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Isolation and Characterization of a Diesel-degrading Bacterium from Waters Near the Langkawi UNESCO Kilim Karst Geoforest Park

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ABSTRACT

This study explored the biodegradation potential of bacterial isolates from various locations in Malaysia, focusing on their ability to utilize diesel as a carbon source. Among the ten isolates tested, Isolate 4, identified as *Pseudomonas* sp. strain UPM-Langkawi 3 based on morphological, cultural, and biochemical properties, exhibited significant growth on 2.5% diesel, indicating rapid activation of diesel assimilation mechanisms. Using ABIS online software analysis, the strain was provisionally identified as *Pseudomonas* sp. UPM-Langkawi 3. Experimental results demonstrated that the optimal conditions for the growth of strain UPM-Langkawi 3 were between 6 and 8 % (v/v) diesel concentration, a temperature of between 28 and 35 °C, and a pH range of between 7.5 to 8.5. The strain showed inhibited growth at higher diesel concentrations and temperatures above 40°C and at a lower pH of 5.7. Among several inorganic nitrogen sources tested, 0.9% w/v ammonium sulphate was found to be the most effective, corroborating previous research. The study underscores the efficacy of *Pseudomonas* sp. strain UPM-Langkawi 3 in degrading diesel, suggesting its potential use in bioremediation of diesel-contaminated environments. Further research should employ molecular identification techniques, such as 16srRNA gene sequencing, to confirm the species identity and optimize biodegradation conditions. This work establishes a foundation for utilizing indigenous bacterial strains in environmental management strategies, particularly for areas impacted by hydrocarbon pollutants.

INTRODUCTION

Soil and water pollution are typically caused by industrial waste. Current physical and chemical treatments are costly and ineffective in eliminating small amounts of pollutants. Several effective remediation approaches have been created and implemented in petroleum-contaminated environments. Indigenous bioremediation is crucial in addressing diesel pollution because of its harmful fumes and strong odor, which require prompt treatment [1–3]. Additionally, utilizing indigenous bioremediation products offers several benefits, including easy maintenance, applicability to vast areas, affordability, and complete degradation of contaminants [4–9]. According to the Department of Environment in Malaysia, oil and grease are considered the most significant sources of industrial pollution [10].

Due to Malaysia's status as a prominent oil and gas producer and its ownership of the Straits of Malacca, one of the busiest waterways globally, oil contamination is inevitable. Contamination in Malaysia is primarily due to human mistake. Two oil tankers collided in the coastal sections of the Straits of Malacca, causing the spillage of about 6000 tons of fuel. This

incident is considered one of the largest hydrocarbon spills ever reported [11]. In addition, there was a spillage of approximately 15 tons of diesel in Seremban due to an overturned lorry tanker, as the New Straits Times reported in 2000. Another incident involved a spill of one ton of diesel into the soil in Gelugor from a 1,000 kW mobile generator unit, as The New Straits Times reported in 2001 [12]. Despite numerous publications on the isolation of diesel-degrading bacteria, the search for the most effective degrader continues in order to isolate bacteria with superior capabilities for enhancing diesel cleanup. This study discusses the isolation of a bacteria capable of decomposing diesel and the subsequent biodegradation investigations conducted in soil. According to the data obtained, the isolate is deemed suitable for application as a bioremediation agent in tropical regions.

Langkawi was designated a UNESCO geopark in July 2007, signifying its shift from a serene Malaysian gem to a popular international tourist spot. The UNESCO designation has significantly stimulated growth in its coastal development and tourist sectors, enhancing its attractiveness to international visitors. The Kilim Karst Geoforest Park has transformed from a tranquil rural area to a popular tourist spot. The park's unique geological formations and natural environments demonstrate the potential of geoparks to enhance local economies and culture. The surge in tourism has led to environmental issues, particularly due to the rise in maritime traffic along the Kilim River and its ecological impact. Increased tourism may lead to the degradation of mangrove habitats and the erosion of riverbanks. Habitats serve to safeguard biodiversity and mitigate coastal erosion. Pollutants including detergents from domestic usage and heavy metals such as cadmium, cobalt, lead, and zinc near Kilim Karst Geoforest Park, maybe from boat emissions and construction activities, endanger the park's ecological equilibrium [13–18].

Hydrocarbon pollution in the waters near Langkawi, Malaysia, is a significant environmental concern, particularly because of its potential impacts on marine and coastal ecosystems. Hydrocarbons, which are primarily composed of hydrogen and carbon, are organic compounds that can be introduced into the marine environment through various sources, including oil spills, industrial discharges, and runoff from land [19]. Studies conducted in the region highlight hydrocarbons' persistent presence and varying concentrations in coastal waters. For instance, in areas like Port Dickson, which faces similar environmental challenges, hydrocarbon levels have been documented to range from 0.77 to 7.87 µg/L. This indicates that hydrocarbons are predominantly introduced into these waters via ballast water from international shipping activities. Although Langkawi is not explicitly mentioned in the detailed data from this study, the scenario is likely similar given the commonality of shipping activities and industrial impacts in coastal regions across Malaysia.

Furthermore, continuous monitoring and studies are essential to understand the full scope of hydrocarbon pollution and its ecological impacts. This involves measuring the concentration of hydrocarbons and studying their sources, seasonal variations, and the effectiveness of existing regulatory frameworks aimed at reducing this pollution. Effective management of hydrocarbon pollution also involves community participation and awareness, ensuring that local populations understand the impact of pollution and engage in efforts to mitigate it [20–24].

Addressing hydrocarbon pollution in waters near Langkawi involves a multifaceted approach that includes monitoring, regulation, and community engagement. Ensuring the health of these marine environments is crucial for the sustainability of local ecosystems and the communities that depend on them. The isolation of xenobiotics-degrading bacteria from this site is crucial for future bioremediation operations. In this study, we report on the isolation of an effective diesel-degrading bacterium from mangrove sediments of the Langkawi UNESCO Kilim Karst Geoforest Park.

MATERIALS AND METHOD

Isolation of Diesel-Degrading Bacteria

Samples of sedimentary soils were collected 5 cm below the topsoil layer from a mangrove located near the Langkawi UNESCO Kilim Karst Geoforest Park. A gram of soil was combined with 100 milliliters of sterile tap water. The soil samples were packed in sterilized plastic bags and preserved on ice throughout transportation from the site to the laboratory. 0.1 mL samples were spread on nutrient agar plates with 1% (v/v) diesel as the only carbon source and cultured at 30 °C for up to 6 days. Several colonies with positive characteristics were isolated and cultured through a series of transfer steps until pure colonies were obtained. Bacterial growth was quantified using the colony count method. A basal salt medium containing fuel as the carbon source was used as the enrichment culture medium.

A modified basal salt medium, pH 7.5, was composed of (per liter of tapwater): KH_2PO_4, 1.360 g; Na_2HPO_4, 1.388 g; KNO_3, 0.5 g; $MgSO_4$, 0.01 g; $CaCl_2$, 0.01 g; $(NH_4)_2SO_4$, 7.7 g. The diesel was bought from an Esso gas station in Seri Serdang, Selangor. The culture medium was enhanced with 1% (v/v) diesel, pH adjusted to 7.5, and autoclaved at 121 °C for 15 minutes before being used [12]. The flasks were incubated at 30 °C and shaken at 150 rpm for six days using a YIH DER shaker from Taiwan. Culture isolation and enumeration were conducted using the spread plate method. The cultures were subsequently placed in an incubator set at 30 °C. Distinct colonies were isolated through repeated subculturing in basal salt medium and solidified basal salt medium to obtain pure strains.

Morphological, physiological and biochemical characterization of the Diesel-degrading bacterium

Biochemical and phenotypic methods were used to characterize microorganisms. On the nutrient agar plate, these features may be determined by looking at the size of the colony as well as its form and color. In line with the Bergey's Manual of Determinative Bacteriology, the Gram staining, bacterial motility, oxidase test (24 hours), beta-galactosidase, catalase production (24 hours), ornithine decarboxylase, and other standard tests were carried out [25]. The results were interpreted via the ABIS online system [26].

Statistical Analysis

One-way analysis of variance or Student’s t-test was utilized for intergroup comparisons, followed by post hoc analysis using Tukey’s test. The values are the mean ± standard deviation (SD) of three repeated experiments. A p-value below 0.05 was deemed significant.

RESULTS AND DISCUSSION

Screening and Isolation of Diesel-Degrading Bacteria

Ten bacterial isolates were obtained from the topsoil layer from a mangrove located near the Langkawi UNESCO Kilim Karst Geoforest Park (**Fig. 1**). Each isolate was assessed for its growth potential on 2.5% diesel (v/v) over a six-day period. Isolate 3 was selected for further investigations due to its highest growth.

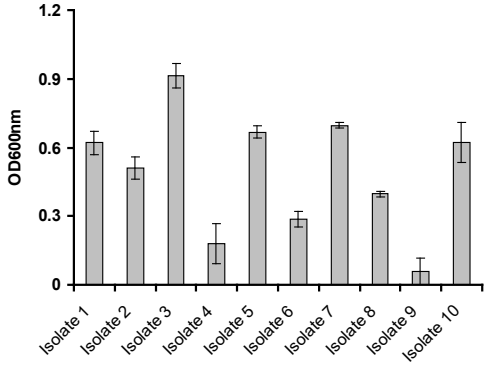

Fig 1. Diesel-degrading bacteria grew after 4 days of incubation on 2.5% (v/v) diesel. Data shows the mean and standard error of the mean (SEM), based on a sample size of 3.

Identification of bacterium

The bacterium was a short rod-shaped, motile, Gram-negative microorganism. The bacteria was identified by comparing the results of culture, morphological, and several biochemical tests to the Bergey's Manual of Determinative Bacteriology. [27] and using the ABIS online software [26]. The software (**Table 1**) provided three recommendations for the bacterial identity with the highest similarity (86%) and precision at 87% as *Pseudomonas putida*. Additional research is required in the future, particularly using molecular identification techniques to compare the 16srRNA gene for further species identification. Currently, the bacterium is provisionally recognized as *Pseudomonas* sp. strain UPM-Langkawi 3.

It is currently impossible to accurately classify individuals based on their species. In the future, additional study, namely molecular identification methods utilizing comparisons of the 16srRNA gene, will be required to identify this species precisely. *Pseudomonas* genus is famous for its ability to degrade toxic compounds such as petroleum sludge and diesel. The genus *Pseudomonas* is renowned for its ability to degrade toxic compounds like petroleum sludge and diesel. Its metabolic versatility and diversity allow it to utilize various organic compounds as energy sources. This capability is largely due to the array of enzymes such as oxygenases and dehydrogenases that they produce, which break down complex hydrocarbons into less harmful substances. *Pseudomonas* species are genetically adaptable, with large genomes that encode numerous catabolic pathways, regulatory networks, and efflux systems, enabling them to adjust to and resist environmental pollutants rapidly.

Additionally, their ability to form biofilms enhances the efficiency of biodegradation processes by stabilizing and concentrating degradative enzymes. They also engage in co-metabolism, where the degradation of one substance is aided by enzymes produced for metabolizing another, and their ecological competitiveness allows them to outcompete other microbes in contaminated environments. These traits make *Pseudomonas* species valuable in bioremediation strategies to clean up sites contaminated with hydrocarbons, aiding in restoring ecological balance [28–36].

Table 1. Biochemical tests for *Pseudomonas* sp. strain UPM-Langkawi 3.

Motility	+	Utilization of:	
Hemolysis	+	L-Arabinose	+
Growth at 4 ºC	–	Citrate	+
Growth at 41 ºC	+	Fructose	+
Growth on MacConkey agar	–	Glucose	+
Arginine dihydrolase (ADH)	+	meso-Inositol	–
Alkaline phosphatase (PAL)	+	2-Ketogluconate	+
Indole production	–	Mannose	+
Nitrates reduction	–	Mannitol	d
Lecithinase	–	Sorbitol	–
Lysine decarboxylase (LDC)	–	Sucrose	+
Ornithine decarboxylase (ODC)	–	Trehalose	–
ONPG (beta-galactosidase)	–	Xylose	–
Esculin hydrolysis	–		
Gelatin hydrolysis	–		
Starch hydrolysis	–		
Urea hydrolysis	+		
Oxidase reaction	+		

Note: + positive result, − negative result, d indeterminate result

Bacterial growth optimization on diesel

The effect of Carbon source

The objective of this study was to identify the optimal concentration of diesel as a carbon source for strain UPM-Langkawi 3. The results suggest that the ideal diesel concentration for the growth of strain UPM-Langkawi 3 is between 6 and 8% (v/v). Higher diesel concentrations were found to inhibit growth.

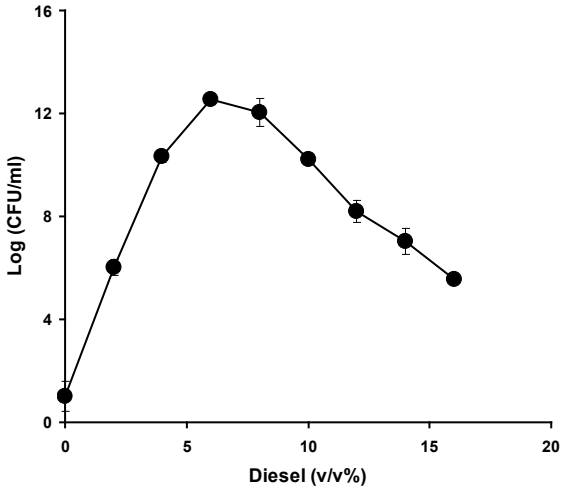

Fig. 2. Studying the impact of different diesel concentrations on the growth of strain UPM-Langkawi 3 over a 3-day period at room temperature using an orbital shaker set at 150 rpm. Bacterial growth was quantified by assessing the colony forming unit (Log CFU/mL). Data shows the mean and standard error of the mean (SEM), based on a sample size of 3.

The effect of Temperature and pHs

The strain UPM-Langkawi 3 was incubated at various temperatures to optimize its growth conditions. Strain UPM-Langkawi 3 demonstrated a growth range of 20 to 40 °C with an optimal temperature of between 28 and 35 °C, as depicted in **Fig. 3**. Growth significantly declines at elevated temperatures. Strain UPM-Langkawi 3 exhibits best growth between pH 7.5 and 8.5 in phosphate or borate buffer, although minimal growth was observed at pH 5.7 and pH 9.0.

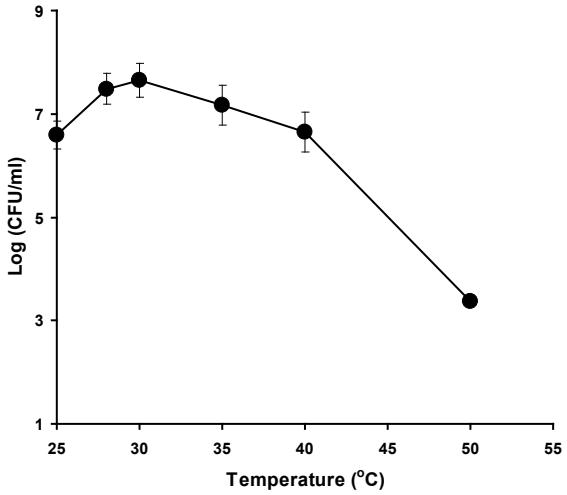

Fig. 3. The effect of temperature on the growth of strain UPM-Langkawi 3. Growth was carried out at pH 7.5 on 4% diesel (v/v) for 6 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.

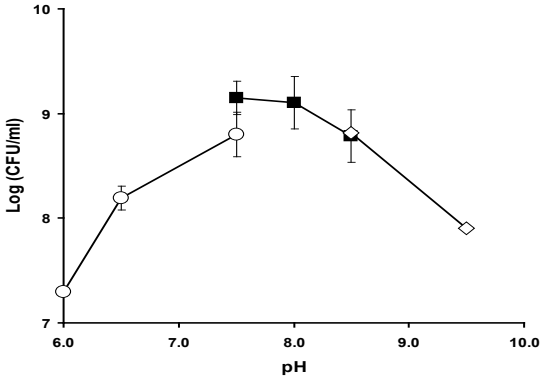

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Fig. 4. The effect of pH on the growth of strain UPM-Langkawi 3 using three overlapping buffers. The buffer system used were phosphate (●), carbonate (○) and borate (▲). Growth was carried out at 37 ºC on 4% diesel (v/v) for 6 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.

The effect of Nitrogen Sources

In the nitrogen sources study, various inorganic nitrogen sources were used, such as $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl and KNO_2. Based on the results in **Fig. 5**, ammonium sulphate was the most effective nitrogen source for the bacteria that degrade fuel. The impact of nitrogen source concentration was examined throughout a range of values from 0.1% to 1.8%. The ideal concentration of ammonium sulphate was 0.9% weight/volume.

DISCUSSION

Generally, the entire carbon source is assimilated by the bacterium for growth and energy and an increase of CFU/mL is regarded as an indicator of degradation, with higher CFU/mL correlating with higher amount of diesel being degraded. Since isolate 3 has the highest bacterial counts than other isolates, it was chosen for further studies in this research. Diesel is needed as a carbon source, but diesel can be toxic to microorganisms at certain concentrations due to the solvent effect of diesel, which destroys bacterial cell membranes.

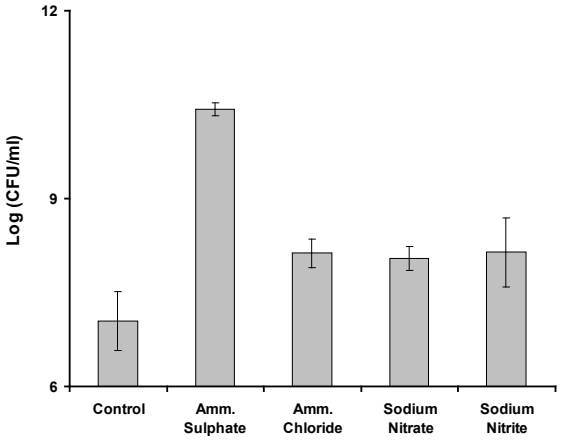

Fig. 5. The effect of different nitrogen sources (0.77% , v/v) on the growth of Strain UPM-Langkawi 3. Bacterial growth was measured by colony forming unit. Growth was carried out at 28 ºC on 4% diesel (v/v) for 6 days on an orbital shaker (150 rpm).

High diesel concentrations can inhibit the growth of bacteria that degrade diesel due to several key factors. These include diesel consisting of complex hydrocarbons mixtures, many of which are inherently toxic to microbial cells. At high concentrations, these hydrocarbons can compromise cell membrane integrity, leading to leakage of cellular contents and eventual cell death. This toxic effect disrupts basic cellular functions, including nutrient uptake and enzyme activity necessary for metabolism and growth [37].

The aerobic degradation of diesel by bacteria requires oxygen. High concentrations of diesel can create a physical barrier around bacteria, limiting the diffusion of oxygen and other essential gases. This results in anoxic conditions that inhibit aerobic microbial activity and thus slow down the degradation process and bacterial growth [23]. While some bacteria can metabolize diesel components, an overload of these substrates can disrupt metabolic pathways. The accumulation of intermediate breakdown products, which can be more toxic than the original substrate, may inhibit further degradation activity and bacterial growth [38]. Diesel predominantly provides carbon when bacteria use it as a substrate. High concentrations of diesel may lead to an imbalance in the carbon to nitrogen ratio, making it difficult for bacteria to access other essential nutrients like nitrogen, phosphorus, and sulfur, which are critical for their growth and reproduction [39].

The degradation rate of diesel can be adversely affected by its toxicity. Bicca et al. [40] established that a 1% (v/v) carbon source concentration was optimal for *Rhodococcus ruber* and *Rhodococcus erythropolis*. Although diesel is required as a carbon source, high concentrations are toxic to microorganisms due to the solvent effects in diesel that can damage bacterial cell membranes. Consequently, many biodegradation studies on diesel use lower concentrations, typically ranging from 0.5 to 1.5% [34,41–48]. Concentrations exceeding 1 or 1.5% have been shown to inhibit degradation processes. However, degradation at significantly higher concentrations (6% v/v) is feasible but requires the addition of glucose (0.2% w/v) and yeast extract (0.1% w/v) [49]. Strain UPM-Langkawi 3 has demonstrated a capability to tolerate higher diesel concentrations, making it an excellent candidate for diesel bioremediation.

Facundo et al. [50] reported that a tropical diesel-degrading bacterium from Mexico thrives optimally at 37°C, which Bicca et al. [40] found also suits *Rhodococcus ruber* well. Temperature plays a critical role in bioremediation, with metabolic rates decreasing when temperatures stray from the optimum, thus reducing the effectiveness of the bioremediation process. Notably, a 10 °C increase in temperature can nearly double the rate of the bioremediation reaction [51]. Other studies have documented growth at varying lower temperature ranges: between 10 and 15 °C [12], 10 and 25 °C [52] and at 20 °C [53,54]. Temperatures ranging from 27 to 37 °C were also favorable [12]. Furthermore, [50] confirmed that 37 °C is a higher growth optimum for similar tropical diesel-degrading bacteria, which is consistent with findings for *Rhodococcus ruber* and *Rhodococcus erythropolis* reported by Bicca et al. [40].

pH is crucial for bacterial growth. Bacterial waste products can alter the pH of a medium. Sepahi et al. demonstrated that the development of Bacillus spp. led to a reduction in the pH of the medium from 6.8 to 6.2. The optimal pH range for bioremediation is often between 6 and 8, with the most favorable pH being around 7, which is neutral [12]. Bacteria typically thrive in a pH range of 5 to 9, with their optimal growth occurring slightly above 7, which is neutral to slightly alkaline [1].

This study underscores the significance of determining nitrogen uptake because limited availability of nitrogen and phosphate can restrict the biodegradation of hydrocarbons, as outlined by Atlas and Cerniglia [55]. Nitrogen supports bacterial growth and serves as an electron acceptor, which is vital for metabolic processes. One of the most notable successes in using nitrogen fertilizers to enhance bioremediation was observed following the Nakhodka tanker oil spill off Oki Island in the Japan Sea.

The application of nitrogen significantly accelerated the biodegradation of over 5000 tons of heavy fuel oil, demonstrating the effectiveness of nitrogen in promoting microbial activity in oil spill scenarios [56]. Additionally, low levels of inorganic nutrients such as nitrogen in soil are known to impede the degradation process of hydrocarbons. Moreover, while nitrites can be used in bioremediation, their application must be cautiously managed. Kang et al. [57] reported that nitrites might inhibit cellular growth during hydrocarbon biodegradation, suggesting a complex interaction between nutrient levels and bioremediation efficacy. Thus, carefully considering and managing nutrient levels, particularly nitrogen, are crucial for optimizing bioremediation processes in contaminated environments. The study of nitrogen sources is important as decreasing it in the bacterial environment will result in limiting the rates of hydrocarbon degradation [58].

CONCLUSION

In conclusion, this study successfully isolated ten bacterial strains from various locations across Malaysia, all of which demonstrated the ability to grow on nearly 10 % diesel as a carbon source over a six-day period. Isolate 4, identified as *Pseudomonas* sp. strain UPM-Langkawi 3, exhibited the most promising growth characteristics and was selected for further detailed study. The isolate showed immediate adaptation to diesel, indicating no lag phase and rapid activation of cellular mechanisms for diesel assimilation. Morphological, cultural, and biochemical tests and ABIS software analysis suggest a high likelihood that this strain is *Pseudomonas* putida. The optimal conditions for the growth and biodegradation activity of strain UPM-Langkawi 3 were determined to be between 6 and 8% (v/v) diesel concentration at a temperature of between 25 and 35 °C and a pH between 7.5 and 8.5. The strain's growth was inhibited by higher concentrations of diesel and elevated temperatures above 40°C and at lower pH levels (5.7). Among various inorganic nitrogen sources tested, ammonium sulphate at a concentration of 0.9% w/v proved to be the most effective, aligning with findings from previous studies. The study highlights the robust capability of *Pseudomonas* sp. strain UPM-Langkawi 3 in diesel degradation, suggesting its potential utility in bioremediation applications, especially in environments contaminated with diesel. Future research should focus on molecular techniques to confirm species identification through 16srRNA gene sequencing and further optimize biodegradation under various environmental conditions. This could enhance the applicability of bioremediation strategies in both contaminated and pristine environments, leveraging the natural ubiquity and resilience of diesel-degrading bacteria.

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