



Degradation of Polycyclic Aromatic Hydrocarbon Pyrene by Biosurfactant-Producing Bacteria *Gordonia cholesterolivorans* AMP 10

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Abstract

Pyrene degradation and biosurfactant activity by a new strain identified as *Gordonia cholesterolivorans* AMP 10 were studied. The strain grew well and produced effective biosurfactants in the presence of glucose, sucrose, and crude oil. The biosurfactants production was detected by the decreased surface tension of the medium and emulsification activity. Analysis of microbial growth parameters showed that AMP10 grew best at 50 µg mL⁻¹ pyrene concentration, leading to 96 % degradation of pyrene within 7 days. The result of nested PCR analysis revealed that this isolate possessed the *nahAc* gene which encodes dioxygenase enzyme for initial degradation of Polycyclic Aromatic Hydrocarbon (PAH). Observation of both tensio-active and emulsifying activities indicated that biosurfactants which produced by AMP 10 when grown on glucose could lower the surface tension of medium from 71.3 mN/m to 24.7 mN/m and formed a stable emulsion in used lubricant oil with an emulsification index (E24) of 74%. According to the results, it is suggested that the bacterial isolates *G. cholesterolivorans* AMP10 are suitable candidates for bioremediation of PAH-contaminated environments.

How to Cite

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INTRODUCTION

One of the major environmental problems today is hydrocarbon contamination which could be derived from natural and anthropogenic process. Although the natural process could contribute to hydrocarbon release into the environment, human activities as it in petroleum and petroleum products industry is a primary cause of pollution of water and soil. Petroleum is a liquid mixture consisting of complex hydrocarbons (Das & Chandran, 2011; Jahangeer & Kumar, 2013).

Oil spillage and oil pollution in the environment have been a threat to the ecosystem and human being through the transfer of toxic organic materials including polycyclic aromatic hydrocarbon (PAH). PAH are organic molecules composed of two or more aromatic (benzene) rings which are fused together in various structural configurations. PAHs contamination is a severe environmental concern since this compounds are the ubiquitous persistent contaminant that highly toxic, mutagenic and carcinogenic. Inputs of PAHs from various sources such as forest fires, volcano eruption, oil spills, ship traffic, burning of fossil fuels, urban runoff manufacture of gas and coal tar, wood processing, escaped automobile gasoline, fuel-burning kitchen stove, and industrial effluents have caused significant accumulation of PAHs in the environment (Luan et al., 2006; Fernandez-Luqueno et al., 2011).

In Jakarta bay Indonesia, the content of PAH at March 2011 ranged from 1.92 to 115.39 ppm in sediment and 104.61 to 474.68 ppm in seawater. The main sources of those PAH was derived from combustion of organic materials, burning of fossil fuels, and the oil spills (Ahmad, 2012). The total concentrations of the 16 PAHs in in sediment samples of an industrial port in the southern Kaohsiung Harbor of Taiwan varied from 4,425 to 51,261 ng g⁻¹, with a mean concentration of 13,196 ng g⁻¹. The PAHs concentration is relatively high in the river mouth region, and gradually diminishes toward the harbor region (Dong, 2012). A similar study by (Wang et al., 2014) showed that PAH concentrations in Coastal sediments in the northern Gulf of Mexico ranged from 100 to 856 ng g⁻¹.

Pyrene which has four benzene rings is included in a list of PAHs priority pollutants by the United States Environmental Protection Agency (US EPA). It has a low biodegradability and high persistence in the environment. Pyrene is a by-product of gasification processes and other incomplete combustion processes. It is highly recalcitrant and resistant to microbial degradation

due to its chemical structure (Ceyhan, 2012). The structure of pyrene is found in the molecule of carcinogenic PAHs.

Bioremediation is the promising method for the treatment of contaminated sites since it is to be an economical and efficient alternative method to other remediation processes such as chemical or physical ones. Bioremediation functions basically on biodegradation by microorganisms. Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants including pyrene. *Mycobacterium* was published as the first bacterium able to completely degrade pyrene in pure culture (Heitkamp et al., 1988). Some other bacteria were also reported as pyrene degrading bacteria, such as *Pseudomonas saccharophila* P15, *Pseudomonas stutzeri* P16, *Bacillus cereus* P21, *Sphingomonas yanoikuyae* R1 (Kazunga & Aitken, 2000), an enteric bacterium *Leclercia adecarboxylata* (Sarma et al., 2004), *Mycobacterium vanbaalenii* PYR-1 (Kim et al., 2007), *Proteus vulgaris* (Ceyhan, 2012), *Bacillus subtilis* C19 (Wijanarko et al., 2012), *Corynebacterium* sp., *Nocardia* sp., *Pseudomonas* sp., *Rhodococcus* sp. and *Micrococcus* sp. (Kafilzadeh et al., 2012).

Nevertheless, biodegradation of PAHs in contaminated sites is a slow process due to the low bioavailability of these persistent contaminants (Das & Chandran, 2011, Shokrollazadeh, 2012). Microorganisms including bacteria may excrete biosurfactants. This compound could enhance the bioavailability of PAHs and accelerate the bioremediation process. In our previous study, nine biosurfactant producing bacteria were isolated from oil contaminated soil. The objective of this study was to determine of the growth and degradation potential of those bacterial isolates on pyrene.

METHODS

Microorganisms and culture conditions

Bacterial isolates used in this study were obtained from oil polluted soil in Jakarta Bay. All isolates were confirmed positive as biosurfactant producing bacteria based on a test performed in the previous study (data not shown). The mineral salt medium (MSM) was used as a basal medium both for growth and degradation experiment. This medium was composed of (g/L): KH₂PO₄ 3.0 g, Na₂HPO₄ 6.0 g, NH₄Cl 1.0g, NaCl 0.5 g and 1 M MgSO₄ 1.0 ml. This medium also contained 2.5 ml of a trace element as follows (g/L): 23 mg of MnCl₂·2H₂O, 36 mg of CoCl₂·6H₂O, 30 mg of MnCl₄·H₂O, 31 mg of H₃BO₃, 10 mg of CuCl₂·2H₂O, 30 mg of Na₂MoO₄·2H₂O, 20 mg of

NiCl₂·6H₂O and 50 mg ZnCl₂ with pH 7.0 (Kumar et al., 2006).

Bacterial growth on solid medium supplemented with hydrocarbon

MSM agar was prepared by added 15 g/l of agar and was sterilised (121°C for 20 min) prior to the addition of PAH as a sole carbon source. The stock solutions of pyrene were made in acetone. The ability of isolates to grow in solid medium was tested by spreading 100 µL of 24 h bacterial culture in Nutrient Broth to MSM agar supplemented with pyrene and incubated at 30°C. Medium without inoculum was used as negative control. Growth on pyrene in the solid media was considered positive by the appearance of the colony at the surface of the medium.

Extraction, PCR Amplification and DNA Sequencing of 16s rRNA Gene

The extracted DNA was used as a template to amplify the 16S rRNA genes by PCR with the universal forward primer 63f (59-CAGGCC TAA CAC ATG CAA GTC-39) and reverse primer 1387r (59-GGG CGG WGT GTA CAA GGC-39) described by Marchesi et al. (1998). DNA was amplified by KAPA Robust 2G (KAPA Biosystem). PCR reaction (25 µl) contained 1.25 µl of each primer, 1.50 µl of template DNA, 12.5 µl KAPA Robust 2G PCR Kit and brought to 25 µl of deionized water. The amplification was performed in a thermal cycler (Takara PCR Thermal Cycler Dice TP600). Amplification conditions included a denaturation step for 5 min at 95°C followed by 35 cycles consisting of 15 s at 94°C, 30 s at 53°C and 60 s at 72°C. The PCR products were analysed by electrophoresis in 1.7% agarose gel.

Determination of bacterial growth and PAH degradation in liquid media

The selected isolates AMP 10 was grown in 250-ml flasks containing 100 ml of MSM supplemented with pyrene at concentration 50 mg/L and 100 mg/L. The flasks were inoculated with 5% (v/v) inoculum at final concentration 10⁴ co-

lony forming unit (cfu/mL) and were incubated at room temperature on a rotary shaker (100 rpm) for 7 days. In order to investigate the growth of isolates, a culture solution were taken out every 24h of incubation and inoculated in plate count agar by pour plate method. The growth of isolates was measured by counting colony forming unit of the culture. The residual PAH from culture flask was determined by taking 1 mL of culture and centrifuged at 10.000 rpm for 10 minutes, and supernatant was separated for GC-MS analysis.

Detection of the gene encoding PAH-degrading dioxygenase

Bacterial DNA was extracted by the DNA extraction kit (Geneaid). The presence of the initial dioxygenase genes in AMP10 isolate was detected based on PCR amplification using PCR kit (KAPA Robust 2G, Kapa Biosystem). The primers for the detection of *nahAc* gene were listed in Table 1.

The PCR conditions were: 94°C for 3 min, followed by 40 cycles consisting of 94°C for 45 s, 55°C for 45 s and 72°C for 45 s, with a final extension period at 72°C for 5 min. The PCR conditions for the nested round were similar to the first, except that 30 cycles were used. The PCR products were checked on 1% agarose gel electrophoresis.

Measurement of emulsifying activity and surface tension

An aliquot (1.0 ml) of inoculum was transferred to 250 ml Erlenmeyer flask containing 50 ml of MSM and 1 % (v/v) carbon sources. Three different carbon source used in the flask culture were glucose, sucrose, and crude oil. Incubation was carried out on a rotary shaker at 100 rpm in room temperature for 5 days. Culture samples were taken at the end of the incubation period, and supernatant of culture was obtained after centrifugation at 10.000 rpm for 20 minutes.

The emulsification index (E24) of culture samples was determined by adding 2 mL of supernatant and 2 mL used lubricating oil to a test

Table 1. PCR primer for the detection of the initial PAH-degrading dioxygenase gene

<i>nahAc</i> - like primer		Sequences	Product size (bp)	Reference
First round	<i>Nah</i> -for	TGCMVNTAYCAYGGYTGG	937	Zhou et al. (2006)
	<i>Nah</i> rev-1	CCCGGTARWANCCDCKRTA		
Nested round	<i>Nah</i> -for	TGCMVNTAYCAYGGYTGG	317	
	<i>Nah</i> rev-2	CRGGTGYCTTCCAGTTG		

tube. The mixture was vortexed for 1 minute and allowed to stand for 24 hours. The height of the stable emulsion layer was measured, and the E24 value was calculated as the percentage of height of emulsified layer (cm) divided by the total height of the liquid column (Bodour et al., 2004).

For surface tension measurements, supernatant was transferred to a glass tube and a capillary tube was dipped in this liquid. This procedure was done at a room temperature. The height reached by the liquid through a capillary tube was measured and surface tension calculated according to the following formula (Viramontes-Ramos, 2010):

$$\gamma = \frac{1}{2} r h \delta g$$

Where :

γ = Surface tension (mN/m);

δ = Density (0.99 g/mL);

g = gravity (980 cm/s²);

r = capillary radius (0.09 cm);

h = height of the liquid column (cm).

Both the surface tension and EI measurements were performed in triplicate.

RESULTS AND DISCUSSION

A four of the nine bacterial isolates which were obtained from oil contaminated soil in Jakarta bay have the ability to grow in MSM agar plate supplemented with PAH pyrene as a sole carbon source (Table 2). This result indicates that these isolates could degrade the PAH compounds. Pyrene is a common PAH obtained from coal tar and frequently used as a chemical intermediate. They were considered as priority pollutant in the environment because of their toxicity. Due to the hazardous effect of PAH, it is necessary to clear up or lowering the concentration of these substances in the environment. Ahmed et al. (2012), described that bacteria have the unique feature in rapidly adapting to toxic environments. The metabolic diversity and plasticity of bacteria allow them to degrade organic pollutants and helps in the mineralisation of these compounds.

As the result in Table 2, four isolates could grow in MSM solid medium with pyrene. Based on the growth rate in PAH medium and performance in the screening of biosurfactant production from the previous study, AMP10 isolates was selected for further analysis. Although RIP43 isolates has the same type of growth with AMP10 isolates (moderate), it was not selected because it has lower emulsification index value than AMA 10 isolates in biosurfactant preliminary screening test (data not shown). The growth of bacteria on hydrocarbons is usually accompanied by the

production of biosurfactants that improve the affinity of the cells to substrates and facilitate the hydrocarbon bioavailability and degradation process. The effect of biosurfactant production by hydrocarbon-degrading bacteria itself may be beneficial, promising and more practical than adding purified biosurfactant for field bioremediation application. A hydrocarbon-degrading bacterium having the ability to produce extracellular biosurfactant can facilitate the oil-microbe contact (Kumar et al., 2006; Sneha et al., 2012).

Table 2. Growth of bacterial isolates on MSM agar plate with pyrene

isolates	Gram type	Growth in pyrene	Type of growth*)
AMA9	negative	-	-
AMP10	positive	+	moderate
CHA60	negative	-	-
CHA63	negative	-	-
CHP64	negative	+	slow
CHP29	positive	+	slow
CRA32	positive	-	-
CRA7	negative	-	-
RIP43	positive	+	moderate

+: positive growth -: no growth

*) Fast: < 7 days, moderate : 7-14 days, slow : > 14 days

Identification of AMP10 isolate was performed by 16S rRNA analysis using forward primer 63f (59-CAGGCC TAA CAC ATG CAA GTC-39) and reverse primer 1387r (59-GGG CGG WGT GTA CAA GGC-39) described by Marchesi et al. (1998). The expected size of the fragment sequenced from the 16S rRNA gene was 1300 bp (Figure 2a). Sequence analysis of 16S rDNA gene showed that AMP 10 was identified as *Gordonia cholesterolivorans* with 99% identity compared to the NCBI databases.

The genus *Gordonia*, belongs to the mycolic acid-containing group of the Actinomycetes and to the suborder Corynebacterineae. Members of *Gordonia* are important in bioremediation processes due to their capacity to degrade hydrocarbons and natural compounds which are not readily biodegradable. Most species of this genus have been known to degrade xenobiotics, environmental pollutants, or otherwise slowly biodegradable natural polymers (Arenskotter, 2004). *Gordonia* sp. strain BS29 has the ability to grow on aliphatic hydrocarbons and produces two different types of surface active compounds (SACs):

extracellular bioemulsans and cell-bound biosurfactants (Franzetti et al., 2009). Some species of genus *Gordonia* has been known able to degrade xenobiotic contaminants or macromolecules such as rubber, (di) benzothiophene, and alkanes. *G. cholesterolivorans* was an aerobic, Gram-positive staining, non-motile and non-spore-forming bacteria (Drzyzga et al., 2009).

Bacterial growth and degradation ability were studied at 50 and 100 mg/L of initial substrate concentrations. Growth of *G. cholesterolivorans* AMP10 both in 50 mg/L and 100 mg/L concentration of pyrene showed a similar pattern (Fig 1). It is observed that the highest cell number obtained on day 5 were 3.3×10^9 cfu/mL and 6.4×10^8 cfu/mL in 50 mg/L and 100 mg/L of pyrene concentration, respectively. Increasing the number of cells indicated that these isolates capable of using pyrene as a source of carbon and energy.

The biodegradation of PAH has been widely studied, and many bacterial strains have been isolated for their ability to degrade PAH. A biodegradation study of PAH by Guo et al. (2010) revealed that more than 80% of each of the three PAHs (Pyrene, Phenanthrene and Fluoranthene) was degraded by *Mycobacterium* after 7 days and pyrene was completely degraded by three *Mycobacterium* strains (SBSW, YOWG and SKEY) after 14 days. *Mycobacterium* showed higher ability in degrading the three PAHs than genera *Sphingomonas*, *Rhodococcus*, *Paracoccus* and *Pseudomonas*. A similar study by Kafilzadeh et al. (2012) reported pyrene degradation value by *Mycobacterium* sp and *Corynebacterium* sp. were 89.1% and 79.4%, respectively. However, according to our knowledge, the degradation of pyrene by *Gordonia cholesterolivorans* has not been reported so far.

The strain *G. cholesterolivorans* AMP 10 showed pyrene degradation about 93.8% at concentration 50 mg/L and 86.5% at concentration 100 mg/L after 5 days and increase to 96.6% and 91.7% after 7 days of incubation (Figure 2). This result was higher than that previously reported. We suggest that the biosurfactant produced by this isolate may assist the bacteria to attach the pyrene for degradation.

Degradation of PAHs was mostly carried out by the dioxygenase enzymes produced by the PAH-degrading bacteria in aerobic conditions. The initial dioxygenases which responsible for attacking the aromatic ring structures of PAH are considered as the key enzymes in PAH degradation activity. The *nahAc* gene that encodes a component of naphthalene dioxygenases can be used

to determine of PAH-dioxygenase genes. This gene serves as a potential biomarker for PAH degradation activity because of its highly conserved (Park & Crowley, 2006; Yuliani et al., 2012)

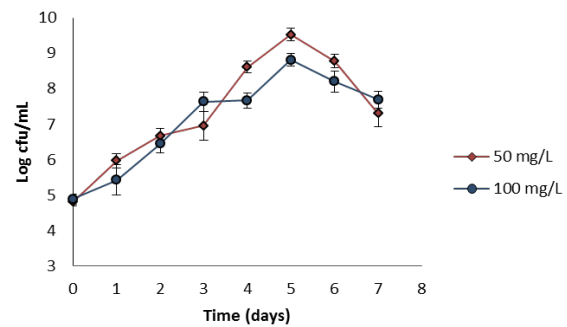


Figure 1. Growth of *G. cholesterolivorans* AMP 10 in liquid MSM supplemented with pyrene

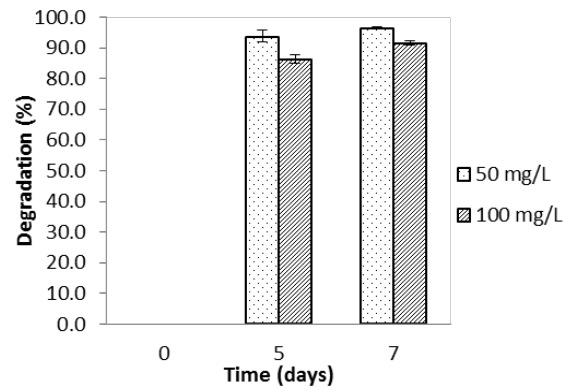


Figure 2. Degradation of pyrene in liquid MSM by *G. cholesterolivorans* AMP 10

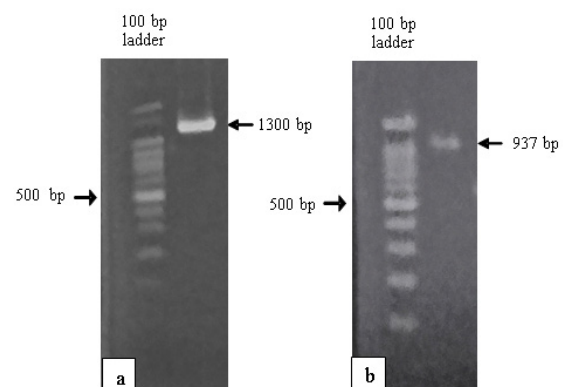


Figure 3. Agarose gel electrophoresis of AMP 10 PCR products. a. 16 S rDNA (1300 bp); b. *NahAc* dioxygenase gene (937 bp)

The presence of the initial dioxygenase gene from *G. cholesterolivorans* was detected by nested PCR amplification using *nahAc*-like primer described by Zhou et al. (2006). These primers amplified DNA fragments of 937 bp (Figure 3b) and 317 bp in nested PCR. However, no PCR

product was detected in nested round PCR in this study. Yuliani et al. (2012) described that nested round PCR was used as a confirmation step to check dioxygenase gene product from the first round PCR. *NahAc* gene is highly conserved among different Gram-negative bacteria (Park & Crowley, 2006). It was obtained from Gram negative bacteria such as *Pseudomonas* and *Burkholderia* species (Guo et al., 2010). This study gives a different result that this gene could obtain from *G. cholesterolivorans* which is Gram-positive bacteria. Park & Crowley (2006) explained that natural horizontal gene transfer could occur between different species that carry this gene. Besides that, the *nahAc* gene was detected not only in naphthalene degrading bacteria but also in bacteria which could degrade higher molecular weight PAHs including phenanthrene, anthracene, fluoranthene, and pyrene.

Surface activity and emulsification capability of bacterial culture give a strong indication of biosurfactant production (Walter et al., 2010). Surface tension reduction, emulsification property and stabilising capacity are the most important properties of microbial surfactants with potential industrial applications (Ferhat, 2011). Biosurfactant contains hydrophobic and hydrophilic groups that confer the ability to accumulate between fluid phases, thereby reducing surface and interfacial tension at the surface and interface regions (Kapadia & Yagnik, 2012). Emulsification activity is the ability for a surfactant to form an emulsion under given condition (Cheng et al., 2008). The excellent emulsification property is critical for biosurfactants to be promising in different environmental and industrial applications (Banat et al., 2000)

To confirm the ability of isolates in biosurfactant production, measurement of surface tension and emulsification test were conducted in this study using three different carbon source i.e. glucose, sucrose and crude oil with 1% concentration each (Fig 4a). Measurement of surface tension and index emulsification (E24) of cell-free culture broth showed that all carbon sources could be used to produce biosurfactant by *G. cholesterolivorans* AMP 10 with surface tension value 26.38-36.64 mN/m and emulsification capacity 62.91-75.38% (Table 2). Used lubricating oil was used in this study to measure emulsification capacity of supernatant (Fig 4b). According to Cooper & Zajic (1980), the major criteria used for selecting microbial biosurfactants is their ability to reduce the surface tension 40 mN/m^{-1} or less, while Wilmsen & Karlson (1997) considered a culture as

promising if it has emulsification capacity at least 50% after 24 h. Biosurfactant is highly variable, depending on the composition of the growth medium that is used to culture the microorganisms.

Table 3. Surface tension and emulsification index (E24) value in different carbon source

Carbon source	Surface tension (mN/m)	Emulsification index (E24)
Glucose	24.7±0.00	78.35±0.38
Sucrose	26.2±0.00	62.91±2.53
Crude Oil	36.4±2.52	68.98±2.06

In this study, glucose was the best carbon source for biosurfactant producing by *G. cholesterolivorans*. It has lowest surface tension and highest emulsification activity compared to sucrose and crude oil (Table 3). The surface tension decreased from 71.3 to 24.7 mN/m and E24 value obtained was 78.54%. Effect of carbon source on biosurfactant production by *Bacillus clausii* 5B was made by Aparna et al. (2012), glucose as a carbon source showed lowest surface tension value (30.23 mN/m) compared to sucrose, molasses and glycerol. Similarly, glucose as a carbon source could decrease the surface tension of *P. aeruginosa* MM1011 cell-free culture up to 20mN/m (Rashedi, 2006). This current result suggesting that *G. cholesterolivorans* AMP 10 was a potential pyrene degrader and a good biosurfactant producer which could played a major role in bioremediation process.

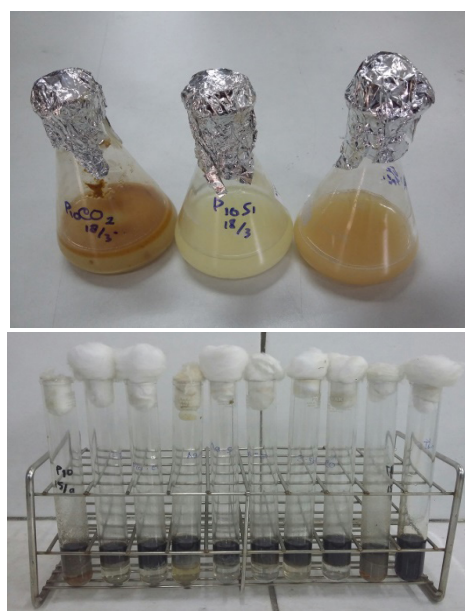


Figure 4. a. Growth of *Gordonia cholesterolivorans* AMP10 in different carbon source; b. Emulsion

of cell-free culture in used oil.

CONCLUSION

The results obtained in the present study showed that the novel biosurfactant-producing bacteria *G. cholesterolivorans* AMP 10 have the ability to degrade pyrene. It has shown a good potential to degrade pyrene as a sole carbon source. This degradation ability was confirmed by *nahAc* dioxygenase gene product obtained by nested PCR analysis. Biosurfactant production of this isolate was indicated by reduced surface tension of culture medium and high emulsification index (E24) value. Therefore, it can be concluded that *G. cholesterolivorans* AMP 10 with the capacity to degrade pyrene and at the same time to produce biosurfactant, can find application in bioremediation.

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