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Media Manipulation with *in Vitro* Developed Biofilms to Cultivate yet-unculturable Bacteria from Soil

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ABSTRACT

More than 99% of bacterial species existing in the soil cannot be cultured using defined media, although they are viable, thus, known as viable, but non-culturable (VBNC) bacteria. They are accumulated in the soil contributing to form a voluminous microbial seed bank. The VBNCs may have functions could deploy for different purposes in biotechnology implying how imperative their isolation is from the soil. Despite the massive setback of traditional methods, currently, they have been combined with co-culture dependent isolation methods, yet reported to be challenging.The wisdom of fungal-bacterial biofilms (FBBs) can be borrowed to culture VBNCs as they have increased soil microbial diversity by creating required conditions mimicking for VBNCs to resuscitate. Thus, the developed biofilm approach shows the potential as a new method to improve the cultivability of diverse VBNCs in laboratory settings, enabling researchers to investigate their functional properties. Once researched and established, this will open a new avenue for microbiologists and biotechnologists to exploit then-VBNCs for different purposes in the biotechnology in the future.

KEY WORDS: Fungal-bacterial biofilm (FBBs), Viable but non culturable bacteria, co-culture dependent cultivation, manipulation of defined culture media, FBBs-mediated media manipulation

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1. INTRODUCTION

Cultivating bacteria is imperative in several fields of biotechnologysuch as drug discovery, biofertilizer, biocontrol, and other microbes-related industries. However, more than 99% of bacterial species existing in the soil cannot be cultured using defined media and traditional techniques[1].The worst issue associates with this is that the culturable 1% show extreme phylogenetic bias, confining predominantly to four phyla (Bacteroidetes, Firmucutes, Actinobacteria and Proteobacteria). It is apparent that uncultured bacterial clades may have some critical roles in different purposes in biotechnological fields. Once detailed metabolism and gene functions of the uncultured bacteria is understood, they could be developed for biotechnological purposes. Culturing them in laboratory settings allows researchers to discover new genes, understand gene functions and mechanisms in functional pathways[2, 3] . However, the question arises here is why it is impossible to culture those bacteria, although they exist in the soil representing as much as 99% of the total bacteria. Thus, this needs further attention to disclose specific growth requirements they want for their germination.

Most of the bacterial cells in the soil remain in a state of reduced metabolic activity, which is known as dormancy[4, 5]. Under the resource-limited, biotic and abiotic stress conditions, live microbial cells transformed into dormant forms, which are reversible forms of active cells[6, 7]. Dormant cells in the soil thus contribute to generating the voluminous microbial seed bank[5], which exist still until meeting favourable conditions to resuscitate[6] (Jones and Lennon, 2010) and maintaining the soil microbial diversity[7]. Although they are viable, dormancy leads to increasing the difficulty of culturing them using defined media[1]. Hence these forms are known as viable, but non-culturable (VBNC) bacteria in the soil.

Developing strategies to cultivate VBNC bacteria in laboratory settings and the challenges and difficulties encountered of finding new techniques have been extensivelyreviewed[2, 3, 1, 8]. However, a fascinating technique based on the knowledge of microbial ecology including cell-to-cell communication and metabolism has been adopted to manipulate defined culture media, creating conditions mimicking their natural environment[9, 1]. Although this knowledge is integrated with new cultivation techniques, yet there is a need of strategies that promote isolation and to preserve isolated microbes from subsequent disappearing during sub-cultivation[2, 3]. Therefore, in this, hypothesis, we introduce a novel technique of manipulating defined culture mediato provide requiring natural conditions for VBNC to grow, with the help of *in vitro* developed biofilms.

2. HOW DOES BIOFILM FORMATION UNDERPIN MICROBIAL DIVERSITY?

In natural environments, apart from defining the community structure, maturation, and niche construction, biofilm microbes are involved in expanding their community[10, 11]. It is common knowledge that, if the community is to be expanded and diversified, new microbial species should emerge or come into the system. For this, communication networks among cells should be strengthened by trading metabolites and exchanging signaling molecules[12, 11, 13]. Generally, cell-to-cell communication is accomplished by generating an array of chemical compounds, which are simply known as public goods that neighbor microbes can utilize [11, 13]. The public goods comprise antibiotics, exopolysaccharide, Quorum sensing (QS) molecules etc., are involved in the survival of the community [14,15,16]. It is now known that the higher public good production means the greater level of communication, favouring a higher relatedness among individuals [17, 11, 18], eventually facilitating the community expansion. Further, a community-expanding resuscitation-promoting factor (growth factor, Rpf), a 17 kDa protein has also shown to be involved in the growth of live microbial cells[19].

When there is a higher relatedness among interacting individuals in a community, kin selection results in enhanced diversity of microbes[10, 11]. Bacteria respond to a wide range of signaling molecules at the intra-species level to help recognizing species-specific compounds, and/or at inter-species levels in recruiting microbes into their pre-existing biofilm[20]. For instance, an electrical signal released by *Bacillus subtilis* in a biofilm has been reported to attract *Pseudomonas aeruginosa* cells to the community[21]. Further, Rpf is capable of initiating resuscitation of dormant forms due to muralytic activity that remodels cell envelope of dormant cells facilitating cell division and regrowth[19,5]. Further, *Micrococcus luteus*[22] and *Mycobacterium tuberculosis*[23] have been shown to increase the growth rate of metabolically inactive vegetative cells as a response to Rpf.

As a recent development, our research has demonstrated an enhanced microbial growth and diversity in agroecosystems following the soil application of *in vitro* developed biofilms[24, 25]. Fungal-bacterial biofilms (FBBs) developed by co-culturing nitrogen-fixing bacteria and fungi isolated from plant rhizospheres were shown to be able to break dormancy of soil microbial seed bank, thus increasing abundance and diversity of microbes[26, 27, 25, 24]. This process was proven by showing the emergence of new cyanobacterial species compared to control soil, although the soil-applied FBBs did not contain any cyanobacteria [28].

FBBs mediated increase of microbial diversity can be attributed to few reasons. First, the FBBs have a higher cell density, ca. 10^{10}, at which an increased cell-to-cell communication of microbes occurs[9] due to a wider spectrum of aforementioned public goods produced more than that of their monoculture bacteria [26, 25]. Once these substrates[5, 7] get attached to endospore surface receptors of VBNC forms, peptidoglycans are hydrolyzed by enzymatic activity, thus turning endospores to active cells[29]. In addition, QS molecules and other growth factors drive spontaneous resuscitation of dormant microbial cells[5]. Thus, the soil application of FBBs can enrich the soil with diverse microbes in general and further by the in-situ activation of dormant forms in particular[25, 28].

3. BIOFILM-MEDIATED MANIPULATION OF CULTURE MEDIA

Currently, for culturing VBNCs, traditional approaches have been combined with co-culture dependent isolation methods[30], which are involvedwith the knowledge of biochemical processes, adaptation and physiology of bacterial monocultures as helper bacteria[31, 32]. This approach offers a potential to cultivate VBNCs since growth factors released by helper bacteria diffuse through the medium allowing VBNCs to utilizefor their growth[33]. However, their sub-cultivation seems to be affected by the media being lack of essential growth factors[3]. Further, it seems that, when there are more bacterial strains in culture media, there is a higher possibility of producing more growth factors required for other microbes to grow. For instance, the growth of *Symbiobacterium thermophilum* has been reported in the presence of growth factors produced by various bacterial strains, yet there is a requirement of a living partner to maintain the optimum level of the growth factor(s) in the medium[32]. Therefore, the optimization of nutrients and growth conditions of helper strains is required to get an optimum yield of these growth factors. However, this seems to be quite challenging[1] as there is a demand for specific growth factors depending on the metabolism of the targeted isolate[2, 3].

Generally, biofilm formation using more than one bacterial strain and a rhizosphere fungal strainis effective than mono or mixed cultures of bacterial strains in kin selection as the inter-kingdom biofilms have developed synergistic interactionsas explained in the aforementioned section[34, 35]. Thus,the mechanism of FBBs, whichlead an increased microbial diversity in the soil can be borrowed to culture VBNCs.

4. FUTURE PROSPECTS

The developed biofilms can be easier to use for the manipulation of defined media than using relatively less efficient bacterial monocultures, which also is time-consuming in the use. In this effort in the future, the defined media can be supplemented with cell-free liquid culture filtrate of biofilms for the possible isolation of VBNC and their subsequent sub-culturing. Thus, FBBs-mediated media manipulationis hypothesized asa potential new technique to improve the cultivability of VBNCs in laboratory settings. As such, this opinion, which now is awaiting further experimental evidence will open a new avenue for microbiologists and biotechnologists to exploit then-VBNCs for biotechnological purposes in the future.

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6. AUTHOR CONTRIBUTION

U.V.A. Buddhika generated the hypothesis and wrote the manuscript; G. Seneviratne contributed to generating the hypothesis and reviewed the manuscript.

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