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Research Article

Validation of the Exposure Time of 90 mm TSA Plates for Environmental Monitoring

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Abstract

Culture media represent the artificial substrates through which the development and *in vitro* growth of microorganisms are observed. They are characterized by specific ingredients (peptones, carbohydrates, salts, indicators, and solidifying agents) that aid in the growth of microorganisms. Furthermore, depending on the presence or absence of certain substances that promote the growth of only certain microorganisms, culture media are differentiated into selective and non-selective types. Currently, pre-prepared culture media are used and available commercially in the form of powders or dehydrated media, as well as ready-to-use media in plates, test tubes, or bottles. In the pharmaceutical field, according to the current regulations of the European Pharmacopoeia and the United States Pharmacopeia, culture media used for environmental monitoring and microbiological analysis of sterile and non-sterile products must undergo a growth promotion test (GPT) and verification of the sterility of the media before use. In this study, the nutritional characteristics of Tryptic Soy Agar (TSA) plates, with a diameter of 90 mm, were verified during passive air monitoring. The latter involves exposing the plates in the environment for a maximum of four hours to collect, by sedimentation, microorganisms carried by solid or liquid particles. The results obtained confirmed that the recovery for each microorganism on TSA plates meets the specifications required by the Pharmacopoeia for all time points tested (from T0 to T5). Furthermore, it was confirmed that, even after a five-hour exposure of the culture media under a laminar flow hood, a recovery rate exceeding 70% can be achieved. However, at this duration, dehydration phenomena and changes in the thickness of the media were observed; therefore, it is preferable to maintain a four-hour exposure time for the plates within clean rooms.

Keywords: Growth Promotion Test; Recovery; Passive Sampling

Introduction

The birth of bacterial cultures

Culture media represent a mixture of biological or synthetic compounds, organic or mineral, capable of providing a suitable environment for the development and *in vitro* growth of microorganisms. The discovery of culture media dates back to the 19th century thanks to the French microbiologist Louis Pasteur, who was able to isolate bacteria by developing an artificial culture medium [1]. About forty years before Pasteur, there was an important discovery by the pharmacist Bartolomeo Bizio regarding the presence of blood-red spots found by a peasant family on the leftover bread from previous days. In this case, the intervention of the Church was inevitable as it was believed that the blood-red spots found could represent the blood of Christ. Thanks to Bartolomeo Bizio and the advances in microbiology, it was demonstrated that those spots did not represent the blood of

Christ but rather a microorganism named Serratia marcescens. The latter appears as red colonies due to the production of a pigment, prodigiosin, and subsequently "decays" rapidly into a mucilaginous fluid mass, hence the name of the species marcescens [2]. The year 1860 represents the year when Louis Pasteur developed a culture medium containing yeast extract, ashes, sugar, and ammonium salts to demonstrate the correlation between fermentative processes (alcoholic, acetic, and lactic) and microorganisms. In fact, the presence of these nutrients allowed the growth or inhibition of specific bacteria but could also favor the appearance of some bacteria over others. Pasteur is also credited with discovering a way to maintain a sterile solution. This occurred through an experiment involving the use of flasks into which a nutrient solution was inserted, the necks of which were heated by placing them in various positions and keeping the end in contact with the air. The solution was then boiled for a few minutes and allowed to cool. However, Pasteur did not observe any growth despite the content of the flasks being exposed to the air. The absence of growth was due to the fact that the germs remained trapped between the walls of the flasks, and in fact, only by breaking them, was it possible to observe immediate growth [1]. The evolution of culture media continues with Robert Koch, who demonstrated optimal bacterial growth following the incubation of a broth containing beef serum or meat extract. Despite this, pure cultures could not be obtained, thus giving rise to the idea of developing a solid culture medium. Initially, albumin, starch paste, potato slice, and finally gelatin was used. Although the latter could be an alternative, Koch observed some problems related to the liquefaction process, which occurred at a temperature >25°C, and its degradation by gelatinase, an enzyme produced by some bacteria. However, thanks to his wife Fannie Hesse, he replaced gelatin with agar, thus obtaining solid media that allowed the isolation of bacteria [3].

Composition of media

Currently available culture media are composed of the following categories of ingredients: i) carbohydrates; ii) peptones and hydrolysates; iii) inorganic salts; iv) indicators; v) solidifying agents [4]. Furthermore, it is crucial that they present specific characteristics such as i) an adequate degree of moisture; ii) an appropriate reaction (pH); iii) sterility; iv) an adequate concentration of nutrients essential for bacterial growth. Carbohydrates. They represent the main source of organic carbon and can be used in identification tests based on fermentation. The most commonly used carbohydrates are glucose, sucrose, and lactose. Peptones and hydrolysates. Mixtures of free and polymerized amino acids easily usable by bacterial cells. They are obtained from enzymatic digestion (peptones) or acid (hydrolysates) of animal proteins (meat, casein, gelatin), plant proteins (soy), and yeast cells. They represent the main source of organic nitrogen essential for bacterial protein synthesis. Inorganic salts. Their function is to supply cells with essential inorganic elements, as well as to ensure adequate osmolarity and buffer the medium. Indicators. They are substances that highlight particular enzymatic activities of microorganisms. The most common indicators are pH indicators, which reveal the production of acids or bases from the degradation of carbohydrates or nitrogen compounds, and oxidation-reduction indicators (methylene blue and resazurin). Solidifying agents. The most commonly used substance for solidifying culture media is agar, a polymer of galactose and galacturonic acid, extracted from various genera of red algae, which has particularly suitable properties for use in bacteriology. It liquefies at a temperature between 90-100 °C and solidifies at temperatures below 45°C [3].

Growth factors

Minimal media are media that exclusively support the growth of autotrophic bacteria, i.e., bacteria capable of autonomously producing the necessary nutrients for their growth. However, it is worth considering that the use of minimal culture media does not promote the growth of bacteria that require specific nutrients to grow. Consequently, it is necessary to add growth factors to culture media to ensure bacterial multiplication. Growth factors are substances that bacteria cannot synthesize, and among these are: i) purines and pyrimidines; ii) amino acids; iii) vitamins; iv) blood and derivatives; v) antioxidants [3]. Purine and pyrimidine bases. They are essential for nucleic acid synthesis and are used by some lactic acid bacteria that require them for their growth [5]. Amino acids. They are essential for protein synthesis [6]. Vitamins. They are coenzymes or coenzyme precursors. In particular, a vitamin is an organic substance necessary for the metabolism of a living organism but cannot be synthesized in sufficient quantities by that organism. Blood and derivatives. Blood and its derivatives promote the growth of some bacteria. Generally, sheep or horse blood is used in culture media, and its role is to act as a protective

agent against oxygen radicals as well as to act as a nutritional supplement. Antioxidants. Some anaerobic bacteria are difficult to cultivate, which is why new strategies have been developed for their isolation. It is necessary to consider that anaerobic bacteria constitute the intestinal microbiota and are sensitive to oxygen, which is toxic. Therefore, antioxidants have been added to culture media to allow the cultivation of strict anaerobic bacteria under aerobic conditions. Examples of antioxidants include glutathione, uric acid, and ascorbic acid [3].

Growth promotion test

Culture media are involved in the execution of numerous microbiological tests. Given their importance for environmental monitoring, sterility testing, process and product validations, and operator qualification, it is important that they are of good quality and function properly. As reported in ISO <11133:2014>, culture media must necessarily meet the criteria established for result accuracy. It is therefore essential to perform tests that demonstrate: i) the acceptability of each batch of medium; ii) if the medium is suitable for its purpose; iii) if the medium can produce consistent results [7]. Furthermore, considering a sterile pharmaceutical environment where microbial growth cannot occur, it is necessary to demonstrate that culture media are capable of supporting microbial growth if present, as failure to do so would result in false negatives that would undermine the validity of any test required by the pharmacopeia. Therefore, to introduce a new medium into analytical routine, according to the regulations of the pharmaceutical industry, it is necessary to test it adequately to ensure consistent and reliable results. Regardless of whether the medium is purchased from an external supplier or produced internally, growth promotion tests (GPT) must be performed on all batches of media before their use. This is referred to as a mandatory requirement of all pharmacopoeias. The GPT, also known as fertility testing or media challenge testing, is used in the pharmaceutical field to demonstrate the ability of microbiological media to support the growth of key microorganisms [8,9].

Microbial air monitoring

Microorganisms, such as bacteria, fungi, and viruses, are entities present in the air and on surfaces but invisible to the naked eye due to their microscopic size. It is worth remembering that airborne microorganisms can have harmful effects on human health. There are numerous sources of potential contamination: operators, clothing, equipment, materials, air conditioning systems, etc. Awareness of this has led to the need to measure and define the presence of possible microbial contamination in the air. Currently, methods for microbiological air monitoring are based on the use of solid media in Petri dishes and are divided into active sampling and passive sampling [11]. Active sampling involves the use of a sampler capable of drawing a volume of air onto an agar-coated surface of a 90 mm diameter Petri dish or a 55 mm Contact plate. As for passive sampling, it is necessary to consider that the number of microorganisms that can come into contact with a pharmaceutical product depends on their concentration in the air as well as the size of the particles carrying them, the surface, and the exposure time of the product to the air. Hence the importance of microbiological environmental monitoring used by pharmaceutical companies to detect trends in changes in microbial counts and the microflora recovered within clean rooms or controlled environments. Passive sampling is performed by exposing a 90 mm diameter Petri dish containing agar nutrient medium for a maximum of four hours to favor the collection of microorganisms carried by solid or liquid particles [12]. However, as it is a sedimentation technique, it does not provide a number of microorganisms present in the air but only those characterized by sufficient weight and size to sediment on the exposed surface.

Material and Methods

Isolation of test microorganisms

The microorganisms selected in this study are conserved and released by the American Type Collection (ATCC) and cited by the European Pharmacopoeia for culture media growth promotion studies (GPT). Furthermore, for each fertility test, two "wild-type" microorganisms found in environmental monitoring were also added (Table 1).

The preparation and standardization of microorganism suspensions were carried out according to laboratory procedures, applying appropriate techniques for maintaining and transferring microbial cultures, and ensuring that the microorganisms used had not undergone more than five passages from the original ATCC

Medium	Test microorganism	Temperature	Incubation time	Acceptance criteria
Tryptic Soy Agar	*S. aureus* ATCC 6538	32,5 +/- 2,5 °C	≤ 3 gg	-50%; +200%
	P. aeruginosa ATCC 9027	32,5 +/- 2,5 °C	≤ 3 gg	-50%; +200%
	B. subtilis ATCC 6633	32,5 +/- 2,5 °C	≤3 gg	-50%; +200%
	**S. epidermidis* **S. hominis*	32,5 +/- 2,5 °C	≤3 gg	-50%; +200%

Table 1: Microorganisms and fertility test for solid general-purpose media. *wild-type microorganisms found in environmental monitoring.

cultures. Starting from the prepared suspensions, serial dilutions were made in peptone buffer pH 7 (VWR Chemicals) until the desired concentration was achieved.

Growth promotion for determining the exposure time of the plates

For each batch of purchased medium, prior to use, a sterility check and a nutritional property control were required (Table 2). Subsequently, a study was conducted to evaluate the growth promotion properties of the medium present in the exposure plate and to assess the recovery of the inoculated microorganisms. Three different batches of Tryptic Soy Agar (TSA) (VWR) were tested. Each plate was exposed, and the recovery was evaluated after different time intervals: time 0 (T0), after one hour (T1), after two hours (T2), after three hours (T3), after four hours (T4), and finally after five hours (T5).

VWR plates	Batch	Composition	Amount per liter
TSA 90 mm	15303 151273 149826	Peptone from Casein Soya Peptone Sodium Chloride Agar	15,0 g/l 5,0 g/l 5,0 g/l 15,0 g/l

Table 2: Composition of the culture medium.

As mentioned previously in paragraph 2.1., once the suspension was prepared, 0.1 mL was taken, and for each batch of medium to be tested, two plates were inoculated with a limited number of microorganisms (< 100 CFU), ensuring that the microbial suspension was spread on the agar surface with an L-shaped spreader (Figure 1). Time 0 was considered the moment the plate was opened and exposed to the laminar flow. The suspension, used for the initial inoculation and kept at a temperature of 2-8 °C for a maximum of five hours [8], was used to assess the recovery at times T1, T2, T3, T4, and T5. Following plate incubation, the results were interpreted. The fertility of the medium can be considered compliant if, within the incubation period (3 days at a temperature of 30-35 °C), the calculated mean for each microorganism does not differ by a factor of 2 (-50%; +200%) compared to the mean of colonies obtained from positive control plates [9].

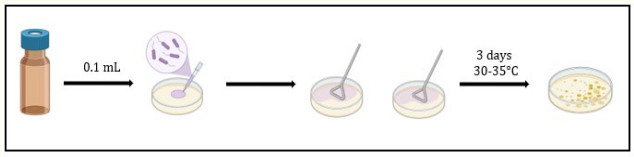

Figure 1: Growth promotion test (GPT).

Results and Discussions

From this study, it was possible to observe that all the media inoculated with standard microorganisms showed microbial growth within the incubation period. Furthermore, in all the tests conducted at different time points (from T1 to T5), following exposure under a laminar flow hood at a flow rate of 0.54 m/s, the following observations were made: i) The recovery for each microorganism, for all three batches of TSA plates at time T0, is in line with the Pharmacopeia standards (Table 3). ii) The recovery of microorganisms for all three batches of TSA medium at different timings (from T1 to T5) compared to T0, used as a reference, meets the Pharmacopeia specifications (Tables 4-8). iii) Following a five-hour exposure (T5), the three batches of tested TSA plates showed a recovery compliant with Pharmacopeia acceptance criteria, whereby the test plates, after the aforementioned exposure period, must recover at least 70% of the sample [10].

	Batch	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*S. hominis*	Isolato 26
T0 TSA	15303	1)90	1)89	1)98	1)67	1)68
	151273	2)91	2)99	2)93	2)62	2)74
	149826	3)88	3)100	3)99	3)82	3)75

Table 3: Exposure time T0.

	Batch	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*S. hominis*	*S. epidermidis*
T1 TSA	15303	1) 85	1) 78	1) 72	1) 70	1) 53
	151273	2) 96	2) 75	2) 64	2) 75	2) 64
	149826	3) 88	3) 69	3) 60	3) 85	3) 56
% recovery	15303	1) 106,3	1) 114,7	1) 102,9	1) 107,7	1) 71,6
	151273	2) 120	2) 110,3	2) 91,4	2) 115,4	2) 86,5
	149826	3) 110	3)101,5	3) 85,7	3) 130,8	3) 75,7

Table 4: Exposure time T1.

	Batch	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*S. hominis*	*S. epidermidis*
T2 TSA	15303	1) 80	1) 90	1) 92	1) 80	1) 80
	151273	2) 71	2) 93	2) 88	2) 94	2) 70
	149826	3) 75	3) 96	3) 95	3) 91	3) 79
% recovery	15303	1) 114,3	1) 105,9	1) 105,7	1) 95,2	1) 112,7
	151273	2) 101,4	2) 109,4	2) 101,1	2) 111,9	2) 98,6
	149826	3) 107,1	3) 112,9	3) 109,2	3) 108,3	3) 111,3

Table 5: Exposure time T2.

	Batch	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*S. hominis*	*S. epidermidis*
T3 TSA	15303	1) 85	1) 99	1) 64	1) 83	1) 60
	151273	2) 78	2) 98	2) 66	2) 80	2) 69
	149826	3) 83	3) 95	3) 64	3) 77	3) 66
% recovery	15303	1) 111,8	1) 117,9	1) 104,9	1) 110,7	1) 103,4
	151273	2) 102,6	2) 116,7	2) 108,2	2) 106,7	2) 119
	149826	3) 109,2	3) 113	3) 104,9	3) 102,7	3) 113,8

Table 6: Exposure time T3.

	Batch	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*S. hominis*	*S. epidermidis*
T4 TSA	15303	1) 73	1) 91	1) 89	1) 77	1) 76
	151273	2) 84	2) 99	2) 91	2) 77	2) 100
	149826	3) 81	3) 97	3) 100	3) 78	3) 77
% recovery	15303	1) 97,3	1) 103,4	1) 106	1) 101,3	1) 108,6
	151273	2) 112	2) 113	2) 108,3	2) 101,3	2) 143
	149826	3) 108	3) 110,2	3) 119	3) 102,6	3) 110

Table 7: Exposure time T4.

	Batch	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*S. hominis*	*S. epidermidis*
T5 TSA	15303	1) 73	1) 90	1) 83	1) 61	1) 57
	151273	2) 77	2) 80	2) 85	2) 71	2) 67
	149826	3) 83	3) 95	3) 90	3) 84	3) 61
% recovery	15303	1) 102,8	1) 107,1	1) 104	1) 101,7	1) 114
	151273	2) 108,5	2) 95,2	2) 106,3	2) 118,3	2) 134
	149826	3) 116,9	3) 113	3) 113	3) 140	3) 122

Table 8. Exposure time T5.

Conclusions

The results from this study have allowed to establish that the recovery obtained for each batch of plates, following a four-hour exposure (T4), complies with the requirements set by Regulatory Agencies regarding microbiological air monitoring [9,11]. Additionally, no dehydration phenomena of the culture medium were observed within the time interval between T0 and T4. The study also considered an additional exposure time point of five hours under a laminar flow hood. In this latter case, it was observed that despite a recovery rate exceeding 70%, thus meeting Pharmacopeia requirements, dehydration of the medium occurred, resulting in a change in its thickness (from 0.5 cm to 0.2 cm) (Figure 2). For this reason, it is preferable to maintain an exposure time frame of four hours for the plates inside cleanrooms. In conclusion, as a future perspective, further studies could be conducted to evaluate a potential increase in the volume of medium contained within the plates. This could lead to overcoming dehydration phenomena and decrease in the culture medium, thereby increasing the maximum exposure time of the plates inside cleanrooms.

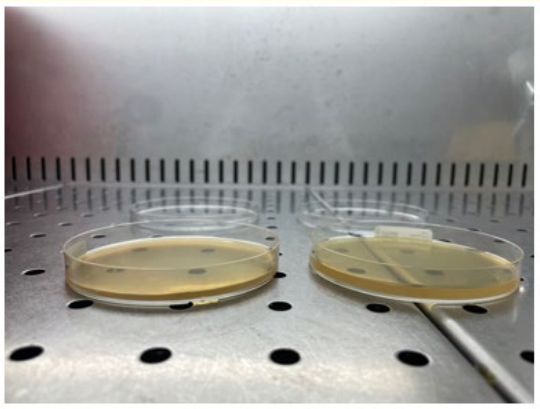

Figure 2: Change in the thickness of the culture medium. T5 (left) and T0 (right).

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