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INHIBITIVE CHEMICAL CUE OF *Pseudomonas pseudoalcaligene* ON BIODEGRADATION OF ANTHRACENE IN SEAWATER MEDIUM

[1]HII YII SIANG; [2]LAW AH THEEM; [2]CHUAH LAI FATT; [2]NOOR AZHAR MOHAMED SHAZILI; [3]MOHAMED KAMIL ABDUL RASHID; [3]YONG JAW CHUEN

[1]*Faculty of Agrotechnology and Food Science,* [2]*Institute of Oceanography and* [3]*Faculty of Maritime and Marine Science Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia.*

** Corresponding author email address: hii@umt.edu.my*

Abstract: This study aims to reveal the self-inhibitive mechanism of an oil degrading bacteria, *Pseudomonas pseudoalcaligene* biodegradation of anthracene in seawater. Biodegradation of anthracene by the oil-degrading bacteria was analogue to the Monod equation and exhibited a typical bacterial growth pattern. Biodegradation of anthracene was retarded when the bacteria approached its stationary growth phase. The results indicated that the level of dissolved organic carbon and hydrogen ion in the seawater medium increased due to anthracene biodegradation. The organic compounds were believed to be secondary metabolites produced by the oil-degrading bacteria. These metabolites seem to inhibit activities of the oil degrading bacteria. The inhibitive effect was confirmed when the newly inoculated oil degrading bacteria failed to grow in the residue medium replenished with nutrients and new carbon source and incubated under optimal condition. It is interesting to note that, this replenished medium supported the growth of other non-oil degrading bacteria, *Erythrobacter citreus.* Presence of *E. citreus* in the medium utilized the inhibitive metabolites and thus resumed activities of the oil degrading bacteria for anthracene. Mixing the oil-degrading bacteria (*P. pseudoalcaligenes*) and non-oil degrading bacteria (*E. citreus*) improved biodegradation of anthracene in the seawater. The bacterial mixture improved anthracene degradation by 36% compared to the oil degrading bacteria alone.

KEYWORDS: Anthracene, n-octadecane, Biodegradation, Mixed bacterial culture, Inhibition.

Introduction

In recent years, world production of crude oil exceeds 3 billion tonnes per year and about half of the crude oil is transported by sea (Harayama et al., 2004). The marine environment, especially those areas with heavy oil tanker traffic are vulnerable to oil spills. The Straits of Malacca is one of the busiest straits in the world. It is located on the primary shipping lane of international crude oil route from the Middle East to Japan. More than 100,000 vessels ply this straits (Chua et al., 2000) and two hundred million tonnes of crude oil (Law and Hii, 2006) are transported through the Straits of Malacca every year. These activities pose the area to the risk of oil pollution. The recommended safety level of oil pollution for marine environment is set at 100 mg kg^{-1} oil in sediment and 100 µg L^{-1} oil in water (Law and Hii, 2006). At present, the average oil concentration in the Malaysian seas is between 100 - 150 µg L^{-1}, and can be increased up to 500 µg L^{-1} during oil spill (Law and Hii, 2006). Oil spill is disastrous to a wide spectrum of marine organisms, the impact of oil spills were widely documented in many reports (IPIECA, 2000; Law and Hii, 2006). Hence, it is important to develop technique for combating oil pollution in the marine environment.

Biodegradation is one of the potential solutions for combating oil pollution. In fact, oil spill will undergo biodegradation processes via microbial activities in the natural environment.

Oil degrading bacteria play a vital role in degradation of hydrocarbons in the marine environment (Chaillan et al, 2004). Zobell first reported the role of marine bacteria in the transformation of hydrocarbon in the marine sediment in 1932 (Zobell and Feltham, 1934). Since then, many studies were conducted to isolate oil-degrading bacteria. Most of the oil degrading bacteria isolated from the marine environment belong to the genus *Pseudomonas* (Bass, 1999). Nevertheless, none of the oil degrading bacteria could solely degrade all the hydrocarbons, especially higher molecular weight polycyclic aromatic hydrocarbons (PAHs). Biodegradation of PAHs is always slow in the marine environment. The slow PAHs degradation could be due to many factors such as nutrient limitation, microbial potential, unfavorable environmental conditions and self-inhibiting chemical cue produced during the compounds breakdown.

This report focused a potential self-inhibiting chemical cue produced during the biodegradation of anthracene by *P. pseudoalcaligenes*. It was found that, addition of non-oil degrading heterotrophic bacteria could enhance the degree of hydrocarbons degradation. This information is useful for formulating new microbial strategy for combating oil pollution in the marine environment. Anthracene and other related PAHs are a large class of ubiquitous environmental pollutants that exhibit toxic, mutagenic and carcinogenic properties. Anthracene was selected for this study because of its carcinogenic property and resistance to biodegradation in the marine environment.

Materials and Methods

Preparation of culture medium

Marine agar 2216 (Difco) was used as solid medium for the bacteria culture. Marine broth 2216 (Difco) was used to mass culture the bacteria. The media were sterilized using autoclave at 121 ºC, 15 psi for 15 minutes. The culture media were incubated at culture condition for 48 hours to ensure that no contaminations occur. All the bacteria produced by the media were harvested and washed thrice with 1% saline medium at 5000 rpm for 15 minutes. Law and Button (1977) medium were used as synthetic seawater medium. The experiments were conducted under optimal condition for the bacteria at 28 °C, salinity 30 g L^{-1} and pH 8.

Hydrocarbon media

Anthracene (Sigma) medium was prepared by adding 1 mL of anthracene standard solution (1 mg/mL dichloromethane, DCM) to a 250 mL conical flask and allowed to dry for 2 days to remove all the DCM. Sterilized synthetic medium (100 ml) was added to make up 10 mg L^{-1} anthracene-enriched medium. Similar procedure was used to prepare 50 mg L^{-1}, 100 mg L^{-1}, 300 mg L^{-1} and 500 mg L^{-1} anthracene medium. The n-octadecane (Sigma) medium was prepared in a similar manner.

Bacterial seeding

The oil degrading bacteria was isolated from the coastal environment of Port Dickson by Law and Teo (1997), while the non-oil degrading bacteria were isolated from Marang river estuary. The non-oil degrading bacteria were selected because of their ability to withstand high concentration of crude oil. However, the heterotrophic bacteria were not able to utilize hydrocarbon as their source of carbon. Based on the identification conducted by Hii et al. (2009), the oil degrading bacteria was identified as *Pseudomonas pseudoalcaligene*s while the non-oil degrading was identified as *Erythrobacter citreus.*

Hydrocarbon analysis

Anthracene and n-octadecane residue in the culture medium was determined by the gravimetric method of Law and Teo (1997). The medium (100 mL) was extracted three times with dichloromethane (50 mL, 25 mL and 25 mL respectively). The water residue and polar compounds in the extract were removed by 5 g sodium sulphate anhydrous (Merck) and 3 g silica gel (Merck) respectively. The solvent was evaporated to dryness in a rotary evaporator at half the atmospheric pressure and 45°C. When the extract reduced to about 1mL in round bottom flask, the concentrated extracts were transferred into a pre-cleaned vial and rinsed with minimum amount of DCM to bring the residue hydrocarbons into the vial. DCM was then removed under a gentle stream of pure nitrogen. Later, the vial was kept in a desiccator for 1 hour. The weight of the sample vial was obtained using a microbalance (Perkim Elmer AD-6). The process (dry in desiccator) was repeated until a constant weight for the sample was obtained. Concentration of the hydrocarbons was calculated based on the weight difference (Sample vial - blank vial) divided by the sample volume.

Determination of total organic carbon (TOC) in the seawater medium

Total organic carbon (TOC) in the seawater medium was determined after the seawater medium was extracted for its hydrocarbons content using dichloromethane. Total Organic Analyzer (Shidmazu, TOC-5000) was used to determine the TOC. The concentration of TOC was obtained from the total carbon (TC) and inorganic carbon (IC) where TOC = TC – IC. The concentration of TC and TIC was obtained by referring the peak areas to the standard curve establish for the TC and IC. Standard curve was established between organic and inorganic carbon versus peak area. Sodium bicarbonate ($NaHCO_3$) and potassium hydrogen phthalate ($C_8H_5KO_4$) were used for inorganic carbon (IC) and total carbon (TC) calibration respectively

Bacterial growth, hydrogen ion and dissolved organic carbon during biodegradation

5 L bioreactor (Sartorius) was used to assess bacterial hydrogen ion production and dissolved organic carbon level during the biodegradation. 2 L of sterilized anthracene enriched medium (100 mg L^{-1}) was added into the culture vessel. *P. pseudoalcaligenes* was inoculated into the vessels at 1 x 10^8 cell ml^{-1} to initiate the experiment. The experiment was conducted under optimal condition at 28 °C, salinity 30 g L^{-1}, pH 8 and dissolved oxygen of greater than 5 mg L^{-1}. Temperature, pH and dissolved oxygen of the medium were regulated and maintained by the reactor ‘s controller at ±1% off set. A controller-operated peristaltic pump would drip 1 N sterilized NaOH into the reactor when the pH of the medium dropped. Hydrogen ion produced during the biodegradation was calculated by using the concentration and volume of NaOH used for maintaining the pH. The bacterial population and total organic carbon were measured every 24 hours for 4 day and hydrocarbons remained in the culture vessel was determined at the end of the experiment. The experiment was repeated using a mixed bacteria culture (*P. pseudoalcaligenes* + *E. citreus*). A blank culture without bacterial inoculation was used as the control. Growth of the bacteria in the synthetic medium was determined by using plate-counting technique (ASM, 1993). *P. seudoalcaligenes* produced creamy and entire colony while *E. citreus* produced orange colour colony.

P. pseudoalcaligenes, E. citreus and mixed bacterial culture in replenished residue medium

Responses of *P. pseudoalcaligenes, E. citreus* and bacterial mixture of these two bacteria in replenished residue medium were assessed in this experiment. Residue medium was the seawater

medium after the biodegradation studies, when the bacteria were at their stationary growth phase. The medium was filtered using a sterilized 0.2 µm filter paper (Whatman) and replenished with nutrients as described by Law and Button (1977) and anthracene at 100 mg L^{-1}. The growth of *P. pseudoalcaligenes, E. citreus* and the mixture of these bacteria were then re-inoculated into the residue medium and the growth of the bacteria was experimented (Figure 1). Briefly, 100 mL of sterilized and filtered residue medium was added into a 250 mL flask. 1 x 10^8 cell mL^{-1} of the bacteria were inoculated into the sterilized medium. The flasks were incubated under optimal condition for 6 days. Cell population, hydrogen ion, hydrocarbons concentration and TOC level in the seawater medium was assessed every 24 hours. The experiments were conducted in three replicates.

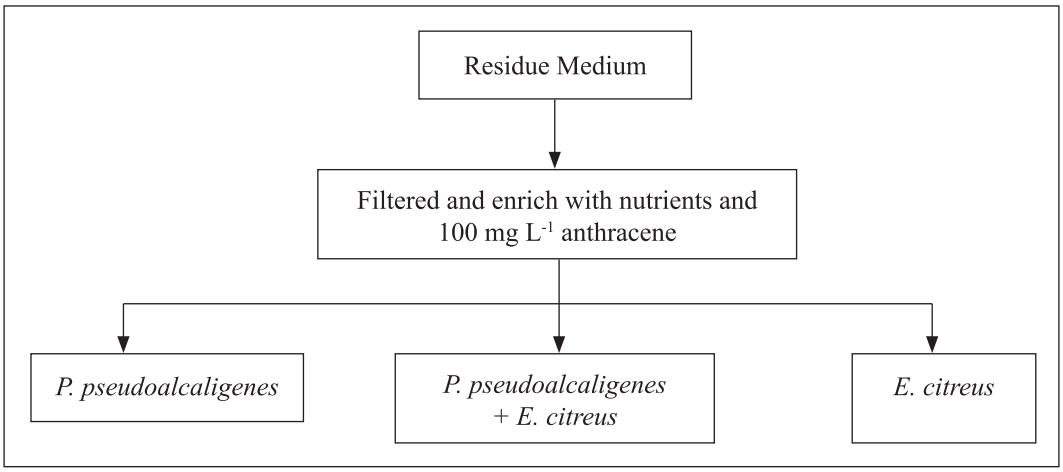


Figure 1 Experimental structure for studying the responses of different bacteria in the replenished residue medium

Data analysis

Specific growth rate (µ) of bacteria in the seawater medium was determined by using the following simple equation (Ray, 2004). Specific growth rate of the cells was reported in $hour^{-1}$. The parameter used in the equation was obtained when the bacteria was in their logarithm growth phase.

$$\mu = \frac{(\ln C_t - \ln C_o)}{t}$$

$$= \frac{\ln (C_t/C_o)}{t}$$

Where,
C_t = Cell population at the end of the log phase, cell mL^{-1}
C_o = Cell population at the beginning of the log phase, cell mL^{-1}
t = Time, hour

The hydrocarbons degradation rate was estimated through the following equation (Law and Teo, 1997). Similar to the bacterial growth curve, the parameters used in the equation were obtained at the logarithm growth phase. Hydrocarbons degraded by *P. pseudoalcaligenes* and the bacteria

mixture (*P. pseudoalcaligenes* + *E. citreus*) was compared by using t-test ($\alpha = 0.05$).

$$\text{Oil-degradation rate, mg L}^{-1}\text{ h}^{-1} = \frac{C_o - C_i}{t_o - t_i}$$

Where,
C_o = hydrocarbons concentration in seawater medium at time t_o, mg L^{-1}
C_i = hydrocarbons concentration in seawater medium at time t_i, mg L^{-1}
$t_o - t_i$ = Incubation time between t_o and t_i, hour

Results and Discussion

Degradation of anthracene and n-octadecane by the oil degrading bacteria, *P. psudoalcaligenes* revealed a typical biodegradation pattern (Figure 2). Biodegradation of anthracene by *P. psudoalcaligene* is a series of enzymatic processes which analogue to the Monod Equation, $\mu = \mu_{max} \frac{S}{K_s + S}$, where μmax is the maximum specific growth rate, S is the anthracene concentration and Ks is the Monod constant. The Monod constant, K_s and μ_{max} for biodegradation of anthracene and n-octadecane in the seawater medium were 17.8 mg L^{-1} anthracene, 0.037 h^{-1} and 137.2 mg L^{-1} n-octadecane, 0.088 h^{-1} respectively. Table 1 lists specific growth rate of *P. pseudoalcaligenes* at different concentration of anthracene and n-octadecane. The bacteria experienced a 48 hours lag phase before the exponential growth and it reached stationary growth phase after 120 hours incubation in the anthracene medium. Biodegradation of anthracene involved a series of biochemical reactions. Hydrocarbons were degraded into organic intermediates in the medium before they can be acquired and assimilated by the bacterial tri-carboxylic acid (TCA) cycle. As shown in Figure 3, the amount of organic metabolites and hydrogen ion increased as anthracene and organic matter degraded by *P. pseudoalcaligenes.* When the crude organic metabolite approached 18 mg C L^{-1}, biodegradation of anthracene by the oil degrading bacteria seems to be inhibited. The inhibition was confirmed when the newly inoculated oil degrading bacteria failed to grow in the nutrients and anthracene replenished residue medium. The inhibitive effect was not observed in the biodegradation of aliphatic hydrocarbon, n-octadecane in fact, biodegradation of n-octadecane was enhanced by the replenishment of nutrients and hydrocarbon source in the residue medium (Table 2). The failure of the oil degrading bacteria to grow in the replenished medium agreed to the presence of inhibitive compounds for biodegradation of the PAHs. The inhibitive compounds were believed to be the secondary metabolite. It is interesting to note that, the non-oil degrading bacteria, *E. citreus* was able to proliferate in the residue medium (Table 2). Growth of *E. citreus* suggested that, mixture of the oil degrading bacteria, *P. pseudoalcaligene* and the non-oil degrading bacteria, *E. citreus* may further enhance the biodegradation of anthracene in the seawater medium. Mixed bacterial culture (*P. pseudoalcaligenes* + *E. citreus*) improved biodegradation of anthracene in the seawater medium (Figure 4). The mixed bacterial culture degraded 30.8±3.2 % anthracene from a 100 mg L^{-1} anthracene enriched medium. Inoculation of single strain of oil degrading bacteria degraded 22.6 ± 1.6 % of anthracene under similar experimental conditions. The mixed bacterial culture showed a significant improvement of 36.3 % anthracene degradation over the single strain ($p = 0.01$, t-test).

Table 1. Specific growth rate of AR3 at different concentration of anthracene and n-octadecane (single inoculation).

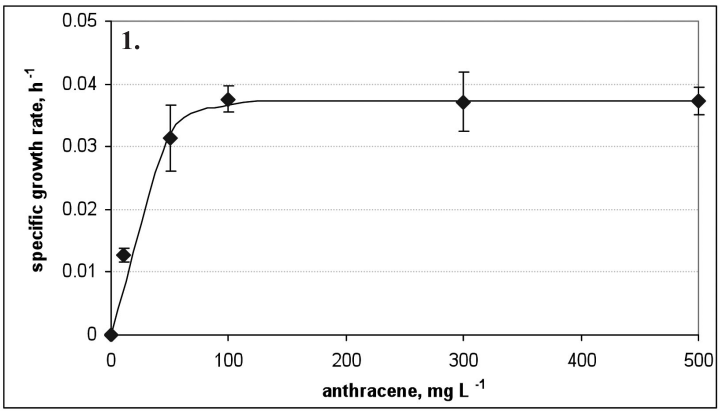
Anthracene	Specific growth rate	n-octadecane	Specific growth rate
10 ppm	0.0127 ± 0.0011 h^{-1}	100 ppm	0.0438 + 0.0056 h^{-1}
50 ppm	0.0314 ± 0.0053 h^{-1}	300 ppm	0.0657 + 0.0087 h^{-1}
100 ppm	0.0376 ± 0.0021 h^{-1}	500 ppm	0.0764 + 0.0101 h^{-1}
300 ppm	0.0371 ± 0.0047 h^{-1}	1000 ppm	0.0854 + 0.0192 h^{-1}
500 ppm	0.0372 ± 0.0022 h^{-1}	1500 ppm	0.0916 + 0.0106 h^{-1}

Table 2 Responses of the oil degrading bacteria (AR3) and non-oil degrading bacteria (OG) in the replenished medium. The plus (+) sign indicates a positive bacteria growth in the medium while the minus (-) sign indicates no/ slow growth.

	Replenished residue medium*	Fresh basal medium
AR3	–	+
OG	+	–
AR3 + OG	+	+

* Residue medium is the residue medium from the biodegradation studies when the bacteria growth approaching stationary phase at day 7. The medium was filtered by using a 0.2 µm filter paper and replenished with nutrients and anthracene at 100 mg L^{-1}

Figure 2. Biodegradation of anthracene and n-octadecane in the synthetic medium. **1.** and **2.** indicates specific growth rate of *P. pseudoalcaligenes* in anthracene and n-octadecane medium respectively.



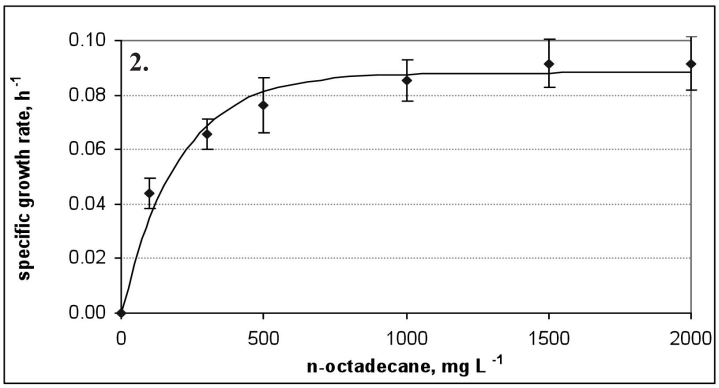
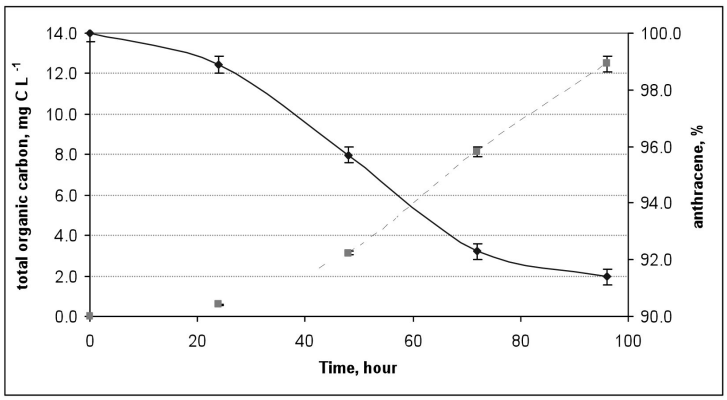
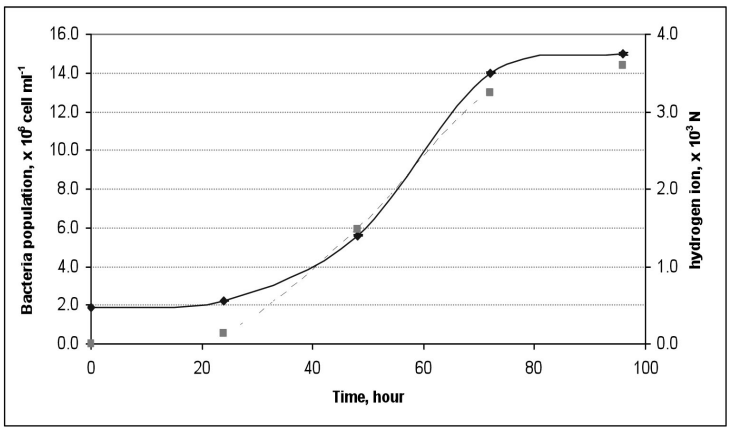


Figure 3. Bacterial population, anthracene remained, hydrogen ion production and total organic carbon in the culture medium.

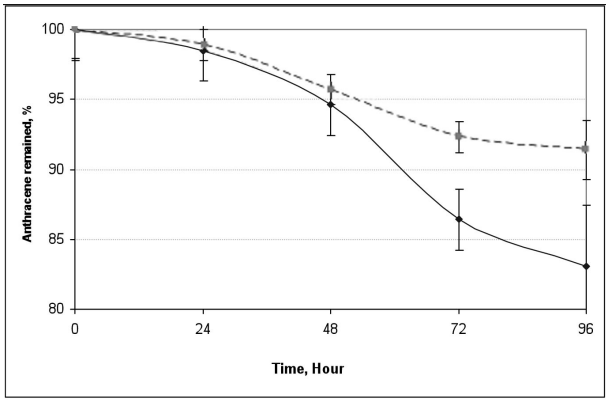


** Anthracene remained in the medium was indicated by solid line while dotted line indicates total organic carbon produced during the biodegradation.*



**Solid line indicates bacteria population while dotted line indicates nett hydrogen ion produced during the biodegradation*

Figure 4. Performance of the mixed bacterial culture in biodegradation of anthracene. The solid line indicates performance of the bacterial mixture while the dotted line indicates performance of the oil degrading bacteria (mono-inoculant).



Bacterial growth phase; lag phase, exponential phase, stationary phase and death phase have been widely reported in many bacteriological studies. The stationary growth phase was normally triggered by exhaustion of available nutrients, accumulation of inhibitory metabolites and lack of space (Madigan et al., 2000). The retarding anthracene biodegradation in this study was clearly not the case for nutrient exhaustion and lack of space as the nutrients and the space was replenished in the residue medium. The retardation was probably due to the presence of inhibitive compounds, which was believed to be the secondary metabolite. Production of inhibitive metabolite by bacteria was widely reported (Boettcher et al., 2000; Hii et al., 2009). In fact, the concept was applied as early as 1966 for production of antibiotics by a marine bacterium during the stationary growth phase (Burkholder et al., 1966) and some of the secondary metabolites nowadays are already used as bio-control agents and are used in aquaculture (Wagner-Döbler et al., 2002; Boettcher et al., 2000). Hii et al., 2009 reported similar observation in the crude oil contaminated sediment but the secondary metabolite was not further examined. This study confirmed the presence of secondary metabolite in the stationary phase. Introduction of other bacteria capable of metabolizing the organic inhibitor would enhance biodegradation of polycyclic aromatic hydrocarbons in the seawater. Interestingly, the secondary inhibitor was not present in the biodegradation of n-octadecane. The results suggested that, the inhibitive organic metabolite is closely bound to the metabolite pathway for the aromatic degradation. Production of the inhibitive metabolite may be compound specific and dependent on the bacterial species. However, further studies are needed to look into the identification of the secondary metabolite.

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