-W-K – fragment just before the iron binding site of the ring-hydroxylating-α domain. Similar to the forward primer, The *Nid-rev1* primer was designed from an area located close to the substrate binding site which codes for amino acids 315–308. The primer of the reverse *nahAc* primers *Nah-rev1* was from the part of gene close to the C-terminal, which codes for amino acids 418–412

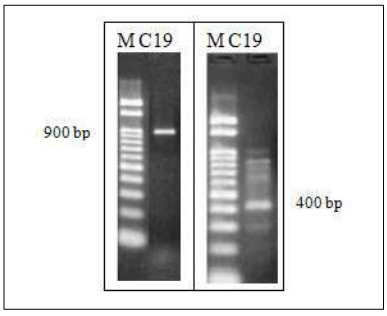

Figure 3.
First and nested PCR of the nahAc primer.

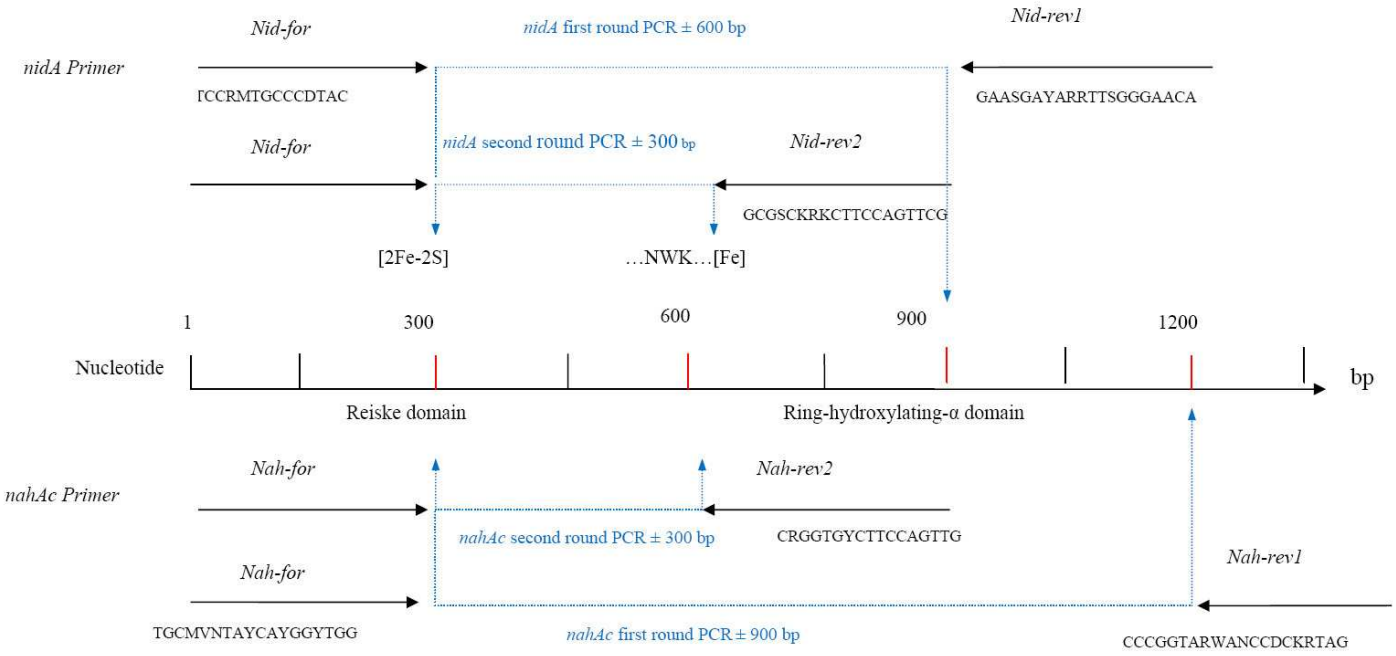

Figure 4.
Nested primer site for the nidA and nahAc primer (Zhou et al,2006)

3.Pyrene Degradation Analysis by Strain C19

Pyrene in the medium was metabolized and was used as carbon and energy source. Strain C19 could completely degrade pyrene in 30 days incubation. The concentration of pyrene in the medium decreased from 200 ppm to 0.986 ppm, the biodegradation rate reached 99.5 %. At higher pyrene concentration pyrene also could be completely degraded. Pyrene concentration decreased from 1000 ppm to 0,927 ppm, and the biodegradation rate reached 99.9 % (Fig. 5). Fig. 6 shows absorbance at maximum wavelength was about 25 for 1000 ppm of pyrene, and ± 0.111 at 0.927 ppm of pyrene.

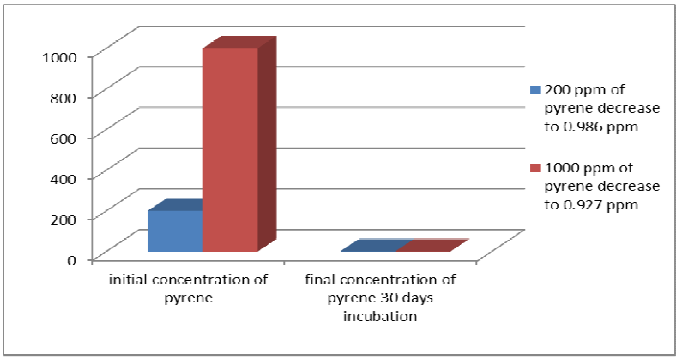

Figure 5.
Pyrene concentration reduction in 30 days incubation by C19 Strain

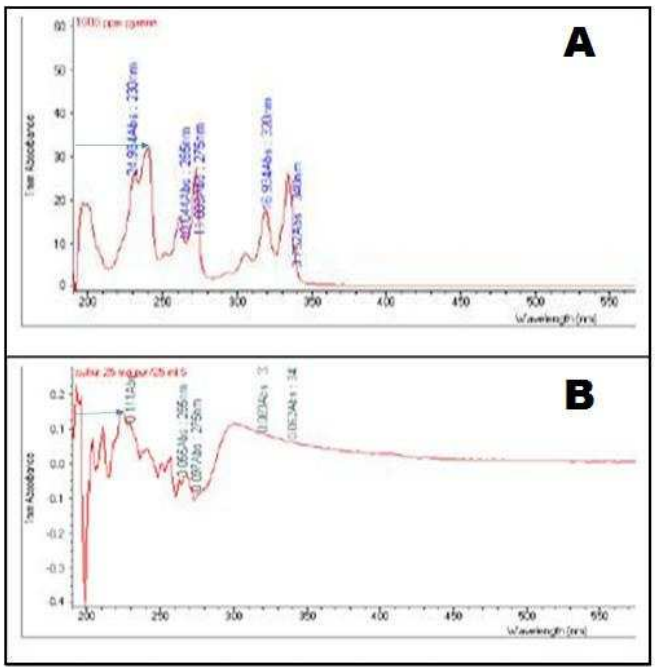

Figure 6.
[a] UV-Vis spectrum of pyrene before degradation (1000 ppm); [b] UV-Vis spectrum of pyrene after 30 days incubation by C19 strain (0.927 ppm)

3. Biosurfactant Production by Strain C19

B. subtilis C19 has potential capability for biosurfactant production when it grows in liquid medium that contains pyrene. In the other hand it produced biosurfactant when it grew in hydrocarbon with limited other general carbon source. Emulsification activity of biosurfactant produced by C19 strain is shown in Fig. 7.

Figure 7.
Emulsification activity of biosurfactant from C19 isolate compared by blank sample.

As the result, C19 strain produced biosurfactant began at 24 h after inoculation and it's continued up to passed stationary growth phase. Furthermore, emulsification activity (E24) of this biosurfactant in supernatant culture reached around 80 % in solar oil.

DISCUSSION

Variety of non-actinomycetes bacteria such as *P. aeruginosa*, *P. putida*, *Flavobacterium sp*, and *Proteus vulgaris* were reported to utilize pyrene when supplemented with other forms of organic carbon such as glucose or sucrose, but *B. subtilis* C19 has the similar ability with soil bacterium *P. aerugenosa* and *B. subtilis* DM04 to utilize pyrene as sole carbon source without being supplemented by the other carbon sources because *B. subtilis* C19 was not only be able to degrade pyrene but also produce biosurfactant. This biosurfactant was produced to enhance the bioavailability of pyrene. Variety of pyrene concentration in growth medium didn't have significant effect on *B. subtilis* C19 growth and its pyrene degradation activity, this result indicating that the *B. subtilis* C19 strain has ability to grow in wide range of pyrene concentration and it also applicable for insitu bioremediation. Toledo *et al* (2006) also have reported the ability of *B. subtilis* to produce biosurfactant when growth on medium contains polycyclic aromatic hydrocarbons naphthalene, phenanthrene, and pyrene which supplemented with supported carbon source such as 1 % glucose. Biosurfactant product contains 33.9 % carbohydrate and 28.2 % protein. Emulsification activity of this biosurfactant was 75.9% in crude oil, surface tension 53 mNm^{-1}. *B. subtilis* C19 also produce the similar emulsification activity (E24) with similar value. Referring to result experiment by Sekhon *et al* (2011), production of biosurfactant by *B. subtilis* which was grown on insoluble carbon source was analyzed genetically. Olive oil was used as inducer and protein associated with the release of the biosurfactant from this strain was found to be an esterase. Biosurfactants produced by *B. subtilis* SK320 and recombinant strains *BioS a*, *BioS b*, *BioS c* were found to be effective emulsifiers. They reduce water surface tension from 72 dynes/cm to as low as 30.7 dynes/cm. Here, as an explanation before *B. subtilis* C19 produced biosurfactant at 24 h incubation and it's activity similar with *B. subtilis* DM04 which also produce biosurfactant after 24 h incubation (Das and Mukherjee, 2006). Abushady *et al* (2005) have also reported production of biosurfactant *B. subtilis* with the optimum incubation time was 72 h (inoculum size 1.5-2 % (v/v) at temperature 30 ^{o}C, at 200 rpm).

Pyrene degradation which is reach 99 % at 30 days incubation shows that *B. subtilis* C19 has higher ability to degrade high molecular weight (HMW) PAHs compared with other strains. *B. subtilis* BMT4i started degrading Benzo [a] pyrene (BaP) after 24 hours and continued up to 28 days achieving maximum degradation of approximately 84.66 % (Lily *et al*, 2009). *Ochrobactrum sp* degraded 20 % of BaP after 30 days incubation at initial concentration 50 ppm (Yirui *et al*, 2008). Furthermore, at initial concentration 100 ppm, *Candida viswanathii* degraded 60 % of pyrene (Hesham *et al*, 2009). As an addition result, Churchill *et al* (1999) have used 500 ppm of initial pyrene concentration, and the degradation rate reached 72% in 14 days. Walter *et al* (1991) and Sarma *et al* (2004) have used 200 ppm of initial pyrene concentration, and the degradation rate reached 61.5 % in 20 days. All of biodegradation activity results revealed that *B. Subtilis* C19's pyrene degradation has the highest result compared to another species' result. Refer to degradation mechanism of pyrene at Fig. 8, determination of dioxygenase gene were clustered and divided into two subgroups surrounding *nahAc* and *nidA* genes. Based on substrate range of these enzymes, the *nahAc* subgroups appeared to be of low molecular weight (LMW) PAH degrading gene, while the *nidA* transformed both LMW and HMW PAHs (Kim *et al*, 2006). In molecular characterization of a phenanthrene degradation pathway, a subclone containing *nidA* and *nidB* converted phenanthrene into *Phenanthrene cis-3,4-dihydrodiol*, suggesting that *NidA* and *NidB* dioxygenase is responsible for an initial attack on phenanthrene (Stingley *et al*, 2004). Kweon *et al* (2010) have also reported roles of the *nidA* for PAHs

degradation, the Reiske non-heme iron aromatic ring-hydroxylating oxygenases (RHOs) *NidAB* and *NidA3B3* from *Mycobacterium vanbaalenii* PYR-1 have been implicated in the initial oxidation of HMW PAHs forming *cis-dihydrodiols*. For pyrene degradation *NidA* converted pyrene only to *Pyrene cis-4,5-dihydrodiols*, but *NidA3* produced both *cis-4,5-dihydrodiol* and *cis-1,2-dihydrodiol pyrene*. In the phenanthrene degradation, *NidA* and *NidA3* have difference conversion rate. *NidA* produce phenanthrene cis-3,4-dihydrodiol (75%), and *Phenanthrene cis-9,10-dihydrodiol* (24%), but *NidA3* produce *Phenanthrene cis-3,4-dihydrodiol* (53%), *Phenanthrene cis-9,10-dihydrodiol* (26%), and *Phenanthrene cis-1,2-dihydrodiol* (21%).

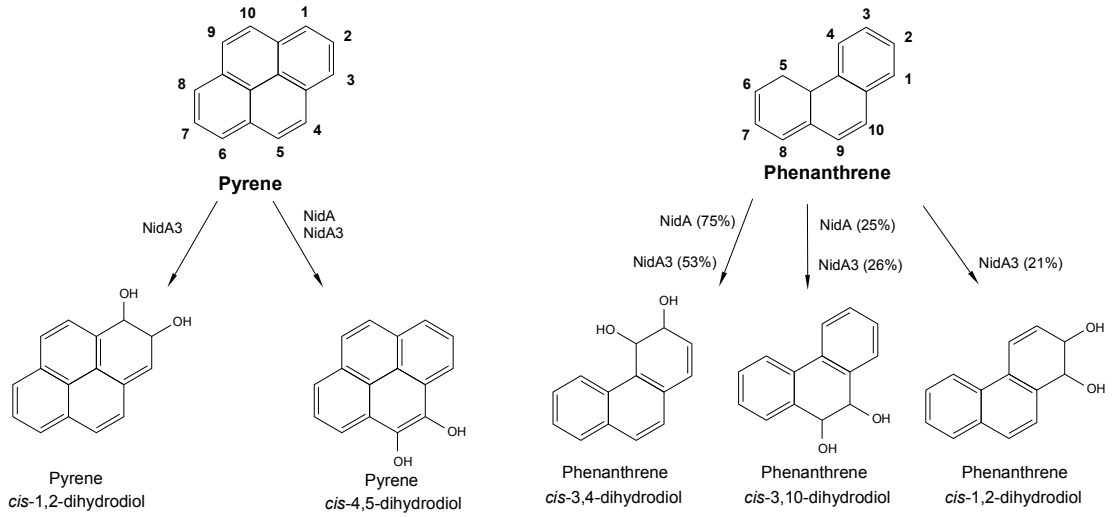

Figure 8.
Metabolite structure produced from aromatic substrates by NidA enzymes. [A] Pyrene [B] Phenanthrene

Finally, as a result the *B. subtilis* C19 have both *nahAc* and *nidA* gene which were responsible to degrade LMW PAHs and HMW PAHs substrate as described in previous explanation, thus *B. Subtilis* C19 could complately degrade PAHs pyrene.

CONCLUSION

Marine bacteria C19 strain utilized pyrene as sole carbon source to grow and produced bioemulsifier. The C19 strain could completely degrade pyrena in 30 days incubation. 16S rDNA sequence analysis of C19 strain revealed that the strain was *B. subtilis*. Analysis of the PAHs-dioxygenase gene using *nidA* and *nahAc* primer revealed that *B. subtilis* C19 has both *nidA* and *nahAc* gene that is responsible for PAH ring-hydroxylating dioxygenase. Therefore *B. subtilis* C19 has ability to transform HMW PAHs pyrene substrate.

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