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Isolation of Marine Bacteria Using Silkworm Pupal Medium

Chengzi Cui[1] and Haocheng Liu[2]

[1]Basis International School Park Lane Harbour
[#]Advisor

ABSTRACT

This study aimed to isolate marine bacteria using a novel silkworm pupal medium, focusing on samples collected from Qixing Bay and Longhua Bay, which included seawater, brittle star, shrimp gut, and sponge. Two types of culture media—basic 2216E and the silkworm pupal selective medium—were employed for bacterial isolation. The process involved bacterial extraction, culture, purification, and subsequent DNA analysis, including PCR amplification, electrophoresis, and sequencing. The results led to the identification of a diverse array of marine bacteria, including Alcanivorax sp. and Verrucomicrobiales sp. Alcanivorax sp. is known for its ability to degrade petroleum hydrocarbons, while Verrucomicrobiales sp. is suggested to be a significant metabolizer in marine ecosystems. The findings highlight the potential of the silkworm pupal medium as a promising tool for cultivating marine bacteria, offering a novel approach to studying the functional diversity of marine microorganisms. This research underscores the importance of innovative culture techniques in the exploration and utilization of marine microbial resources for biotechnological and environmental applications.

Introduction

The earliest bacterial culture media consisted of liquid broths created through infusion or enzymatic digestion of meat from various sources. These foundational methods enabled the cultivation of bacteria and catalyzed significant advancements in microbiology. The introduction of solidifying agents such as gelatin and agar transformed broths into solid media, a break-through pioneered by Pasteur and his contemporaries [1]. This innovation marked the golden age of medical microbiology in the late 19th century, during which many pathogenic bacteria responsible for serious human diseases were identified. How-ever, this success inadvertently narrowed the focus of microbiologists, as modern sequencing techniques have revealed that fewer than 2% of bacterial species can be cultivated under laboratory conditions [2].

This limitation stems from various challenges, including the inability of traditional media to replicate the natural environments necessary for bacterial growth. Common issues include the absence of essential nutrients, the presence of toxic compounds in the medium, or the inhibitory effects of substances produced by other bacteria in the sample. Furthermore, some bacterial species depend on symbiotic relationships, which prevent their independent growth in vitro [3]. These obstacles highlight the need for novel cultivation techniques to broaden the spectrum of bacteria that can be studied.

Marine bacteria, in particular, offer immense potential for applications in medicine and biotechnology. For example, species like Alcanivorax degrade petroleum hydrocarbons, significantly mitigating the environmental and health risks associated with oil spills [4]. These bacteria's ability to transform harmful pollutants into benign substances demonstrates their ecological importance in maintaining environmental balance and ecosystem health [5].

This study explores the use of silkworm pupae as a novel culture medium for isolating marine bacteria. Samples were collected from sponges, brittle stars Ophiactis savignyi, white-leg shrimp Penaeus vannamei, and seawater in Qixing Bay. The bacterial isolates were purified and identified through 16S rDNA amplification and sequencing.

The findings highlight the efficacy of silkworm pupae as a selective medium, offering new insights into the functional diversity of marine bacteria and their potential applications in bioremediation and pest control [6].

Materials and Methods

The goal of this experiment was to identify bacteria from marine organisms and cultivate them using a self-designed medium. Two culture media were employed: the standard 2216E medium, widely used for cultivating marine bacteria, served as a baseline, while the second was a custom medium formulated to selectively grow bacteria capable of decomposing nutrients in silkworm pupae. By carefully controlling the nutrients in the custom medium, the screening process was refined to isolate target bacteria.

Samples, including purple sponges and shrimp gut tissues, were collected from marine environments and processed to extract bacteria. Each sample was cultured in both the basic 2216E and the selective silkworm pupae-based media. These cultures were then incubated to allow bacterial colonies to develop.

Once visible colonies formed, they were purified to isolate individual bacterial strains. DNA was extracted from the colonies to enable genetic analysis. The 16S rRNA gene was amplified using polymerase chain reaction (PCR), followed by electrophoresis testing to confirm the integrity and quality of the DNA. Finally, sequencing was performed to identify the bacterial species.

This approach demonstrated the utility of the custom silkworm pupae-based medium in isolating unique marine bacteria. It allowed for the identification of bacteria with specialized ecological functions, including the ability to utilize silkworm pupae nutrients. The use of a selective medium alongside a standard medium enabled a comparative analysis, advancing the study of marine bacterial diversity and functionality.

Collection of samples

Seawater samples were collected from Qixing Bay (N 22.56786070677839, E 114.5312209755567), while seawater prawns were procured from the Xiangmei Seafood Market in Shenzhen, Guangdong, China. Specimens of brittle stars and an unidentified purple sponge species were gathered from the fouling community on ropes attached to a fish farm raft in Qixing Bay. All samples were placed into 30 mL centrifuge tubes filled with seawater and transported to the laboratory.

To preserve the integrity of microbial communities, careful measures were taken to ensure the survival of small organ-isms during transportation. Seawater was collected from the same environments as the organisms to maintain ecological consistency. Temperature was carefully monitored, and when necessary, samples were transported in insulated containers with oxygenation to simulate natural conditions, minimizing microbial disruption and improving experimental accuracy.

Extraction of bacteria in the samples

To extract bacteria, samples were processed immediately upon returning to the laboratory. For each extraction, 0.05 g of the sample was placed into a 2 mL centrifuge tube containing 1 mL of pure water. The sample was physically disrupted and homogenized using a mechanical stirrer. The homogenized mixture was then combined with seawater and left to stand, allowing the sediment to settle. The clear supernatant, which contained the bacteria, was carefully extracted to avoid contamination by the sediment, which could interfere with subsequent cultivation.

The extracted supernatant was first introduced into the basic medium (2216E) to promote rapid bacterial growth and ensure observable colony formation. Once the bacteria grew into visible colonies, purification was performed to isolate individual strains, as bacteria often grow in mixed colonies. The purified bacteria were then transferred to the selective silkworm pupae-based medium for further screening.

If the crushed sample could not be processed on the same day, it was stored in a refrigerator at 4°C to maintain stability and prevent contamination, ensuring the integrity of the bacterial communities for future analysis.

Selective and basic culture medium

Marine Agar 2216E served as the basic culture medium in this study. A selective medium was also developed, with live silkworm pupae as the primary nutritional source, to cultivate bacteria capable of decomposing insect pupae. This approach aimed to harness marine bacteria for pest control on land, presenting a safe, efficient, and easily manageable method with minimal risks to human health.

The selective medium comprised peptone (10 g), glucose (50 g), phosphate (1 g), magnesium sulfate (0.5 g), live silkworm pupae (30 g), and agar (15 g), with pure water added to reach a total volume of 1,000 mL. Pure water was used instead of seawater to promote bacterial adaptation to non-salty environments.

Prior to incorporation into the medium, silkworm pupae were frozen at -80°C and ground with frozen carbon dioxide to maintain their frozen state during grinding. The finely processed pupae were thoroughly mixed with the other components of the medium. Residues were carefully scraped and evenly distributed by stirring the mixture. The final medium was sterilized in a pressure cooker at 121°C for 30 minutes to eliminate any contaminants, ensuring a sterile and controlled environment for bacterial cultivation.

Spreading and Streaking

Samples were collected from Qixing Bay and Longhua Bay, and biofilm samples were used to produce eight culture plates for each medium type. This included both the basic medium and the selective medium supplemented with live silkworm pupae. Four samples were taken from fishing ropes, while the other four were collected from seawater.

Bacteria isolated from a shrimp sample collected in Longhua Bay exhibited slow growth on the selective medium but eventually formed two colonies. Another bacterial strain from seawater successfully adapted to the selective medium, indicating its ability to transition from a salty marine environment to a non-salty medium.

The culture plates were incubated at 37°C for approximately five days to allow bacterial growth. However, only one strain of white bacteria demonstrated sufficient growth speed for use in further experiments.

DNA extraction

The first step in identifying bacterial species was to extract their DNA. Bacterial colonies were carefully picked using an autoclaved toothpick and transferred to a 2 mL centrifuge tube, followed by the addition of 1 mL of water. The mixture was thoroughly mixed to ensure even distribution. The tube was then heated in a boiling water bath for 10 minutes to lyse the bacterial cells, breaking down their membranes and releasing the DNA.

After boiling, the tube was placed diagonally in a centrifuge and spun at high speed for 15 seconds to separate the components. This rapid centrifugation caused the heavier materials, including cellular debris, to settle at the bottom, while the clear supernatant containing the bacterial DNA remained in the upper layer. The supernatant was carefully extracted, as it contained the DNA of the target bacteria, which was then used for subsequent analysis.

PCR Amplification

1 μL of each DNA sample was added to a 2 mL centrifuge tube, along with 1 μL of the 27F primer (forward, 5'-AGAGTTTGATCCTGGCTCAG-3'), 1 μL of the 1492R primer (reverse, 5'-GGTTACCTTGTTACGACTT-3'), 12.5 μL of Taq polymerase, 7.5 μL of double-distilled water, and 3 μL of the DNA extract. This mixture created a total volume of 25 μL, which was briefly centrifuged for 5 seconds to ensure thorough mixing.

The PCR conditions were set as follows: an initial denaturation step at 95°C for 2 minutes to separate the DNA strands, followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The final extension step was performed at 72°C for 10 minutes. The PCR products were analyzed by gel electrophoresis, and the DNA bands were visualized by comparing the migration of the samples with a DNA marker to determine the presence and size of the amplified fragments.

Electrophoresis Test

Gel electrophoresis was performed to confirm the successful extraction and amplification of DNA. The gel preparation involved mixing 1.2 grams of agarose powder with 80 mL of TAE buffer. After heating the mixture to dissolve the agarose, 4 μL of nucleic acid dye was added. The solution was then poured into a gel mold and allowed to solidify for 30 minutes.

Once the gel had set, 5 μL of each PCR product was carefully pipetted into individual wells in the gel. The first well was loaded with 5 μL of a DNA marker to serve as a reference for determining fragment sizes. The gel was then subjected to electrophoresis at 120 V for 20 minutes, allowing the DNA fragments to separate based on size.

DNA Sequencing

After completing the electrophoresis test, the PCR products that showed distinct bands were selected for further analysis. These samples were then sent to Tsingke Biotech Company for DNA sequencing using the Sanger method. This technique is widely used to determine the nucleotide sequence of DNA by synthesizing DNA fragments with chain-terminating nucleotides. The resulting fragments were separated by size using capillary electrophoresis, and the sequence was read based on the fluorescent labels attached to the nucleotides. The obtained sequences were compared to reference databases to identify the bacterial species. DNA sequencing provided critical information for confirming the bacterial identity and gaining insights into the genetic characteristics of the isolates.

Results

Bacteria cultured from environment samples using Marine Agar 2216

For sea water, Brittle star, and shrimp gut samples, bacteria inoculation was performed with 100 times dilution on Marine agar 2216 medium as the basic medium (Fig. 1). In the sample, at least four types of bacteria colonies can be observed. One type with circular form, relative medium size,flat elevation, entire margins,smooth surface, completely opaque and in white color could be observed in shrimp gut samples plate (Fig. 1c); Second type with irregular form, relative small size, raised elevation, entire margins, smooth surface, relatively opaque and in pink color could be observed in brittle Star samples plates (Fig. 1b); Third type with circular form, relative small size,raised elevation, entire margins,smooth surface,completely opaque and in bright yellow color could be observed in seawater samples plates (Fig. 1a); the fourth type with large size, flat elevation, entire margins, smooth surface, transmitting the light in orange color could be observed in sponge samples plates .

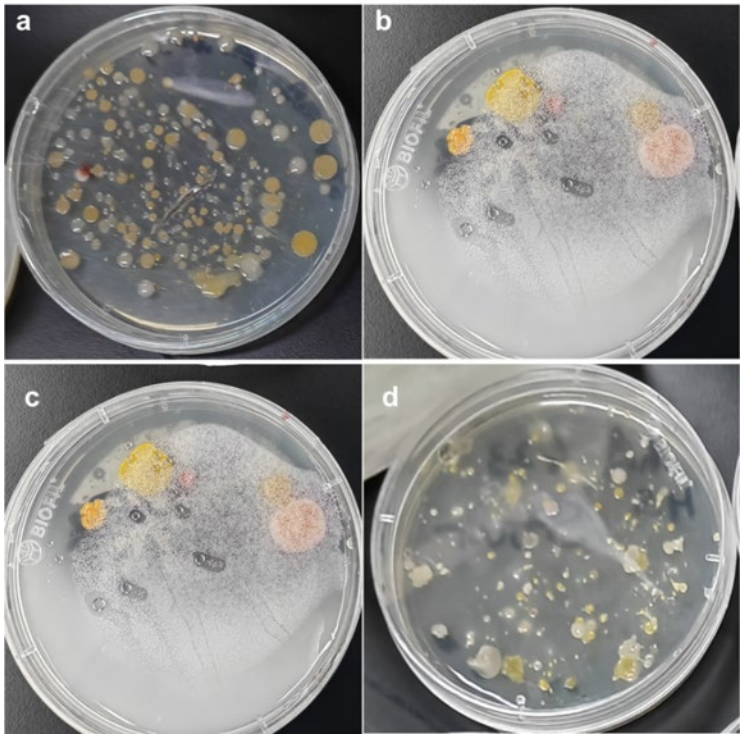

Figure 1. Bacterial inoculation of a. seawater, b. brittle star, c. shrimp gut and d. purple sponge samples on Marine Agar 2216E.

Bacteria culturing from environment samples using silkworm pupae selective medium

For sea water, Brittle star, and shrimp gut samples, bacteria inoculation was performed with 100 times dilution on the live silkworm pupae medium as the selective medium. However, the Brittle star did not grow on the silkworm pupae selective medium and the plate which used shrimp gut has only two observable colonies. They only grew single type of the bacteria and had less numbers of the colonies (Fig. 2). The colonies were bigger than it on the 2216E plate. Also, it looked like an irregular circle, the surface seemed a little bit dry and a bit rough, as if it's not as smooth and moist.

Figure 2. The shrimp gut on live silkworm pupae

Purification and identification of bacterial isolate from Marine Agar 2216E medium

Using the Marine Agar 2216E medium, four bacteria isolates from sponge (Fig. 3), four from shrimp gut (Fig. 4), two from brittle star (Fig. 5) and four from seawater samples (Fig. 6) were purified.

For the purified isolates from sponge, one isolate was bright yellow opaque regular linear colonies, seems moist and sticky (Fig. 3a); one was somewhat blurry, it seems to be yellow (Fig. 3b); one was yellow and white color that seems to be somewhere in between, with a circular shape. It looks very moist (Fig. 3c); and the final one was some pink moist and sticky linear colonies (Fig. 3d).

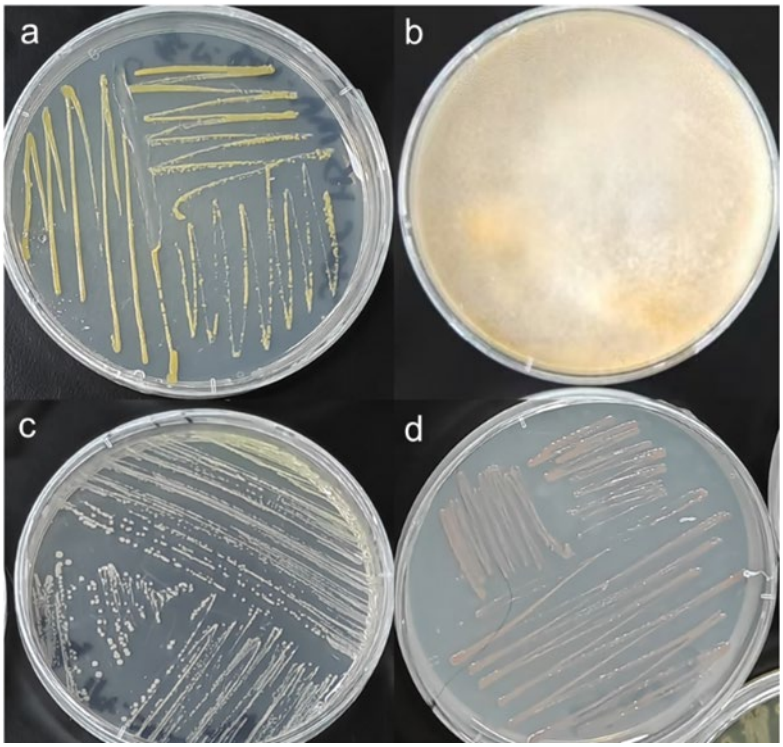

Figure 3. Marine Agar 2216E purified bacteria isolates from purple sponge sample.

For the purified isolates from shrimp gut, one isolate was white opaque linear colonies, appearing relatively dry on the surface (Fig. 4); one was some are transparent yellow linear, some are moist, and at the same time, they are quite sticky; one was Growing into a wet linear patch colonies; one was wide opaque white lines with a relatively dry surface.

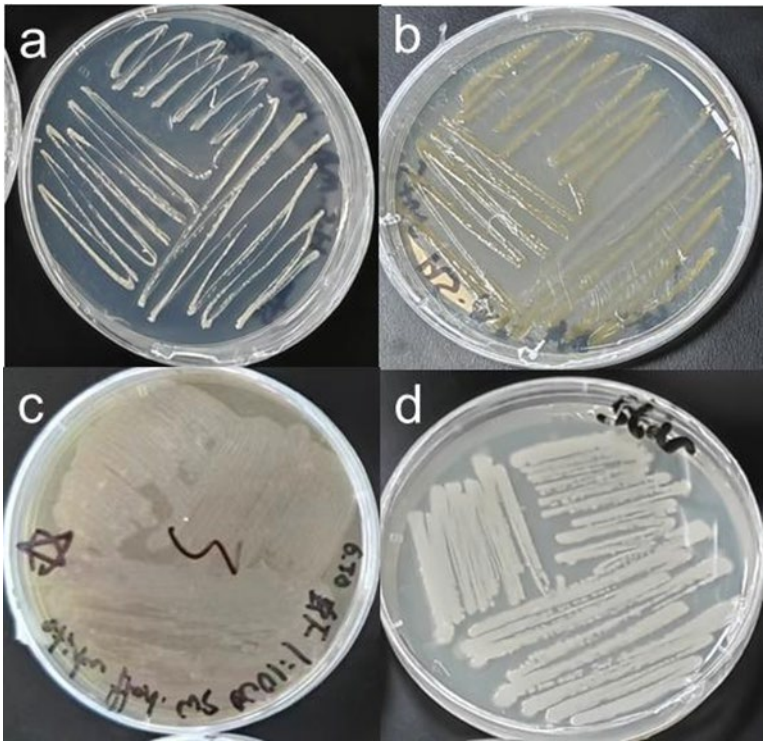

Figure 4. Marine Agar 2216E purified bacteria isolates from shrimp gut sample.

For the purified isolates from brittle star, one isolate was the light green transparent lines are relatively narrow, and the surface is relatively moist (Fig. 5); one isolate was the light yellow transparent lines are relatively narrow, and the surface is relatively moist.

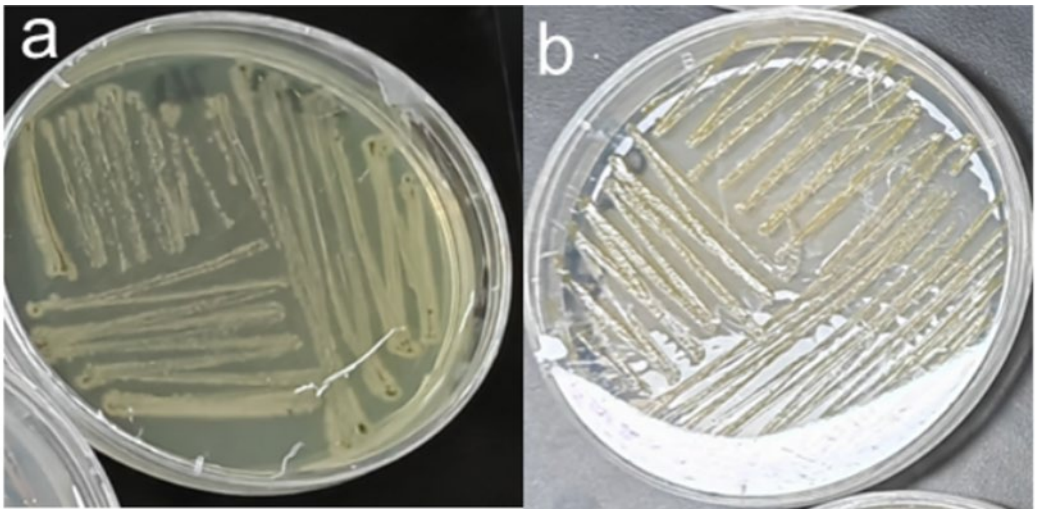

Figure 5. Marine Agar 2216E purified bacteria isolates from brittle star sample.

For the purified isolates from sea water, one isolate was the red dots and white lines appear smooth and moist on the surface, suggesting that they are symbiotic bacteria growing together, and therefore cannot be purified separately (Fig. 6); one isolate was the narrow opaque black lines with a relatively dry surface and thorns like growths; one isolate

was the dark green opaque surface is a smooth and moist circular shape that seems to have grown from lines; one isolate was the orange transparent surface has smooth and moist lines, with an incomplete and irregular shape.

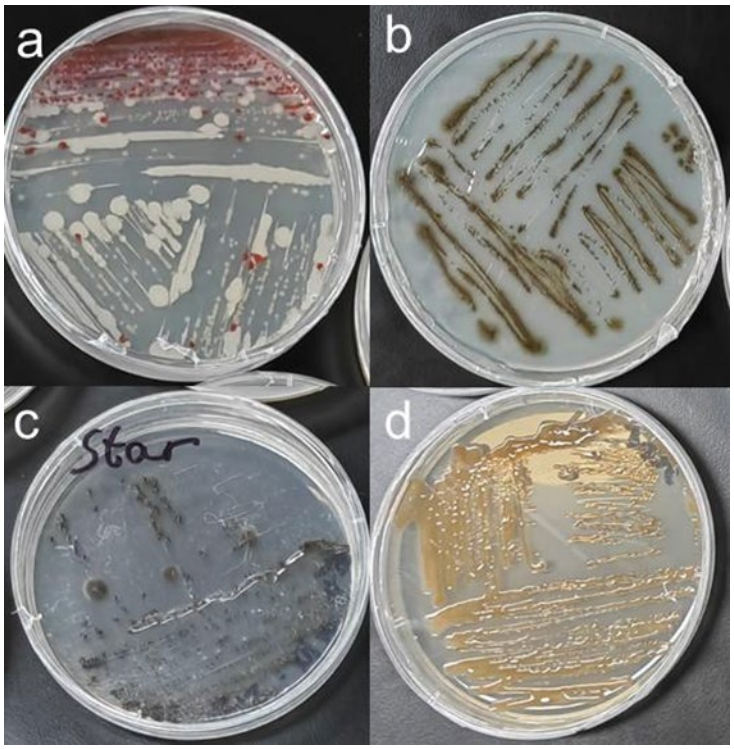

Figure 6. Marine Agar 2216E purified bacteria isolates from seawater sample

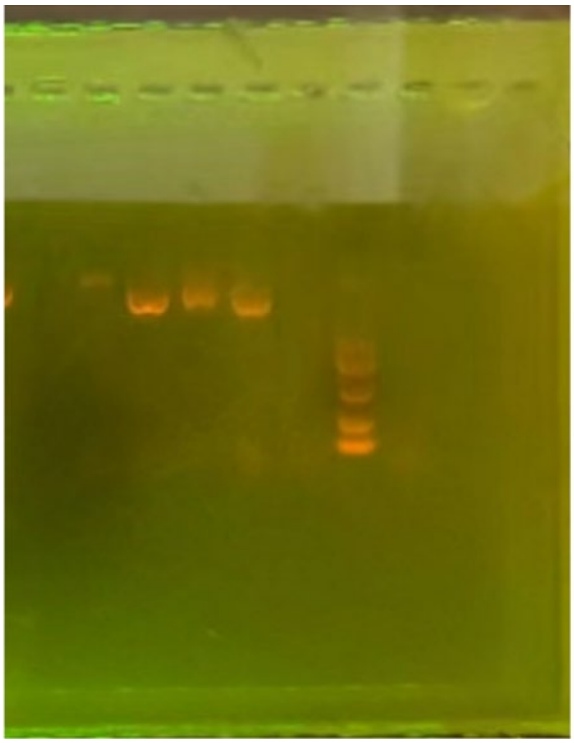

Figure 7. Gel electrophoresis of PCR of 16S rDNA of purified bacteria

Purification and identification of bacteria isolate from Marine Agar 2216E medium

Using the live silkworm pupae medium, 1 bacteria isolates from shrimp gut, 1 from seawater samples were purified. For the purified isolate from shrimp gut, the isolate was white opaque regular linear colonies, seems to have dry surface and sticky (Fig. 8). For the purified isolate from sea water,the isolate's growth is somewhat slow, with regular yellow translucent lines (Fig. 9).

Isolate from purple sponge sample using 2216E medium which species identity undetermined

In this sample came from a purple sponge, which grew in the 2216E medium (Fig. 3d), found only 78 present of similarity with the known Uncultured bacterium clone Liv16S-L273 16S ribosomal RNA gene (JN087494.1), partial sequence which means it may be a new type of bacteria in the ocean. At the same time, it may because there are multiple organisms overlapping together, cause the impure DNA. That's the reason of the low similarity.

Isolate from seawater sample using 2216E medium which species identity undetermined

In this sample, came from sea water grew in 2216E medium (Fig. 6b), there is no specific reference to a particular species, but many organisms with the same DNA have been detected, which was 100 present same. This may be because their genes are too conserved, or this method cannot detect their DNA. Anyway, this shows the maintain of the genetic situation.

Isolation, purification and identification of bacteria from marine samples using live silkworm pupae selective medium

Isolate Alcanivorax sp. from shrimp gut sample using live silkworm pupae medium

One of the white colonies isolated from the shrimp gut grew in the live silkworm pupae medium, was successfully purified. After purification, I obtained more samples, and their morphology also changed. At first, due to being coated, the bacteria grew very regular, two standard circles, but after purification, they grew into slender white lines (Fig.8). The PCR of 16S rRNA of the bacteria isolate was successful. DNA sequencing results showed that the species 16S rRNA has 98.85 percent of similarity to that of Alcanivorax sp. (HM598194.1).

Figure 8. Alcanivorax

Purification and identification of Verrucomicrobiales sp. from sea water using live silkworm medium

One of the orange colonies isolated from the sea water grew in the live silkworm pupae medium, was successfully purified. After purification, the speed of bacterial growth became slower and the shape of the bacteria colonies has changed. After purification, the bacteria colony grew into slender orange lines (Fig. 9). The PCR of 16S rRNA of bacteria species was successful. DNA sequencing results showed the species 16S rRNA has 99.76 percent of similarity to the Verrucomicrobiales.

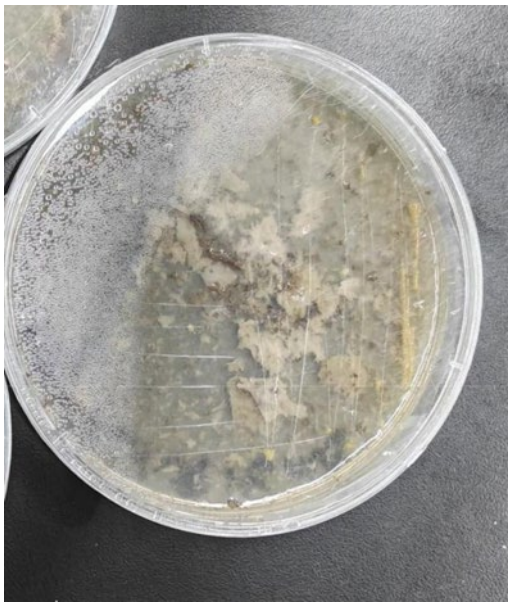

Figure 9. Verrucomicrobiale

Discussion

In this study, Alcanivorax sp. was isolated from a shrimp gut sample using a live silkworm pupae medium. This obligate hydrocarbonoclastic bacterium, which relies primarily on petroleum hydrocarbons as its carbon and energy source, is widely distributed across marine environments. Alcanivorax sp. is capable of degrading a wide range of petroleum hydrocarbons and typically dominates oil-degrading microbial communities due to its efficient central metabolic pathways and diverse hydrocarbon degradation abilities. Because crude oil is toxic to most organisms, oil spills cause significant ecological damage, particularly in marine environments. Fortunately, much of the petroleum that enters the sea is naturally degraded by microbial communities. Bacteria like Alcanivorax sp. play a crucial role in breaking down these pollutants, thereby helping to maintain environmental balance. Among all Alcanivorax species, Alcanivorax borkumensis is one of the most globally important. Whole-genome sequencing and subsequent functional analyses have revealed the exceptional adaptive capacity of A. borkumensis. Key traits that enable A. borkumensis to rapidly adapt to oil and thrive in marine environments include its oligotrophic lifestyle, high affinity for hydrocarbons, biofilm formation at the oil-water interface, and its ability to manage nutrient imbalances typical of oil spills, particularly through specialized systems that scavenge organic and inorganic nitrogen.

DNA analysis from seawater samples grown in the silkworm pupae medium revealed an uncultured bacterium belonging to the Verrucomicrobiales cluster. This organism was previously identified by its 16S rDNA sequence as a dominant species in Dutch Drentse A grassland soils. The potential metabolic activity of this bacterium was assessed by direct ribosome isolation and partial amplification of the 16S rRNA gene using RT-PCR with bacterial-specific primers. Temperature gradient gel electrophoresis separated the amplicon sequences into distinct, reproducible patterns. One of these fingerprint bands matched the signal from clone DA101, and Southern blot hybridization using a DA101-specific V6 probe confirmed the sequence identity. This is the first indication that a member of the Verrucomicrobiales cluster may play a significant role in environmental microbial communities, suggesting that this bacterium is capable of surviving in saline environments.

There is currently no precedent for using live silkworm pupae as a culture medium, especially for cultivating marine bacteria. However, the nutritional value and functionality of silkworm pupae have been proposed in other contexts, such as for biopesticide bacteria like Bacillus thuringiensis. It is the most widely used biopesticide against lepidopteran pests and is mass-cultured using molasses-based or wheat bran-based media through submerged and semi-solid fermentation methods. Commercial production of thuringiensis has spurred the search for cheaper, locally available media alternatives. The successful cultivation of marine bacteria on silkworm pupae medium could offer a cost-effective and sustainable solution, reducing the consumption of traditional media ingredients while achieving similar results.

The ability of marine bacteria to grow on the silkworm pupae medium suggests that salinity is not a limiting factor for their growth. While bacteria grown in Marine Agar 2216E generally exhibited faster growth and more colonies, bacteria in the silkworm pupae medium were not negatively affected by changes in salinity. This indicates that as long as sufficient nutrients from silkworm pupae are available, they can replace the saline environment of the 2216E medium. Furthermore, this suggests that these bacteria not only thrive on the nutrients provided by silkworm pupae but may also be capable of decomposing them, highlighting the potential for silkworm pupae as an effective and environmentally friendly culture medium.

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