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EVALUATION OF CABIN-SEQUESTERING METHOD TO ELIMINATE BACTERIAL CONTAMINATION FROM AQUATIC FUNGAL CULTURES

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Abstract: Bacterial contamination is a major problem that needs to be addressed in fungal culture. Therefore, this is the first study that successfully eliminated bacterial contamination from aquatic fungal cultures using the Cabin-sequestering (CS) method. To assess its effectiveness, two treatment groups were established: Group A, employing the CS method and Group B, using subculture on antibiotic-free media. The bacteria-fungal mixture, comprising *Aspergillus* sp. and *Aeromonas* sp. isolated from the Kuala Ibai mangrove area, was prepared with a ratio of 2:1 (bacteria: fungi). *Aeromonas* sp. was grown in Tryptic Soy Broth (TSB) and *Aspergillus* sp. spores on Potato Dextrose Agar (PDA). In Group A, the CS method was applied by piercing a square hole in the growth medium, referred to as a "cabin," and covering it with a coverslip. After incubation, fungal hyphae that grew beyond the coverslip were transferred onto new PDA plates. This growth was morphologically characterised and confirmed as *Aspergillus* sp., demonstrating the CS method's efficacy in ensuring pure fungal growth. In contrast, Group B, which utilised subculturing on antibiotic-free media, showed bacterial growth. This bacteria was streaked on Glutamate Starch Phenol-red (GSP) agar, resulting in yellow colonies that were presumptively identified as *Aeromonas* sp., highlighting the presence of bacterial contamination in the control group. In conclusion, the CS method, particularly with the 2:1 bacteria-to-fungus ratio, proved to be highly effective in eliminating bacterial contamination and maintaining the purity of fungal cultures. The study's findings advocate the CS method as a reliable and efficient alternative to traditional subculturing techniques in managing bacterial contamination in fungal cultures. Future research is recommended to explore diverse species and ratios, further validating the CS method's versatility in different microbial settings.

Keywords: Aquatic fungi, bacteria, contamination, Cabin-sequestering.

Introduction

The issue of bacterial contamination in fungal culture is a significant hurdle, particularly given the economic importance of fungi. Visible bacterial contamination not only disrupts cell cultures but also leads to misinterpretations in research, as these contaminants often result in the altered morphology of fungi. This change in physical appearance can lead to invalid and unreliable experimental results (Cother & Priest, 2009).

Over the years, various methods have been developed to separate bacteria from fungal cultures. These methods range from physical separations, such as centrifugation, to chemical treatments that selectively target bacteria. Despite these efforts, each method has its limitations. For instance, physical methods can be labour-intensive and might not completely segregate bacteria from fungi. Chemical methods, particularly antibiotics, are standard but come with their challenges.

The use of antibiotics to control bacterial contamination is widespread. However, this approach has several drawbacks, including the incomplete removal of bacteria, a limited range of bacterial species that can be targeted, the risk of recontamination, adverse effects on fungal strains, and environmental concerns (Serwecinska, 2020). These limitations highlight the need for a more effective method.

In response to these challenges, the Cabin-sequestering (CS) method has been developed (Shi *et al*., 2019). This method stands out for its simplicity, efficiency, and stability in eradicating bacterial contamination from solid fungal cultures. Unlike traditional methods, the CS method does not require intricate techniques or handling of large agar plates, which can introduce new contaminants. It is user-friendly, quick, and cost-effective, as it eliminates the need for antibiotics. Furthermore, it has proven effective against bacteria that are difficult to separate from fungal colonies using conventional methods. It has also been observed to enhance fungal characteristics such as sporulation, melanin production, and virulence. The method involves strategic positioning of coverslips and growth mediums, enabling fungi to grow uncontaminated (Shi *et al*., 2019).

Given the frequent occurrence of bacterial contamination in laboratory environments, our study utilises the CS method to effectively address and eliminate such contamination, potentially offering significant advantages for future research and applications.

Materials and Methods

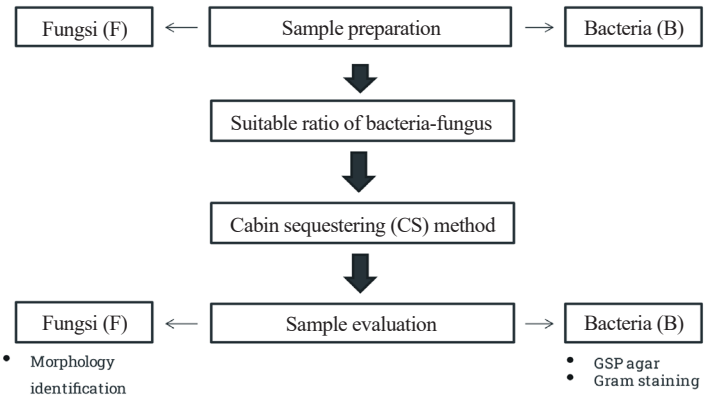

Figure 1: Overview of methods and experimental design

Sample Preparation

Aspergillus sp. and *Aeromonas* sp. were selected for sample preparation of a mixed solution of fungus and bacteria. Both were obtained from water collected in the Kuala Ibai mangrove area (5.279460, 103.168940). Fungal species of *Aspergillus* sp. were pure cultured using Potato Dextrose Agar (PDA) supplemented with 300 µg/ml of Chloramphenicol. The pure culture of *Aspergillus* sp. was morphologically identified until the genus level. Then, *Aeromonas* sp. was streaked on Glutamate Starch Phenol-red **(**GSP) agar for bacterial growth confirmation. Further gram staining was conducted to confirm the bacteria's shape and gram colour.

Selection of Suitable Ratio of Bacteria-fungus Mixed Solution

Aeromonas sp. was cultured in Tryptic Soy Broth (TSB) overnight to obtain the bacterial culture in liquid form. The spore was scrapped from the fungal growth in a solid medium and mixed with saline water for fungal culture. Sample preparation of bacteria-fungus was made through contact assay, where each of the microorganisms' aliquots was mixed according to three different ratios (Table 1). The initial solution was referred to as 0.5 McFarland standard for both bacteria and spore solution of fungi (Akinpelu *et al*., 2015).

This mixture was inoculated onto PDA using a sterilised cotton swab. Three replicates

of each co-culture were made. The Petri dishes were then incubated at 25°C for three days. One ratio of bacteria-fungus mixed solution mimicking bacteria contamination in fungus growth was selected for the CS method.

Table 1: Bacteria-fungus culture using three different ratios

Ratio	Co-culture
A	1 (bacteria) : 1 (fungi)
B	2 (bacteria) : 1 (fungi)
C	1 (bacteria) : 2 (fungi)

Experimental Design

Two groups with CS treatment and control were prepared to evaluate the effectiveness of the CS method in eliminating bacterial contamination in aquatic fungal cultures. The two groups are Group A with CS treatment and Group B consisting of a control group without CS treatment where the mixed aliquots were inoculated directly on PDA (Table 2).

Table 2: Groups of culture medium with two different treatments

Group	Treatment
A	CS treatment
B	No CS treatment (control)

Operational Procedures of Cabin - sequestering Method

The agar plates, sterilised coverslips, and inoculation needles were prepared on a sterilised bench. A sterile inoculation needle was used in the PDA medium to make a square hole around 3-5 mm, called the "cabin." Then, sample preparations of bacteria-fungus mixed solution were inoculated into the cabin using a sterile inoculation needle. Next, a sterilised coverslip was cautiously placed over the inoculated cabin using sterile forceps. After that, the coverslip was gently patted against the medium to ensure the absence of air bubbles between it and the medium. The culture plate was then sealed and incubated at optimum temperature for fungal growth at around 25°C for five days. Any growth on the agar was monitored regularly.

Sample Evaluation

The growth of fungi and bacteria on the agar was observed and evaluated. The growth was evaluated to ensure that the correct fungi and bacteria were isolated and not other contaminants. The fungus was morphologically identified up to the genus level, and the bacteria were streaked on GSP selective agar to obtain a yellow colony, presumptively identified as *Aeromonas* sp. In the collection process from the culture plates, the fungal and bacterial species were isolated using aseptic techniques. The fungal species were transferred with a sterilised loop to a fresh agar plate conducive to fungal growth. Post-transfer, these species were allowed to proliferate and were subsequently identified based on morphological characteristics. Concurrently, bacterial colonies were streaked onto GSP selective agar plates. This medium is tailored for the growth of *Aeromonas* sp., known to form distinct yellow colonies. The streaking process on this selective medium facilitated the presumptive identification of *Aeromonas* sp. based on the unique colouration and morphology of the colonies.

Results and Discussion

Bacteria and fungi are microorganisms that can coexist in the same environment, including in cultures intended to grow only one of them. However, in fungal cultures, the presence of bacteria can be problematic as it can lead to contamination and interfere with the growth and development of fungi. Poor aseptic techniques during culture preparation, including not washing hands, not using gloves or masks, and not properly cleaning the work area, can lead to bacterial contamination. Bacteria can also contaminate fungal cultures through cross-contamination. This can occur when instruments or equipment for handling bacteria are also used for handling fungal cultures without proper sterilisation (Ko *et al.*, 2001).

The cabin sequestration method used in this study reverses contaminated solutions. To prepare contaminated solutions as a starting solution for evaluating the effectiveness of the method CS, a mixture of fungi and bacteria was prepared. However, choosing an appropriate ratio of bacteria and fungi in this method is important to ensure optimal efficiency.

Selection of Suitable Ratio of Bacteria-fungus Mixed Solution

A suitable ratio is necessary for the bacteria-fungus mixed solution to ensure the bacteria or fungus does not overpower each other and thus interfere with the result (Frey-Klett *et al*., 2011). Ratios A and C resulted in only fungus growth. Meanwhile, the bacteria and fungus grew simultaneously for Ratio B, mimicking the fungal contamination situation. For that reason, Ratio B proceeded with the CS method (Figure 2).

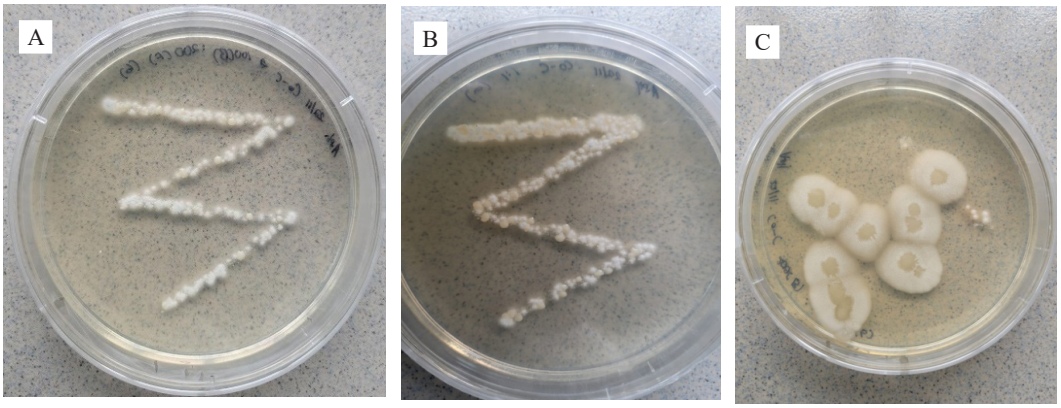

Figure 2: Growth of bacteria-fungus mixed culture with the ratio of (A) 1 (bacteria): 1(fungi), (B) 2 (bacteria): 1 (fungi), and (C) 1 (bacteria): 2 (fungi)

The optimal ratio may vary depending on various factors, such as nutrient competition and environmental factors. As a factor of nutrient competition, bacteria and fungi may compete for nutrients in the contaminated site. If the ratio of bacteria to fungi is not optimal, one may dominate and inhibit the growth of the other, leading to reduced bioremediation efficiency (Nottingham *et al*., 2018). Meanwhile, for environmental factors, the optimal ratio of bacteria and fungi may vary depending on the type of contaminants, soil pH, temperature, and moisture content (Ismail *et al*., 2023). Therefore, it is important to consider the environmental factors of the contaminated site when selecting the appropriate ratio of bacteria and fungi.

CS Method

The CS method effectively eliminated bacterial contamination in aquatic fungal cultures, particularly by employing the Ratio B (2 bacteria: 1 fungi). After a five-day incubation

period, the CS method successfully facilitated exclusive fungal growth on the agar, effectively eliminating bacteria. This outcome was in stark contrast to the negative control (Group B), where bacterial growth dominated, overshadowing the fungal component in the mixed culture. This is shown in Figure 3 below.

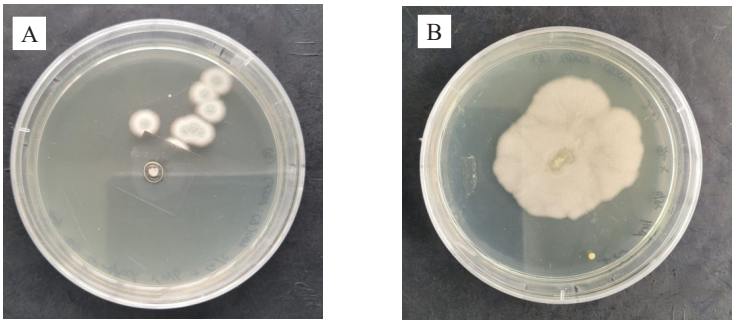

Figure 3: CS method (A) and negative control (B) after five days of incubation

The study found that Ratio B was better than other tested ratios. This ratio's effectiveness can be attributed to the larger proportion of bacteria in the overall culture biomass, which played a crucial role in the dynamics of the co-cultures. The bacteria's dominance in the mixture likely facilitated an environment where only the healthiest and most vigorous fungal cells could thrive. These fungal cells, growing in the cabins, were required to navigate past the physical constraints imposed by the coverslip and the culture medium. This necessity acted as a natural screening mechanism, allowing only the most robust fungal cells to emerge and grow.

Additionally, the morphological characteristics of the newly grown fungal strains under the CS method were observed to be as healthy as the original strains. This observation further validates the CS method's capability to eliminate bacterial contamination and preserve the integrity and health of the fungal cultures. As documented by Mensah-Attipoe *et al.* (2019), the findings provide a deeper understanding of the interaction dynamics in microbial co-cultures and underscore the CS method's potential to achieve pure fungal growth in contaminated settings.

Sample Evaluation

Fungal growth from the CS method was further characterised morphologically and confirmed as *Aspergillus* sp. Meanwhile, the bacteria growth from the control group was streaked on GSP agar to obtain the yellow colony presumptively identified as *Aeromonas* sp.

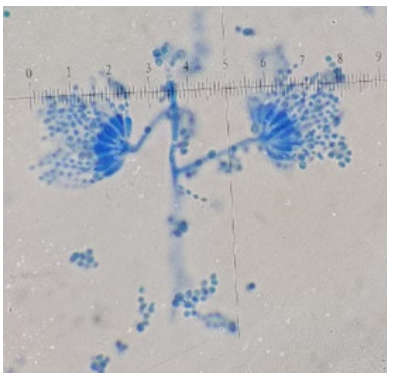

Figure 4: *Aspergillus* sp. from CS method under the microscope (1,000x magnification)

Fungal colonies were isolated and grew on PDA. As for microscope view under 1,000x magnifications (Figure 4), these colonies were found to be dark green, wolly to cottony, rugose, surface with an outer white apron as the macromorphology meanwhile elongated vesicle, hyphae septate and hyaline, conidial heads are strongly columnar, conidiophores are smooth-walled, conidia are smooth to finely roughened, subglobose a for the micromorphology (Zulkifli & Zakaria, 2017). Conidial head shape, colour, shape, texture, and the dimension of stipes, vesicles, and conidia are phenotypic features of aspergillum-like spore-bearing structures (Samson *et al*., 2014).

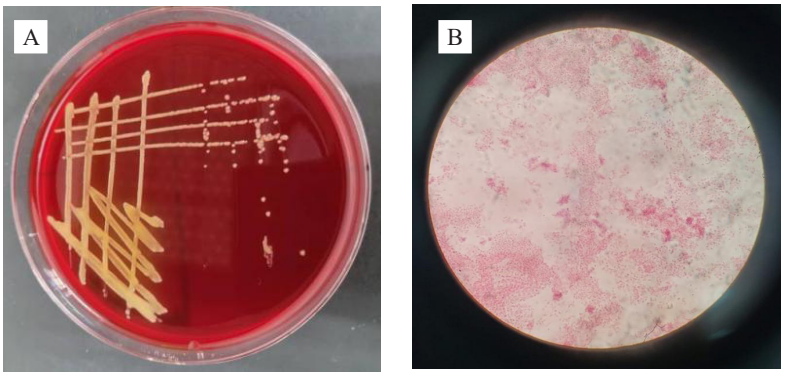

Figure 5: *Aeromonas* sp. on GSP agar (A) and gram staining of the isolates showed a pink colour, indicating gram-negative bacteria under the microscope (100x) (B)

Bacterial colonies were streaked on GSP agar, and yellow colonies were seen growing. As for the microscope view under 100x magnifications, as shown in Figure 5 above, these colonies were rod-shaped and pink in colour. They produced large, opaque, and flat colonies with irregular margins. GSP agar is a selective and differential agar used to isolate and identify *Aeromonas spp*. GSP agar contains two different indicators, bromothymol blue and acid fuchsin. Bromothymol blue is a pH indicator that turns yellow at acidic pH, while acid fuchsin is a dye that inhibits the growth of most bacteria except for *Aeromonas spp*. *Aeromonas spp*. can produce acid from glucose, which causes the pH of the medium to become acidic, which turns the bromothymol blue indicator yellow. Thus, the yellow colour on GSP agar indicates acid production by *Aeromonas spp*. during the fermentation of glucose (Popowska *et al*., 2017). GSP medium was used because this bacteria was presumed to be inhabited by members of the *Pseudomonas* or *Aeromonas* genera, where bacteria that can degrade starch grow as yellow colonies. In contrast, bacteria that cannot degrade starch grow as red colonies (Gomez-Gil *et al*., 2007).

Conclusion

In conclusion, this study effectively validates the CS method as an efficient and simple solution for removing bacterial contamination in aquatic fungal cultures. The study highlights its particular effectiveness with a bacteria-to-fungi ratio of 2:1, balancing bacterial elimination and fungal growth. Looking ahead, it is essential to broaden the scope of this method to include various aquatic fungi and bacteria. Such expansion is expected to assess the CS method's adaptability and efficacy across different microbial ecosystems and proportion variations. This progression will deepen the understanding of microbial interactions and enhance microbial culture practices.

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Conflict of Interest Statement

All authors declared that they have no conflicts of interest.

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