

Gas Generation by Egg-White-Enclosed DNA Crown Cells in Agar Culture

Shoshi Inooka*

*Japan Association of Science Specialists.

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ABSTRACT

Self-replicating artificial cells, referred to as DNA crown cells, have been shown previously to generate antibiotic-producing cells when cultured with egg white powder and various partners. However, the mechanisms underlying the emergence of these antibiotic-producing cells remains unclear. To better understand this phenomenon, this study examined whether gas production occurred during the cultivation of egg white powder enclosing DNA (bovine meat) crown cells with a blueberry extract on agar. Agar cultures of the powder produced four distinct gases: dimethyl disulfide (DMS), 5-(2-methylpropyl)nonane, 1-3-dichlorobenzene, and dodecane. These findings suggested that gas production may be associated with the transformation or activity of DNA (bovine) crown cells in conjunction with blueberry extracts. The resulting powder was named antibiotic Crown-Bovine meat-blueberry-ex-cells-P.

Keywords: DNA (bovine) crown cells, Sphingosine-DNA, antibiotic Crown-Bovine meat-blueberry-ex-P, monolaurin, gas production, dimethyl disulfide

INTRODUCTION

Artificial cells capable of self-replicating within egg white were first reported in 2012. These cells were initially contrasted from Shingoshine, DNA and Ascidian (**Sea Squirts**) extracts (1). In 2016, it was described that the active components of the Ascidian extracts are the nucleosides uridine and thymidine, which are also present in Ascidian extracts (2). When the effects of various nucleosides on cell proliferation was investigated, adenosine was found to be particularly effective. Moreover, additional characteristics of artificial cells were described, including artificial cell seeds (Sphigoshine-DNA-adenosine), their overall morphology, and plasmid-enclosed protein expression (3).

When adenosine was added to cultured egg white, adenosine-lipid conjugates that promote the production of artificial cells was identified. These findings illustrated the importance of nucleoside-lipids in the production of artificial cells (4).

In that study, the lipid moiety of this conjugate was found to correspond to monolaurin. When adenosine-monolaurin (A-M) compounds were prepared, artificial cells could be produced (4). Subsequent analysis revealed that the outer membrane of artificial cells are coated with DNA, resulting in the designation of these cells as “DNA Crown Cells” in 2016 (4).

DNA crown cells, which have been shown to develop into fully self-replicating cells after incubation in egg white of artificial cell seeds or synthetic DNA crown cells which can be prepared with sphingosine, DNA, adenosine, and

monolaurin, all of which are commercially available compounds. A comprehensive procedure for generating DNA crown cells with synthetic DNA crown cells was reported in 2017 (5), and numerous DNA crown cells and associated cell strains have been produced to date (6–14). These cells have been prepared and stored stably at 4°C for nearly a decade.

When cultured in agar with egg white, monolaurin, salt or microorganisms, synthesized DNA crown cells formed assemblies or diverse morphological objects (15–20).

Antibiotic-producing cells were also isolate from beer produced using co-cultures of DNA crown cells and yeast (21). Based on these findings, egg white powder enclosing different DNA crown cells in conjunction with different substances has been prepared, and numerous antibiotic-producing cells have been produced using agar cultures of these powders (22–27). Such a culture system (i.e., using egg white powder) can be regarded as being equivalent to an egg-white-based culture system, which obviates the need to use eggs for culture.

Corresponding author: Shoshi Inooka, Japan Association of Science Specialists, 3-8-2 Nakada, Taihaku-ku, Sendai 981-1104, Japan. E-mail: s3inooka@aol.com; Tel: +81-22-241-1146

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Despite these extensive observations, the mechanism by which such self-replicating antibiotic-producing cells arise remains unknown.

Therefore, to better understand the emergence of self-replicating antibiotic-producing cells, this study examined whether gas production occurs when egg white powder enclosing DNA (bovine) crown cells with a blueberry extract is cultured.

MATERIALS AND METHODS

Materials

DNA (bovine meat) crown cells that had been prepared previously and stored in a refrigerator at approximately 4°C were used in this study (5, 22). However, the methods are described here again for clarity.

The materials used in the present study were the same as those employed in previous studies (5): Sph (Tokyo Kasei, Japan), DNA (from bovine meat), adenosine (Sigma-Aldrich; Wako, Japan), monolaurin (Tokyo Kasei), and adenosine-monolaurin (A-M), a compound synthesized from a mixture of adenosine and monolaurin (5). Sorbent tubes (SIBATA, Japan). Monolaurin solutions were prepared to a final concentration of 0.1 M in distilled water. Agar plates; agar medium (SMA) (AS ONE, Japan). Blueberries were obtained from a local market.

Gas chromatography-mass spectrometry (GC-MS) and gas chromatography (GC) analyses were performed using a GCMS-TQ8030, a GC-2010 Plus and NIST11 Mass Spectral Library, respectively (Shimazu, Japan).

Methods

Preparation of DNA (bovine meat) crown cells (6, 22).

Step 1 A total of 180 μL of Sph (10 mM) and 50 μL of DNA (1.7 $\mu\text{g}/\mu\text{L}$) were combined, and the mixture was heated and cooled twice.

Step 2. A-M solution (100 μL) was added and the mixture was incubated at 37°C for 15 min.

Step 3. A total of 30 μL of monolaurin solution was added, and the mixture was incubated at 37°C for another 5 min.

Step 5 Then, 0.3–0.5 mL of the suspension was injected into egg white and incubated for 7 days at 37°C. Egg white was subsequently recovered and used as DNA (bovine meat) crown cells.

Preparation of blueberry extract

Berries (~50 grains) were ground using a mortar and pestle and suspended in 3 mL of distilled water.

Preparation of powder

1. First, 3 mL of blueberry extract was mixed with 3 mL of egg white.

2. The mixture was incubated for 5 h at 37°C.
3. Then, approximately 25 mL of fresh egg white was added to the mixture.
4. The fluid was poured into two Petri dishes and dried for 1–2 days at 37°C.
5. Dried materials were collected and powder was prepared using a mortar and pestle.
6. The powder (Fig. 1), named Crown-Bovine-meat-blueberry-ex-P, was stored at room temperature until use.

Preparation of samples for GC-MS/GC analysis

A small amount (~40–50 mg) of powder (Crown-Bovine-meat-blueberry-ex-P) was added to an agar plate and incubated for 2 days at 37°C. Approximately 1.5 mL of 0.1 M monolaurin was dispensed onto a plate, together with two gas assay tubes, and the plate was sealed in a bag. The samples were then incubated for 2 days at 37°C. After incubation, the tubes were sealed and stored in a freezer until gas detection

Gas detection

- 1) The tube was cut open, and the activated carbon was removed. It was transferred to a new brown bottle
- 2) Then, 1 mL of carbon disulfide was added to the bottle and briefly shaken.
- 3) After 2 h, the gasses had been extracted.
- 4) The sample was subjected to GC-MS analysis.

Antibiotic-producing cells:

Objects within the frame shown in Fig. 6 that were grown on the plate were suspended in approximately 1 mL of distilled water. Then, approximately 200 μL of the suspension was cultured on an agar plate at 37°C for 1 day.

General observations

Objects on the plates were observed directly with the naked eye and under a light microscope.

RESULTS AND DISCUSSION



Figure 1. Powder used in this study.

Figure 2 shows a photograph of an agar plate immediately after the start of culture with the powder (Crown-Bovine-meat-blueberry-ex-P). Powder particles of various sizes were observed to cover the entire Petri dish.

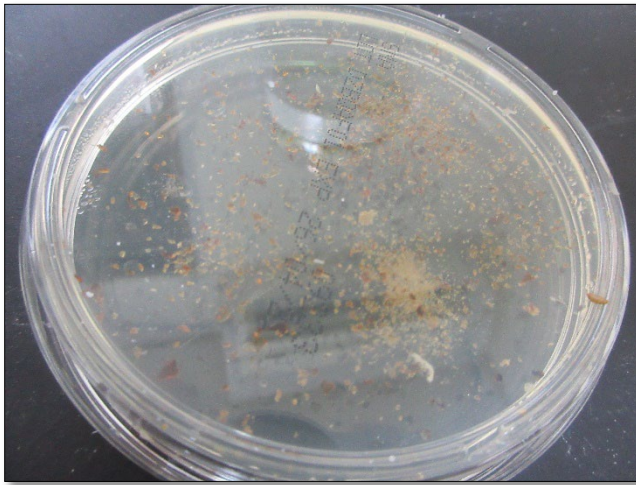


Figure 2. Photograph of an agar plate immediately after initiating powder culture (Crown Bovine meat-blueberry-ex-P). Powder particles of various sizes were observed covering the entire Petri dish.

Figure 3 shows a photograph of an agar plate after 1 day of powder culture. Large objects of various sizes and shapes were observed on the Petri dish.

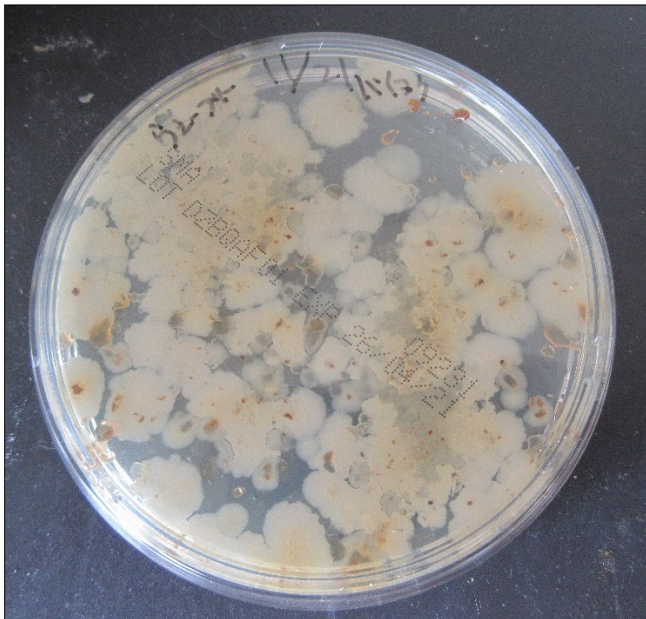


Figure 3. Photograph of an agar plate after 1 day of powder culture. Large objects of various sizes and shapes were observed on the Petri dish.

Figure 4 shows a photograph of an agar plate after 2 days of culture using the powder. The appearance was similar to that after 1 day.

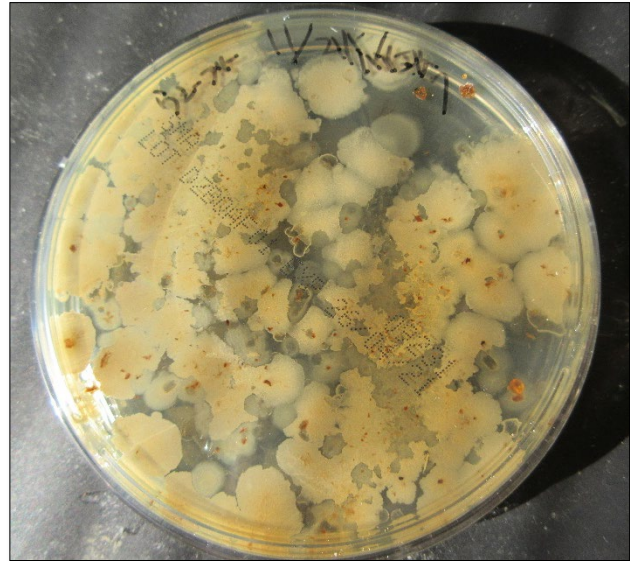


Figure 4. Photograph of an agar plate after 2 days of culture using the powder. The appearance of the plate was similar to that after 1 day.

Figure 5 shows a photograph of an agar plate with powder cultures at 1 day after the addition of monolaurin. Brown objects were observed.

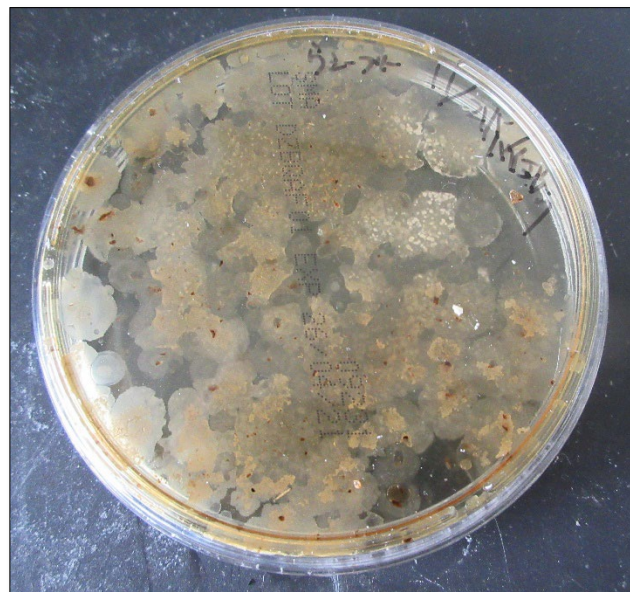


Figure 5. Photograph of an agar plate with powder cultures at 1 day after the addition of monolaurin. Brown objects were observed.

Figure 6 shows a photograph of an agar plate at 2 days after the addition of monolaurin to the powder. Various brown

objects of different sizes and shapes were observed. Objects within the frame were collected.



Figure 6. Photograph of an agar plate at 2 days after the addition of monolaurin to the powder. Various brown objects of different sizes and shapes were observed. Objects within the frame were collected.

Figure 7 shows the microscopic appearance of objects within the frame shown in **Figure 6**.

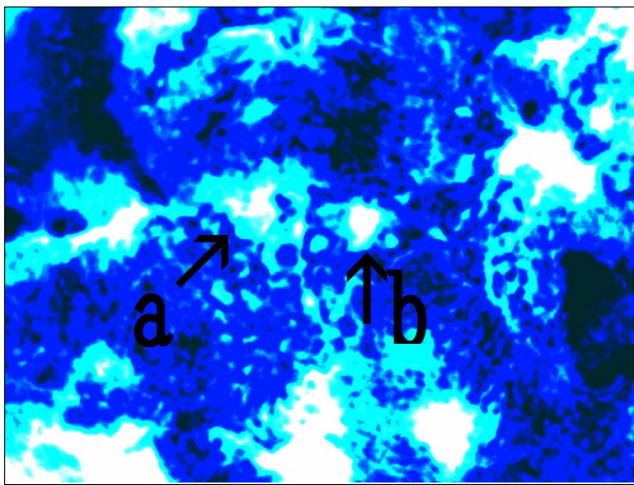


Figure 7. Microscopic appearance of objects within the frame shown in **Figure 6**. Various amorphous objects were observed (**Figure 7a**). The approximate size of the object in **Figure 7b** is 120 μm .

Various amorphous objects were observed (**Figure 7a**). The approximate size of the objects shown in Fig.7b is 120 μm .

Figure 8 shows the growth obtained on an agar plate after culture of the material within the frame in **Figure 6**.

Microorganism-like objects were observed over the entire plate.

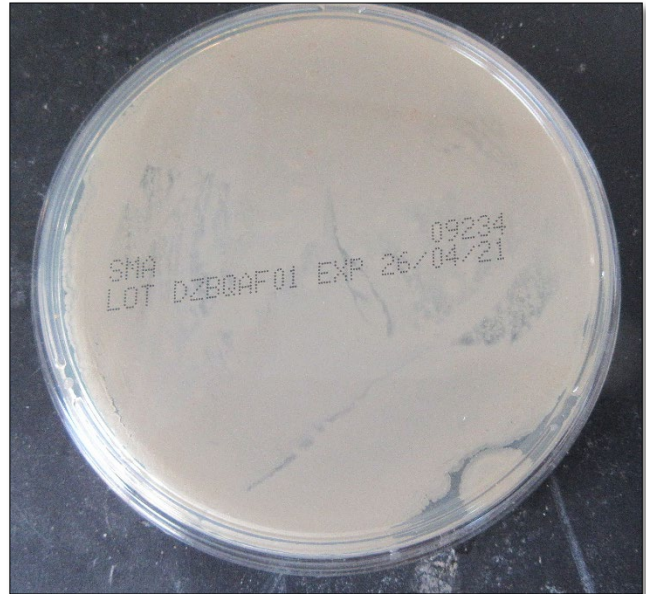


Figure 8. Photograph of objects grown on an agar plate from a culture of the objects collected within the frame shown in **Figure 6**. Microorganism-like objects were observed over the entire plate.

Figure 9 shows a photograph of the experimental setup for the gas assay. After gas collection, the tubes were sealed.



Figure 9. Photograph of the equipment used for gas assays. After gas collection, the tubes were sealed.

Figure 10 shows a gas chromatogram of the sample used for gas analysis. The red trace represents the sample, and the black trace represents the blank. Four peaks were observed.

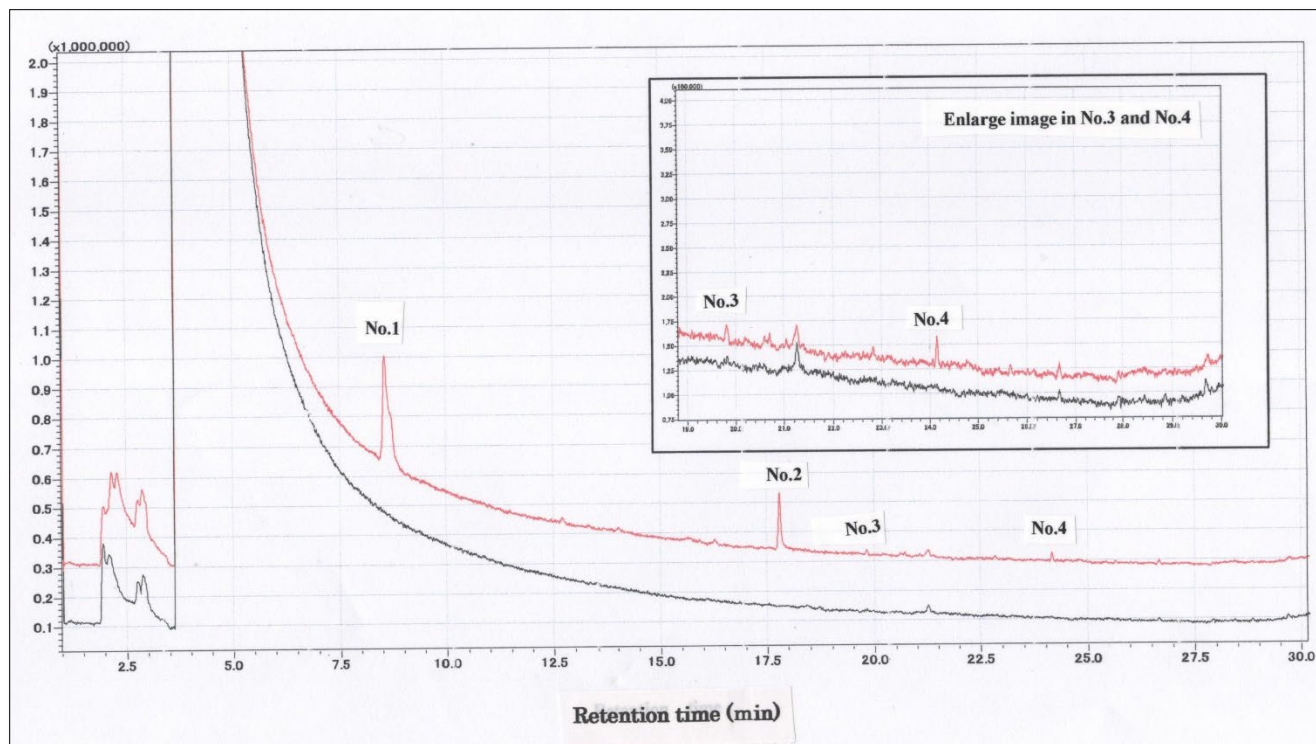


Figure 10. Gas chromatogram of the sample used for gas analysis. The red trace represents the sample and the black trace represents the blank. Four peaks were observed.

Figure 11 shows the search results obtained using the mass spectrometry library. For each entry (No.), the upper panel shows the mass spectrum of the sample, and the lower panel shows the corresponding library spectrum.

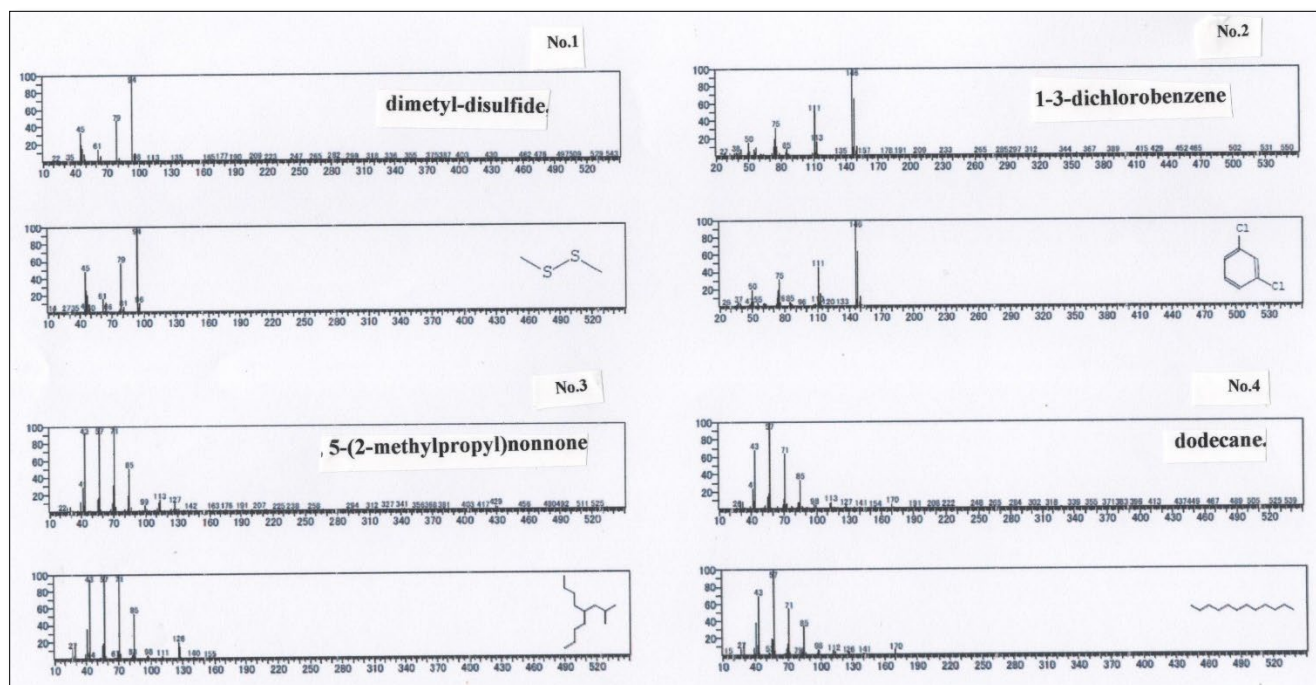


Figure 11. Search results using the mass spectrometry library. The upper panel shows the mass spectrum of the sample and the lower panel shows the corresponding library spectrum.

Table 1 summarizes the compounds identified by GC-MS analysis.

Table 1. The compounds identified by GC-MS analysis.

No.	RT	Compounds
1	8.55	Dimethyl disulfide
2	17.79	1-3-dichlorobenzene
3	19.81	5-(2-methylpropyle)nonane
4	24.14	Dodecane

For each detected compound, the table provides No (the entry number) and RT (retention time in minutes).

In previous studies, it was demonstrated that antibiotic-producing cells were generated through the combination of DNA crown cells and partners (22–27). Importantly, the generation process was found to common across all combinations tested.

At the initial stage of the experiments, the powder was spread onto agar culture medium and the plates were incubated (Figures 2, 3 and 4). Following incubation, monolaurin was added to the culture. Two days after the addition of monolaurin, the developing objects exhibited a slightly brownish coloration (Figures 5 and 6), appearing amorphous under microscopic examination (Figure 7)

When the monolaurin-treated objects were further cultured, numerous self-regenerating antibiotic-producing cells subsequently emerged (Figure 8).

Therefore, the observed increase in objects appear to be stimulated by monolaurin.

However, no conclusions can be drawn at present as slight increases in object number have also been observed in the absence of monolaurin. It is possible that such increases may be attributed to the egg white-derived lipids in powder, some of which may exhibit monolaurin-like activity.

Since the process underlying the generation of antibiotic-producing cells is shared across all combinations of DNA crown cells and partners tested, the use of powder prepared from the combination of DNA crown cells (bovine meat) and blueberry extract in this study is not particular unique. If anything, it merely demonstrates that this specific combination can also be used to produce DNA crown cells.

As noted earlier, numerous antibiotic-producing cells emerged during the culture period following the addition of monolaurin. However, the reason such a phenomenon occurred and the processes involved during the course of cultivation, remain unclear.

To clarify this point, gas production was investigated during cultivation. Gas was collected over two days of cultivation after the addition of monolaurin using the setup shown in Figure 9.

The collected gas was analyzed by GS-MS and the resulting gas chromatograms showed four distinct peaks (Figure 10). The MS spectrum of each distinct peak, along with the corresponding-library search results, are shown in Figure 11. The library results identified the peaks as DMS (No. 1), 5-(2-methylpropyl) nonane (No. 2), 1-3-dichlorobenzene (No. 3), and dodecane (No.4) (Table 1).

How gases were generated and whether they have any relationship to the proliferation of antibiotic-producing cells remains unknown. Nevertheless, the discovery that gas is produced in this type of culture system is considered significant when the nature of the detected gases is considered.

Several of these gasses are commonly associated with biological activity, which raises important questions about the chemical processes occurring within the culture systems.

In particular, DMS is used in a variety of fields, including the food industry

It is also produced by marine microorganisms and is associated with biological processes in sulfur-rich environments.

Moreover, it is considered one of the most significant and promising gases in astrobiology and is regarded as a potential indicator of extraterrestrial life. Indeed, its presence on some planets, has been interpreted as a possible indicator of life.

Likewise, the presence of methyl-containing compounds is often interpreted as an indication of biological or pre-biological chemical processes. Similarly, the gases detected during the generation of antibiotic-producing cells are considered to be suggestive evidence of potential biological activity.

Further studies should clarify whether gas production is directly linked to the emergence of antibiotic-producing cells, to the formation of the unique cellular structures, or to the synthesis of antibiotics themselves.

In addition, it will be important to clarify how these gases are produced and which gases are generated under different combinations of DNA crown cells and partners.

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