Validation of a microbial enumeration method in a non-sterile product

Summary of dissertation for the degree of Master in Biological Engineering

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Abstract

The work here presented had the purpose of creating and validating an analytical method of microbial screening and enumeration in a non-sterile anti-infective product of tetracycline family. The method will be used in the analytical microbiology lab in CIPAN, to search for biological contaminations of the product. To achieve this, the chosen procedure needs to be able to neutralize the antimicrobial activity of the product without compromising microbial cell integrity.

The validation process involves the verification of the microorganism growth capacity in the presence of the non-sterile product. Therefore, after the product analysis, a reference microorganism suspension indicated on the pharmacopeia is added and, subsequently, is compared to a control assay without anti-infective product.

Several neutralizing efficacy tests were done, using the cellulose membrane filtration method and varying the culture medium composition, the volume and number of rinses and the sample division between membranes. The microorganisms used in this work were Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus aureus, where their microbial growth represents an effective neutralization. At the beginning of this work, the enumeration method used in CIPAN was already validated to Aspergillus brasiilensis and Candida albicans and, for this reason, these two reference microorganisms were not used.

These tests resulted in the proposal of an experimental procedure, which was subjected to a triple validation in order to demonstrate the robustness of the method. This way, the analytical method became validated for four of the reference microorganisms, considering it a capable way of detecting microbiological contamination from different origins.

Keywords

Microbial enumeration, Non-sterile products, Reference microorganisms, Filtration method, Cellulose membrane, Anti-infective

1. Introduction

In an industrial process there are some possible microbial contamination sources, such as water, air and the human being. A microbial contamination is particularly important in fermentation processes, because of opportunistic organisms that compete for carbon and nitrogen sources, thus reducing the process yield. These contaminations are avoided during the production and purification process, through water and air treatment. However, some products, in their composition, may contain microorganisms within specific limits. These are called non-sterile products.

CIPAN (Companhia Industrial Produtora de Antibióticos) produce and sell anti-infectious products of tetracycline family and these products need to be approved by the regulatory authorities before they can be sold to their clients. To be approved, the anti-infectious composition, physicochemical properties and the presence of microorganisms need to be known. In a way that all products are characterized uniformly, there are documents in which it is described the specific quality and quantity parameters of different products and the permitted tests to evaluate them, known as pharmacopeia. Each regulatory agency has its own pharmacopeia, but these documents don’t change from one another, because they create harmonized methods, allowing product commercialization by the same rules in different countries. The United States Pharmacopeia (USP) (1) and the European Pharmacopeia (EUP) (2) are the ones that CIPAN needs to follow, because it sells their products mainly to the United States of America and to European Union member states.

The objective of this work is to create and validate an analytical method of microbial enumeration in a non-sterile, anti-infective tetracycline family produced in CIPAN, according to USP (1) and EUP (2).

USP (1) and EUP (2) establish that it’s needed a distinction between bacteria and mycelial fungus (named moulds to simplify) and yeasts, so it’s done a total aerobic microbial count (TAMC) and a total
combined yeasts/mould count (TYMC). To determinate TAMC and TYMC the non-sterile product is inoculated in TSA (Tryptic Soy Agar) and SDA (Sabouraud dextrose agar) medium, respectively, and incubated at 30°C–35°C and 20°C–25°C, respectively.

The enumeration method can be done by directly placing the non-sterile product in the medium (pour-plate or surface-spread method) or by performing a membrane filtration, with a 0.45 µm diameter pores cellulose membrane.

The non-sterile product used in this work has antimicrobial activity. To certificate that the method can detect different types of contamination, the analytical method is subjected to a validation where five reference microorganisms are used. The method is considered valid when it allows the growth of Aspergillus brasiliensis, Bacillus subtilis, Candida albicans, Pseudomonas aeruginosa and Staphylococcus aureus to be demonstrated. These species are representative of different genus with contaminant potential through air, water or by human contact.

To demonstrate the capacity of the method to be able to grow microorganisms, three test groups are performed:

- Negative control – the solution used is tested for the presence of microbial contamination, to guarantee solution sterilization;
- Growth promotion/Control – the reference microorganisms are inoculated in triplicate, using the same analytical method. The microorganism solution volume shouldn’t have more than 100 cfu (colony forming units). The inoculation conditions for which reference microorganism are inoculum are in Table 1. After the inoculation time, an average of the results is calculated and the control limits are established (equation (1) where $\bar{x}$ is the average of the cfu).

$$\frac{x}{2} \leq \bar{x} \leq 2\bar{x} \quad (1)$$

<table>
<thead>
<tr>
<th>Species</th>
<th>Conditions</th>
<th>TAMC</th>
<th>TYMC</th>
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<tbody>
<tr>
<td>Aspergillus brasiliensis</td>
<td>Medium</td>
<td>TSA</td>
<td>SDA</td>
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<td>Temperature</td>
<td>30°C–35°C</td>
<td>20°C–25°C</td>
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<td>Inoculation time</td>
<td>5 days</td>
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<tr>
<td>Bacillus subtilis</td>
<td>Medium</td>
<td>TSA</td>
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<td>Temperature</td>
<td>30°C–35°C</td>
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<td>Inoculation time</td>
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<tr>
<td>Candida albicans</td>
<td>Medium</td>
<td>TSA</td>
<td>SDA</td>
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<td>Pseudomonas aeruginosa</td>
<td>Medium</td>
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<td>Staphylococcus aureus</td>
<td>Medium</td>
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Suitability of the counting method in the presence of product – the non-sterile product is diluted and then tested in duplicate as proposed. In the end, a microorganism solution volume is added, with no more than 100 cfu, and the plates are inoculated according to Table 1. After the inoculation time, the results average is compared with the control limits. If the results lie within the range, the method is considered validated for the detection of the microorganism in question. The diluted solution and the culture medium may contain in their composition a neutralizer agent, to suppress the antimicrobial activity. These agents are added before sterilization. The diluted volume/diluted factor can also be raised. The membrane filtration method is preferred when the antimicrobial activity needs to be neutralized. If needed, a combination of these neutralizing methods can be performed.

The non-sterile product selected to be used in this work is a tetracycline family antibiotic used in several respiratory and skin diseases and, recently, as an anti-inflammatory and immunosuppressant used in patients with cardiovascular disease, rheumatoid arthritis, cancer, etc. (3, 4, 5)

2. Related work

Presently, the method used in CIPAN to detect microorganisms in the non-sterile anti-infective is the membrane filtration method. The membrane filtration is performed under vacuum, using a system composed by a filter funnel manifolds with several filtration cups (where the cellulose membrane is placed), hosing to a dumping flask. The solution used to dilute the product is a phosphate buffer pH 7.2 solution with a neutralizer agent, named neutralizer buffer solution (STN) to text simplification. The test solution is filtrated and rinsed with STN. This procedure is performed in duplicated for
TAMC and TYMC. The membranes are placed in TSA and SDA (two membranes on each medium) and inoculated. The absence of toxicity in the cellulose membrane was tested for all the reference microorganisms, allowing the use of the membrane filtration method.

In previous validation processes, several solutions with different neutralizer agent concentration were tested in assays with and without the product to prove its efficacy and absence of toxicity. STN simultaneously allowed the neutralization of the antimicrobial activity and cell growth. The method used with this solution concentration was validated for Aspergillus brasiliensis and Candida albicans.

3. Proposed work

Since CIPAN already had a method validated for two of the five reference microorganisms, it was proposed to continue this validation work. This means that the membrane filtration method and the STN composition remained the same.

It was proposed to perform serial dilutions of 1:10, change the rinses number and volume and introduce neutralizers to the culture medium, to achieve neutralization of the non-sterile product antimicrobial activity.

The serial dilutions were executed in a way that allowed the detection of microorganisms between the established limits for the used non-sterile product (Table 2).

According to the USP (1) and EUP (2), the microbial limits can be interpreted as:
- When the microbial limit is $10^1$ cfu, the maximum count allowed is 200 cfu;
- When the microbial limit is $10^2$ cfu, the maximum count allowed is 2000 cfu.

| Table 2 – Established microbial limits (6) |
|-------------------------------|----------------|
| **Analysis** | **Limit (cfu/g)** |
| TAMC | 1000 |
| TYMC | 100 |

In this validation process KH$_2$PO$_4$ and a neutralizer agent were employed to prepare the STN, tryptone salt (TS) for the microorganisms reconstitution and ready-to-use plates of SDA, TSA and TSAn (Tryptic Soy Agar with neutralizers).

The volume of TS solution adopted in microorganisms’ reconstitution should allow less than 100 cfu per plate. The TS solution volume depends on the microorganism lot. For each lot of used microorganism, a control test is performed. The average of the control tests results is calculated and the control limits are established (equation (1)), which will be compared to the tests results.

Before the initiation of any test, a validation procedure needed to be delineated (scheme presented in Figure 1).

Initially, a non-sterile product is weighted to a sterile flask and STN is added. Then serial 1:10 dilutions are performed, until the desired concentration. The chosen diluted solution is equally divided in several filtration cups, filtrated and rinsed. The filtrated volume and rinses number and volume may change according to neutralization needs. The last rinse volume is divided in two. In the first half, a microorganism solution volume is added. To guarantee that the entire microorganism volume is removed from the filtration cup, the remained rinse volume is added. Finally, the membranes are placed in TSA/TSAn plates and incubated for 48 hours.

4. Results discussion and conclusions

Different combinations of several types of neutralization were done, and the one that proved to be the most efficient and less expensive was considered to create an analytical protocol. It was shown that the variation of the number and volume of rinses was not significant as well as the division of the test solution volume.

Both the cfu number and the results average need to be between the control limits to consider the method valid to detect the reference microorganisms.

The results for Pseudomonas aeruginosa and Staphylococcus aureus show that the adopted method allow Gram-positive and Gram-negative bacteria detection and, therefore, is valid of this reference microorganisms. Regarding Bacillus subtilis, a plate does not respect the control limits, having less cfu than
the inferior limit. Although every test recovery percentage is higher than 50% and lower than 200%, it cannot be stated that the method is capable to detect spore Gram-positive bacteria.

Different neutralizations were performed to all the microorganisms and, even so, the tests done to Bacillus subtilis were not satisfying. It can be concluded that if the product had some spore Gram-positive bacteria, the antimicrobial activity would be enough to eliminate the contamination (1, 2).

Since the validation of the method was not possible to Bacillus subtilis, the less expensive method was chosen. This is very important in an economic context, considering it will be applied in CIPAN’s microbial analytical laboratory. Since it is the method that uses less resources, it can be considered the most environmental friendly.

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