

Evaluation of the Nutritive Capacity of Soybean Tryptic Agar for Environmental Control in the Pharmaceutical Industry

Evaluación de la capacidad nutritiva del agar tríptico de soja para control ambiental en la industria farmacéutica

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ABSTRACT

Introduction: An environmental monitoring program is key to ensure aseptic production areas where sterile pharmaceutical products can be produced under environmental conditions that prevent the introduction, generation and retention of contamination.

According to Brazilian guidelines, the exposure time of agar plates for environmental monitoring should be ≤ 4 h and industries should evaluate whether the culture medium used presents satisfactory nutritive capacity for use in this period of time.

Objective: To evaluate the nutritive capacity of tryptic soybean agar for environmental monitoring in the pharmaceutical industry in different exposure periods.

Methods: Seven microorganisms *Aspergillus brasiliensis* ATCC 16404, *Bacillus spizizenii* ATCC 6633, *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538; and *Klebsiella oxytoca* and *Staphylococcus epidermidis* (in house strains) that were previously characterized by VITEK®2 and complete sequencing of the 16S rRNA gene were used. Microorganism suspensions were prepared to achieve less than 100 colony forming unit. Plates were exposed inside biological safety cabinet for 2, 3, 4 and 5 h and then transferred to another biological safety cabinet where each suspension was spread on two tryptic soy agar plates. Plates inoculated with *Aspergillus brasiliensis* and *Candida albicans* were incubated at 22.5 ± 2.5 °C/5 days and the others were incubated at 32.5 ± 2.5 °C/3 days. Two uninoculated plates were used as controls. Colony forming units were counted and compared with positive controls.

Results: The percentage recovery of strains was ≥ 50 % in all periods (50 to 189 %).

Conclusions: The nutritive capacity of soy tryptone agar was adequate for use in environmental monitoring tests up to 4 h.

Keywords: environmental monitoring; pharmaceutical industry; aseptic production; classified areas; sediment agar plates.

RESUMEN

Introducción: Un programa de monitoreo ambiental es clave para asegurar áreas de producción aséptica donde los productos farmacéuticos estériles puedan producirse en condiciones ambientales que eviten introducción, generación y retención de contaminación. Según directrices brasileñas, el tiempo de exposición de placas de agar para monitoreo ambiental debe ser ≤ 4 h y las industrias deben evaluar si el medio de

cultivo utilizado presenta capacidad nutritiva satisfactoria para su uso en este período de tiempo.

Objetivo: Evaluar la capacidad nutritiva del agar tróptico de soja para control ambiental en la industria farmacéutica en diferentes períodos de exposición.

Métodos: Se utilizaron siete microorganismos *Aspergillus brasiliensis* ATCC 16404, *Bacillus spizizenii* ATCC 6633, *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538; y *Klebsiella oxytoca* y *Staphylococcus epidermidis* (cepas in house) que se caracterizaron previamente por VITEK®2 y secuenciación completa del gene 16S rRNA. Las suspensiones de microorganismos se prepararon para lograr menos de 100 unidad formadora de colonias. Las placas se expusieron dentro de cabina de seguridad biológica durante 2, 3, 4 y 5 h y luego se transfirieron a otra cabina de seguridad biológica donde cada suspensión se extendió en dos placas de agar tróptico de soja. Las placas inoculadas con *Aspergillus brasiliensis* y *Candida albicans* se incubaron a $22,5 \pm 2,5$ °C/5 días y las demás se incubaron a $32,5 \pm 2,5$ °C/3 días. Se utilizaron dos placas no inoculadas como controles. Se contaron las unidades formadoras de colonias y se compararon con controles positivos.

Resultados: El porcentaje de recuperación de las cepas fue ≥ 50 % en todos los períodos (50 a 189 %).

Conclusiones: La capacidad nutritiva del agar soy tryptone fue adecuada para su utilización en pruebas de monitoreo ambiental hasta 4 h.

Palabras clave: monitoreo ambiental; industria farmacéutica; producción aséptica; zonas clasificadas; placas de agar de sedimentos.

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Introduction

Pharmaceutical industries must comply with good manufacturing practice (GMP) requirements in order to reduce the chances of contamination during production processes. Therefore, environmental monitoring programs are one of the key elements in ensuring the maintenance of aseptic processes, allowing contamination control at acceptable levels.⁽¹⁾

Maintaining low levels of contamination in environments where production and quality control activities are performed in pharmaceutical industries is a critical factor to ensure product quality, especially when it comes to areas that perform aseptic processes and sterile product control.⁽²⁾

The quality of an aseptically produced product is achieved when equipment and facilities are designed in such a way as to minimize or eliminate potential contamination hazards.⁽³⁾

Thus, the manufacturing of sterile products must be performed in clean areas, classified according to the current version of ISO 14644-1.⁽⁴⁾

Clean areas are areas with defined environmental control of particulate and microbial contamination, constructed and used in such a way as to reduce the introduction, generation, and retention of contaminants within the area.⁽⁵⁾

A well-planned and executed environmental monitoring program provides greater assurance of product sterility, so in clean areas environmental monitoring should be done routinely, using methods such as sedimentation plates, volumetric and surface air sampling (e.g. swabs and contact plates).^(3,4)

Air sampling can be active, when microorganisms can be quantified by air sample volume, and passive, when the environment is monitored by determining the number of microorganisms that settle on a petri dish with culture medium by gravity. In this way, the ambient air can be assessed for microbiological particles.⁽⁶⁾

In passive (sedimentation) air sampling, petri dishes with culture media should be exposed for a certain period of time, and the result of microbial growth is expressed as number of colony forming units (CFU) per plate exposed per time.⁽⁷⁾

The culture media that are used in environmental monitoring must be pre-tested for sterility and growth promotion, so that their nutrient capacity is checked before use, and they must be capable of maintaining microbial growth when inoculated with less than 100 CFU, using reference strains predefined in the official compendia, as well as environmental microorganisms that are representative of an industrial environment because they are commonly found in environmental monitoring, called autochthonous or in house strains.^(3,8)

Therefore, obtaining information regarding the identification of microorganisms can be of great importance in the investigation of contamination sources in industrial processes, especially when specified limits are exceeded.⁽¹⁾

According to the WHO document Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities⁽³⁾ and the EU Guidelines to Good Manufacturing Practice,⁽⁹⁾ the recommended exposure time limit for sedimentation plates is four hours. If longer exposure is required, the plates should be replaced.

However, these same documents recommend that validation studies be conducted to determine how long a sedimentation plate can be left in the specific conditions of use and still maintain growth promotion for the microorganisms of interest. In industrial environments, where areas with high airflow, high turbulence, high temperatures, or low humidity are common, exposed sedimentation plates may dry out or suffer changes in their properties, which may make it totally or partially impossible to recover microorganisms.⁽³⁾

The performance of these studies proves that the culture medium used is capable of maintaining its nutrient properties during the entire incubation period, thus allowing the recovery of microorganisms present in the tested environment.⁽¹⁰⁾

In Brazil, the quality control of medicines is performed by the National Health Surveillance Agency (Anvisa) of the Ministry of Health. According to the Collegiate Directive Resolution (RDC) No. 658/2022, which provides for the Good Manufacturing Practices (GMP) of medicines, it is recommended that the performance of all culture media should be checked before use.

In addition, Anvisa's Normative Instruction No. 35 of August 21, 2019(4), which provides for complementary GMP for sterile medications, advocates that sedimentation plates used in monitoring should be exposed in the environment for up to 4 h, a guideline equal to that of international compendia.^(9,11)

Thus, in view of the need to meet the regulatory requirements for the production of immunobiological in Brazil, the objective of this work was to test the nutrient capacity of casein soy agar (TSA) used in microbiological environmental monitoring when subjected to different exposure times.

Methods

Preparation of work batches and cryopreservation

Seven microbial strains were used in this study, being five reference strains, which are recommended in the Brazilian Pharmacopoeia for the growth promotion test,⁽⁸⁾ from a Biological Collection and two autochthonous strains described in table 1.

Table 1 - Microbial strains used in this study

Strain	Origin	Isolation Source	Culture medium (incubation temperature/incubation time)
<i>Aspergillus brasiliensis</i>	ATCC ¹ 16404	Fruit (blueberry)	SDA ² (20 ± 2,5) °C, 5-7 days
<i>Bacillus spizizenii</i>	ATCC 6633	Unknown	TSA ³ (30 ± 2,5) °C, 18-24 h
<i>Candida albicans</i>	ATCC 10231	Clinic	SDA (20 ± 2,5) °C, 2-3 days
<i>Pseudomonas aeruginosa</i>	ATCC 9027	Clinic	TSA (30 ± 2,5) °C, 18-24 h
<i>Staphylococcus aureus</i>	ATCC 6538	Clinic	TSA (30 ± 2,5) °C, 18-24 h
<i>Klebsiella oxytoca</i>	B892/21 - Autochthone	Investigation Swab	TSA (30 ± 2,5) °C, 18-24 h
<i>Staphylococcus epidermidis</i>	B1225/21 - Autochthone	Intermediate product bioburden testing	TSA (32,5 ± 2,5) °C

¹American Type Culture Collection; ²sabouraud dextrose agar; ³soy casein agar.

The reference strains were purchased freeze-dried and were rehydrated in 2.0 mL volume of soybean casein broth (TSB, BioCen, São Paulo, Brazil) and incubated for 5 m at (30 ± 2.5) °C until complete dissolution.

For the autochthonous strains, growth was performed in TSB (32.5 ± 2.5) °C for 24 h. After incubation, 0.1 mL of the strain suspensions were transferred to six TSA (BioCen, São Paulo, Brazil) or Sabouraud dextrose agar (SDA) (Millipore, Ontario, Canada) plates, the inoculum was spread with a Drigalsky loop (OLEN, China), and incubated at the temperature/time recommended in table 1.

After this period, 5.0 mL of Skim Milk 10% (BD Biosciences, Le Pont de Claix, France) with glycerol 30% v/v (Merck KGaA, Darmstadt, Germany) was added to the plates and the colonies were detached with the help of Drigalsky loop.

This suspension was added to a sterile vial, homogenized, and then 2.0 mL were transferred to cryotubes (Corning, Reynosa, Mexico). Three cryotubes of each strain (working lot) were selected to verify viability, purity and morphological identity.

To verify viability, serial dilutions were performed at factor 10 (10^{-1} to 10^{-10}), and 0.1 mL of the dilutions were inoculated onto two plates of the culture media recommended in table 1.

Subsequently, the count was performed to determine the number of CFU/mL, considering the average obtained from the three cryotubes. To verify purity, seeding was performed in sheep blood agar (BioCen, São Paulo, Brazil) by the depletion technique and the plates were incubated at (32.5 ± 2.5) °C for 24 h to detect possible contaminants.

Identification of the microbial strains used

The morphological identity of the bacterial strains used in this study was verified by the observation of the colonial morphology and morphotintorial characteristics by Gram staining. The strains of *Candida albicans* and *Aspergillus brasiliensis* were evaluated by cotton blue staining.⁽¹²⁾

All strains were analyzed by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) with the equipment VITEK® MS RUO (Bio Mérieux, France) according to the manufacturer's recommendations. In addition, the strains, with

the exception of the *A. brasiliensis* strain, were submitted to phenotypic characterization using the semi-automated VITEK®2 system (Bio Mérieux, Craaponne, France).

Test to evaluate the nutritive capacity of casein soy agar associated with exposure time

The TSA plates used were from the same batch and presented 90 mm in diameter and 25 mL of culture medium per plate. The batch had previously passed the growth promotion test and sterility test (5% of the batch) according to the Brazilian Pharmacopoeia.⁽⁸⁾

From a cryotube of the working batches of each strain, dilutions were made in phosphate buffered saline (PBS, Gibco, Paisley, UK) to obtain a suspension with a concentration ≤ 1000 CFU/mL.

These suspensions were used for the test, and seeding of 0.1 mL/plate (corresponding to an inoculum ≤ 100 CFU) was performed.

For confirmation of the inoculum, 0.1 mL of the suspensions were seeded, in duplicate, in TSA at the beginning and at the end of the assay (after 5 h).

Sixty-four TSA plates were positioned inside a biological safety cabinet (BSC) type II (Thermo Scientific, Langensfeld, Germany) and the lids were removed to start time counting. After 2 h of exposure, 16 plates were closed with the covers, removed from the BSC, and in another BSC, inoculation of 0.1 mL of the working batch of each of the seven strains was performed in duplicate. The inoculum was spread evenly using a Drigalsky loop.

The plates referring to *Aspergillus brasiliensis* and *Candida albicans* were incubated at (22.5 ± 2.5) °C for 5 days, and the others were incubated at (32.5 ± 2.5) °C for 3 days. The same procedure was repeated at time intervals of 3, 4, and 5 h of exposure.

Calculation of Results Evaluation

The plates were counted and the average of the duplicates was calculated. The nutritive capacity of the TSA for each strain studied, in each evaluated period, was considered

satisfactory when the count obtained was $\geq 50\%$ in relation to the number of CFU of the control of each strain.

The percentages of recovery of the strains were statistically evaluated using the analysis of variance (Anova) single factor and differences were considered statistically significant when P-value obtained was less than 0.05. The tests were performed in Biostat 5.0 software (Federal University of Pará, Brazil, 2007).

Results

After characterization of the autochthonous strains, strain B892/21 was identified by VITEK®2 and MALDI-TOF MS as *K. oxytoca* with identification percentages of 99% and 88.8 %, respectively, while strain B1225/21 was identified as *S. epidermidis* with 99% by both methodologies. All reference strains presented the expected results.

The counts of the suspensions used as positive control, performed at the beginning and end of the assay, are shown in table 2. All strains presented initial inoculum ≤ 100 CFU, as recommended by the Brazilian Pharmacopoeia.

Table 2 – Controls counts of the strain suspensions

Strain	Initial Counting (1 h)			Final Counting (5 h)			Final average
	Plate 1	Plate 2	Average	Plate 1	Plate 2	Average	
<i>A. brasiliensis</i>	72	79	76	77	90	83	80
<i>B. spizizenii</i>	49	53	51	*	37	37	44
<i>C. albicans</i>	29	40	35	35	33	34	34
<i>K. oxytoca</i>	4	11	7	7	11	9	8
<i>P. aeruginosa</i>	48	58	53	57	60	58	56
<i>S. aureus</i>	70	82	76	73	88	81	78
<i>S. epidermidis</i>	89	95	92	78	*	78	85

* These plates were not counted due to confluent growth.

The results of the counts of each strain evaluated on TSA plates during exposure for 2, 3, 4, and 5 h are presented in table 3.

For all strains studied, at each time interval, the percentage of the count obtained was $\geq 50\%$ relative to the number of CFU obtained when counting their controls, and no significant differences were observed between the percentages of recovery ($p = 0.70$).

Table 3 – Counts of the seven strains evaluated on casein soy agar at exposure intervals of 2 to 5 h

Strain	Exposure time (h)							
	2		3		4		5	
<i>Aspergillus brasiliensis</i>	Plate 1	77	Plate 1	81	Plate 1	67	Plate 1	67
	Plate 2	92	Plate 2	67	Plate 2	89	Plate 2	66
	Average	85	Average	74	Average	78	Average	67
	%	106	%	93	%	98	%	84
<i>Bacillus spizizenii</i>	Plate 1	56	Plate 1	50	Plate 1	47	Plate 1	54
	Plate 2	71	Plate 2	48	Plate 2	46	Plate 2	41
	Average	64	Average	49	Average	47	Average	48
	%	145	%	111	%	107	%	109
<i>Candida albicans</i>	Plate 1	40	Plate 1	40	Plate 1	37	Plate 1	46
	Plate 2	50	Plate 2	62	Plate 2	60	Plate 2	32
	Average	45	Average	51	Average	49	Average	39
	%	132	%	150	%	144	%	115
<i>Klebsiella oxytoca</i>	Plate 1	18	Plate 1	13	Plate 1	2	Plate 1	10
	Plate 2	7	Plate 2	8	Plate 2	5	Plate 2	8
	Average	13	Average	11	Average	4	Average	9
	%	163	%	138	%	50	%	113
<i>Pseudomona aeruginosa</i>	Plate 1	28	Plate 1	52	Plate 1	56	Plate 1	56
	Plate 2	32	Plate 2	42	Plate 2	55	Plate 2	78
	Average	30	Average	47	Average	56	Average	67

	%	54	%	84	%	100	%	120
<i>Staphylococcus aureus</i>	Plate 1	93	Plate 1	122	Plate 1	89	Plate 1	75
	Plate 2	99	Plate 2	*	Plate 2	106	Plate 2	94
	Average	96	Average	122	Average	98	Average	85
	%	123	%	156	%	126	%	109
<i>Staphylococcus epidermidis</i>	Plate 1	143	Plate 1	144	Plate 1	164	Plate 1	83
	Plate 2	132	Plate 2	135	Plate 2	158	Plate 2	116
	Average	138	Average	140	Average	161	Average	100
	%	162	%	165	%	189	%	118

* No counting was performed on these plates due to confluent growth.

Discussion

Microbiological testing should be performed on culture media used for environmental monitoring to ensure the reliability of results that indicate whether or not an environment is within acceptable parameters for aseptic production.⁽⁸⁾

Official compendia mandate the preparation of a low-level inoculum (< 100 CFU), serving as a microbial challenge standard for the evaluation of growth promotion.^(8,13) The percentage of adequate microorganism recovery advocated by the compendia available in the literature is $\geq 50\%$ compared to initial inoculum.^(8,13,14)

The results of the present study demonstrated that recovery for all strains tested was $\geq 50\%$ for all time periods, and $> 80\%$ at a time greater than the established exposure time for the passive environmental monitoring plates (5 h).

These results demonstrated the satisfactory nutritive capacity of TSA at all time intervals evaluated, conforming to parameters determined by the compendia.^(8,13)

The percentage of recovery of the strains ranged from 50 to 189%, with the lowest and highest percentages identified in the autochthonous strains *Klebsiella oxytoca* and *Staphylococcus epidermidis*, respectively (table 3). These percentages vary because this is a microbiological counting assay, which is subject to variation due to operator influences and colony counting range.⁽¹⁵⁾ The 50 % recovery percentage in the 4 h period identified in the *Klebsiella oxytoca* strain may be associated with the lower counting range in relation to the other strains studied (table 3).

The use of autochthonous strains in growth promotion assays, cleaning validations, identification method validation, and others, is increasingly encouraged in the pharmaceutical industry, since these strains represent the real microbiota that is found on site.^(1,16,17)

In the present study, *Klebsiella oxytoca* and *Staphylococcus epidermidis* strains were selected because they are some of the most commonly isolated species in environmental monitoring of productive areas, each representing the Gram-negative and Gram-positive group, respectively.

In agreement with what is recommended by international compendia^(3,18) and Brazilian legislation,⁽⁵⁾ in the present study, the evaluation of the nutrient capacity of TSA culture media, used for passive environmental air monitoring, was tested in different time periods, and it was shown that exposure of the culture media to environmental conditions, even above the recommended 4 h, did not cause significant nutrient losses to the point of preventing or reducing microbial growth, remaining during all time intervals evaluated, within the specified $\geq 50\%$ (table 3).

Similar results to the present study were observed by *Sandle* in 2015, in a study conducted in England.⁽¹⁹⁾ The authors evaluated the effect of dissecting TSA plates under exposure to unidirectional airflow in a clean area.

The study analyzed the weight loss of the sedimentation plates, associated with the loss of fluid that can occur due to exposure, and may lead to decreased growth-promoting ability.

The results indicated that there was weight loss of approximately 6 grams on average from initial exposure to the end of the incubation period.⁽¹⁹⁾

The maintenance of growth-promoting capacity was also evaluated after exposure of the plates for more than 4 h, the time at which the plates suffered the maximum weight loss. The work was conducted using ATCC reference strains, with two more microorganisms isolated from clean areas of a pharmaceutical industry. At the end of the two experiments, it was demonstrated that all the sedimentation plates tested were able to recover $> 70\%$ of the tested strains, meeting the acceptance criteria. It is worth noting that despite the weight loss observed on the plates over the exposure time, the nutrient capacity of the TSA remained adequate, promoting microbial growth as specified.

Conclusion

The data obtained in this study showed that the nutrient capacity of the TSA culture medium used for passive environmental air monitoring remained satisfactory in the time interval from 2 to 5 h for all strains evaluated, both those recommended in the Brazilian Pharmacopoeia (n=5) and autochthonous strains (n = 2).

These results may serve to support the development of environmental air monitoring programs and also as a means of stimulating this type of study to be carried out in other pharmaceutical units.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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