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Determination of concentration of the pseudomycelles from fermentation broth via spectroscopy and Neubauer chamber

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ABSTRACT: The present work presents an alternative technique to obtain the concentration of pseudomycelles as number of pseudomycelles/sample volume, obtained by Neubauer chamber, as function of absorbance from spectroscopy. Samples of broth from fermentation via bacteria *Streptomyces tsukubaensis* were analyzed, making it possible to obtain a linear relationship between the number of pseudomycelles/ sample volume and absorbance, with determination coefficient of 0.995. In addition, a morphological analysis of the unfermented pseudomycelles was performed using scanning electron microscopy (SEM) at the magnifications of 20, 2000, 5000, 10000 and 20000 x.

Keywords: Neubauer chamber; Pseudomycelles concentration; Spectroscopy; Absorbance; Fermentation.

1. INTRODUCTION

Bacteria of genus *Streptomyces* in fermentation process is essential to obtain tacrolimus [1-3], an important immunosuppressant recommended for therapy of the treatment of various types of transplants [4, 5] and in several autoimmune and ocular diseases [6], as well as in dermatological disorders [7]. In view of the need to purify this drug, the first processing step, after fermentation, is associated with the separation of pseudomycelles, which adds microbial biomass in its constitution. A widely used technique for separating such pseudomycelles is filtration [8-10]. However, in order to evaluate the performance of the filter or other equipment intended for separation, it is necessary to know the concentration of pseudomycelles in the broth to be subjected to filtration as well as in the filtrate [11, 12].

In the case of particulate material suspended in a liquid medium, its concentration can be obtained in the form of number of particles/sample volume, in which this sample is stored in equipment intended for such measure, such as the particle counters KL-04A (ORION Co., LTD), HIAC 9703+ (Beckman Coulter) and APSS-2000 (Particle Measuring Systems). Due to the high cost, these equipments are not always available for analysis. The counting chamber (or Neubauer chamber) is a method aimed at obtaining the number of particles/sample volume with proven efficacy, and much lower cost compared before counters mentioned [13, 14]. The great disadvantage of the counting chamber is the time to perform such counting, and the inherent complexity of the technique, especially when this counting must be done systematically. However, it is not

uncommon to find spectrophotometers in laboratories that work with biotechnological processes. Spectroscopy or spectrophotometry is a technique used in the quantification of chemical and biochemical species, based on absorption of electromagnetic radiation in the visible and ultraviolet regions from species in solution. The attenuation of the incident radiation beam by the spectrophotometer is proportional to the amount of chemical species contained in the sample [15, 16].

The objective of the present work, therefore, is to show an alternative technique to obtain the concentration of pseudomycelles, in the form of number of pseudomycelles/sample volume, from the construction of a calibration curve (for the spectrophotometer), in which presents the relationship between such concentration, from counting of particles by Neubauer chamber, and absorbance, which comes from the spectrophotometer.

2. MATERIALS AND METHODS

2.1. Materials

This work was developed at Department of Process Engineering (DEPro), School of Chemical Engineering (FEQ), University of Campinas (UNICAMP). The liquid medium used for pre-inoculation, inoculation and fermentation was based on the detailed analysis of literature works, and the components used, corroborate in three studies, in which they obtained good drug yield [9, 10, 17]. The samples analyzed were fermented broth via *Streptomyces tsukubaensis*, whose medium for fermentation consists of soy peptone, steep corn liquor, phosphate, sulfate and carbonate salts, malt and yeast extracts, glucose and maltose. Fermentation occurred in an Erlenmeyer vial of 500 mL for 144 hours, maintained at 28°C, under agitation of 130 rpm, using orbital agitator. At the end of the fermentation, an equal volume of acetone was added to the fermented broth, aiming to interrupt the fermentation process [9, 10, 18]. It should be noted that, in addition to the original composition of the fermentation medium, there was production of bacterial biomass as well as organic materials such as proteins, reducing sugars and tacrolimus drug [8, 9]. Then, the broth fermented with acetone went through the vacuum filtration process to retain the particulate material described above.

2.2. Spectroscopy

The spectroscopic analysis was performed on a spectrophotometer (NanoPhotometer UV/VIS, IMPLEN brand), whose wavelength is between 200-950 nm. Four previous tests were carried out with the sample to determine the wavelength range, respecting the wavelength range of equipment. Considering that the biomolecule of interest, tacrolimus, is detected at 210 nm. In addition, in previous studies, an important concentration of proteins was found in the fermented broth, constituting part of the pseudomycelles [9] and, in Literature, one of the methods to quantify proteins is through absorption in the ultraviolet , which is based on the fact that proteins present absorption in the region of 280 nm and that below 220 nm, the first due to the presence of amino acids in their constitution and the second due to the peptide bond presents in them [19]. The objective is to identify at which wavelength the maximum absorbance between 0 and 1 is obtained. Wavelengths equal to 200, 250, 300 and 350 nm were evaluated, resulting in absorbance values equal to 0.107; 0.444; 1.207 and 1.651, respectively. The chosen wavelength range was 200 to 300 nm, with an interval of 10 nm, the absorbances being read in this scanning range. Subsequently, sample dilutions and absorbance measurements were made for each diluted sample. The original sample was diluted until the absorbance was zero, indicating the absence of pseudomycelles, thus presenting only acetone (Absorbance = 0 $\equiv \lambda_{ultraviolet}$ = 330 nm). The prepared dilutions were 10, 15, 20, 25, 30, 35, 40, 45 and 50 times. The original sample, at each dilution, was homogenized on a magnetic stirrer bar (Fisatom, model 752

A) at 25 rpm. The tests were performed in triplicate. It should be noted that, for each fermented broth analyzed, it is necessary to perform a new procedure. In other words, the results to be presented here refer to specific fermentation conditions of this work.

2.3. Pseudomycelles count

Pseudomycelles counting was carried out with the aid of the Nikon optical microscope (Model Alphaphot - 2 - YS2) and the Neubauer chamber (Olen brand). Pseudomycelles are composed of hydrophobic and hydrophilic components. The pseudomycelles can be spherical, ellipsoid, cylindrical or unilamellar nanodimensional structures [20]. In this case, the pseudomycelles are aggregates of molecules present in the fermentation medium, such as proteins, sugars, lipids, microbial biomass etc [9, 10]. The protocol used for counting pseudomycelles in the five grid blocks of the Neubauer chamber was based on the study by [21] with modifications. In the present study, trypan blue was not used as mentioned by [21], since the aim of the alternative technique is not to distinguish viable cells. In addition, [21] used volume of 5 x 0.1 µL for counting, while the present technique, due to the differences in the measurements of the Neubauer chamber with these researchers, was applied 5 x 3.2 µL.

Pseudomycelles were counted from broth fermented with acetone (crude sample), and for each dilution prepared from this fermented broth. The crude sample aliquot was placed on the grid by touching the end of a capillary tube of the Pasteur pipet, so the sample flowed between the coverslip and the counting chamber. The chamber was positioned on the adjustable stage of the optical microscope and waited 2 min for the pseudomycelles to settle, then the microscope was focused. The procedure for handling and focusing the microscope was based on [22]. The 10 x objective was used to count the pseudomycelles. In addition, the pseudomycelles that were in upper and left limits were counted, unlike the pseudomycelles that touched the lower and right limits, which were not taken into account, according to the protocol. Due to the high concentration of pseudomycelles in the original sample of the fermented broth and at low dilutions, the zig-zag counting technique was adopted, avoiding errors in determining the number of pseudomycelles [21]. The counting chamber of the present study has 16 squares in each grid block, each with 1 mm^2 of area and depth of the chamber equal 0.2 mm. Considering the transformation of the unit from mm^3 to mL, and the volume of each grid block of the chamber equal 3.2 mm^3, the pseudomycelles counting over each of the five grid blocks were made according to:

$$\frac{(n^0 \text{ of pseudomycelles})}{mL} = \frac{(n^0 \text{ of pseudomycelles counted in the mesh})}{3.2} \times 10^3 \quad (1)$$

2.4. Morphology of fermented broth pseudomycelles

The morphological analysis of the pseudomycelles present in the fermented broth was carried out using scanning electron microscopy (SEM), in the LEO 440i equipment, at Biomass Characterization, Analytical and Calibration Resources Laboratory (LRAC), School of Chemical Engineering (FEQ), University of Campinas (UNICAMP).

Before the morphological analysis of the fermented broth pseudomycelles, the sample was pre-processed. Initially, the biomass retained in the filter papers (filter medium) with biomass were washed with deionized water and kept at 80°C for 24 hours for drying (drying and sterilization oven model 315 SE) [9]. Then, the dry biomass was inserted into the desiccator for 24 hours and taken inside a closed box for the SEM analysis.

The first stage of the SEM consisted of cutting two regions of the filter paper with scissors, small maceration in the sample, and fixing it on the sample holder with double-sided carbon adhesive tape. Afterwards, the samples were metalized with gold and taken to SEM. The magnification performed were 20, 2000, 5000, 10000 and 20000 x. Coating samples with gold is necessary, as samples may have insulating characteristics and tend to accumulate electrical charge, causing unwanted artifacts in the image. Gold improves the emission level of electrons and ground charges [23].

3. RESULTS AND DISCUSSION

In the wavelength range determined between 200 and 300 nm, the maximum absorbance, considering it less than 1, was equal to 0.979 at 280 nm. Therefore, all dilutions were read at a wavelength of 280 nm. The graph relating the number of pseudomycelles/mL vs absorbance is shown in Figure 1. Thus, through the analytical curve, other samples of the same fermented broth and more diluted (due to a filtration process, for example), can be inserted in the spectrophotometer to read the absorbance, and later obtain the number of pseudomycelles /sample volume by the Equation 2, and without using the Neubauer chamber again. The data revealed a straight line with determination of coefficient of 0.995, in the form

$$y = 894.36 + 49503x \quad (2)$$

with y as number of pseudomycelles /mL, and x, absorbance.

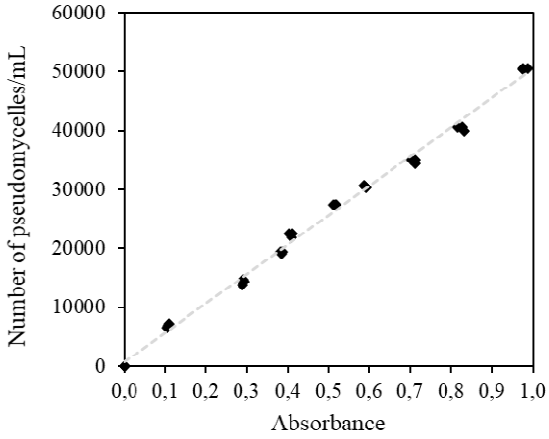

Figure 1. Determination of pseudomycelles concentration from absorbance.

Figure 2 shows the grid blocks of the Neubauer chamber during focusing the microscope for reading the samples. Figures 2a and 2b show the pseudomycelles of the fermented broth (without dilution). The number of pseudomycelles were counted in the 10 x objective (right column), because it is possible to visualize an entire grid block with good quality and distinguish one micelle from the other, in contrast to the 4 x objective (left column). Figures 2c and Figure 2d show the pseudomycelles of the fermented broth diluted 50 times. In this dilution the sample is absent from pseudomycelles. In the case of filtration, this represents total retention of pseudomycelles by the filter medium [11, 12].

In the analysis of the morphology of the pseudomycelles from fermented broth, via SEM, images were captured with micrometric dimensions, 1 and 3 μm, as shown in Figures 3 and 4. In Figure 3, the pseudomycelles are circled in red and behind, the most uniform surface, are like cellulose fibers of the filter paper. In Figure 3, a 5000 x magnification was used. Figure 4 shows the aggregate of compounds that characterize the pseudomycelles at a magnification of 10000 x. The microstructures around the micelle may have come from the maceration process during the analysis.

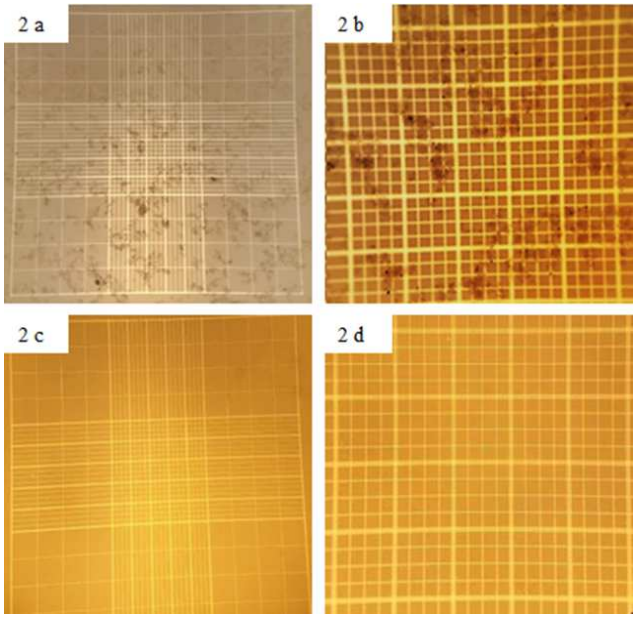

Figure 2. Observation of pseudomycelles in the optical microscope (2a) Fermented broth: 4 x objective. (2b) Fermented broth:10 x objective. (2c) Fermented broth diluted 50 times: 4 x objective. (2d) Fermented broth diluted 50 times: 10 x objective.

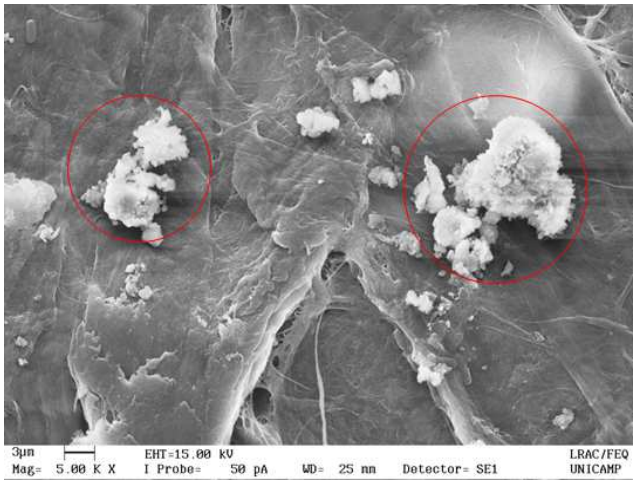

Figure 3. Pseudomycelles from fermented broth with a micrometric dimension of 3 µm and a magnification of 5000 x.

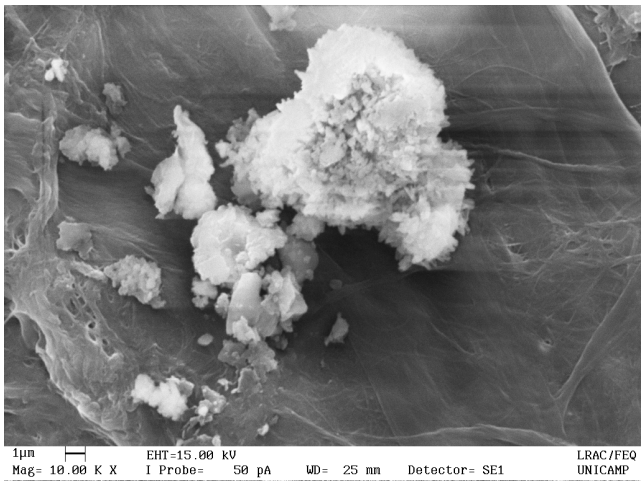

Figure 4. Pseudomycelles from fermented broth with micrometric dimension of 1 µm and magnification of 10000 x.

4. CONCLUSIONS

One of the challenges when processing a certain fermented broth is the separation of pseudomycelles, aiming at further purification of the desired component. However, in order to assess the performance of this separation, it is essential to know the concentration of pseudomycelles, particularly particulate material. There are equipments for this purpose, however they have high cost, causing the search for cheaper alternatives, made by counting particles through the Neubauer chamber. Such method, despite its low cost, proves tedious and demands time from the user, especially when used continuously. This study presents an alternative technique that associates the counting of particles through the Neubauer chamber with the spectroscopy technique, providing a substantial reduction in the analysis time to obtain the concentration of pseudomycelles, mainly for samples considerably more diluted than the original sample.

Figure 5 shows a schematic of the proposed alternative technique. Initially, the researcher assesses the appropriate wavelength for the analysis of his sample. In the second stage, the researcher counts the pseudomycelles of the fermented broth (without dilution), and after the dilutions from the original sample in the Neubauer chamber. With the same samples, perform the absorbance reading on the spectrophotometer. With data on the number of pseudomycelles/sample volume vs absorbance, the analytical curve is constructed. From this stage, it is not necessary to use the Neubauer chamber to count sample pseudomycelles from the same original broth, because the spectrophotometer is already calibrated. Therefore, in step three the researcher only reads the absorbance and obtains the number of pseudomycelles/sample volume through the equation of the straight line, already obtained.

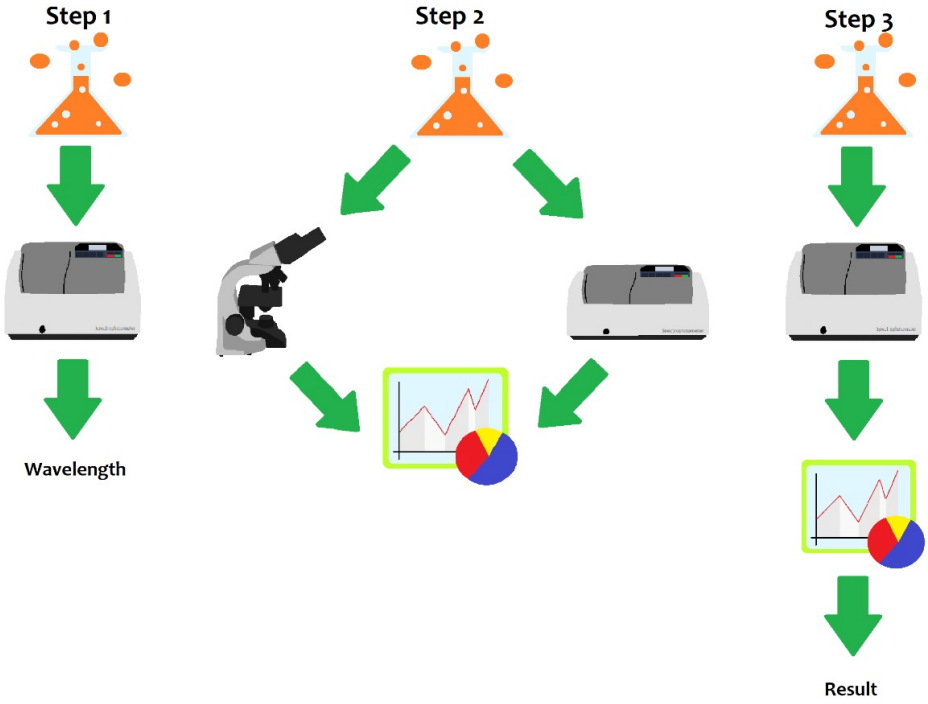

Figure 5. Scheme of the proposed alternative technique.

Authors' Contributions: ASB investigated, conducted the experiments, analyzed the results and wrote the manuscript; and MAC conceptualized, supervised the project, analyzed the results and reviewed the manuscript. The final manuscript has been read and approved by all authors.

Conflict of Interest: The author declares no conflict of interest.

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REFERENCES

1. Wong SHY, Sunshine I. Handbook of analytical therapeutic drug monitoring and toxicology. Boca Raton, CRC Press, 1996.
2. Kaplan B, Burckart GJ, Lakkis FG. Immunotherapy in transplantation: principles and pratice. Nova Jersey, Wiley-Blackwell, 2012.
3. Nakamura K. Tacrolimus (Prograf). In: Nagaoka S, ed. Drug discovery in Japan. New York, FL: Springer, 2019: 147-167.
4. Broen JCA, Van Laar JM. Mycophenolate mofetil, azathioprine and tacrolimus: mechanisms in rheumatology. Nat Rev Rheumatol. 2020; 16: 167-178.
5. Liu Y, Zhang C, Li L, Ou B, Yuan L, Zhang T, Fan J, Peng Z. Genome-wide association study of tacrolimus pharmacokinetics identifies novel single nucleotide polymorphisms in the convalescence and stabilization periods of post-transplant liver function. Frontiers Genet. 2019; 3: 1-9.
6. Erdinest N, Ben-Eli H, Solomon A. Topical tacrolimus for allergic eye diseases. Curr Opin Allergy Clin Immunol. 2019; 16: 535-543.
7. Dähnhardt D, Bastian M, Dähnhardt-Pfeiffer S, Buchner M, Fölster-Holst R. Comparative the effects of proactive treatment with tacrolimus ointment and mometasone furoate on the epidermal barrier structure and ceramide levels of patients with atopic dermatitis. J Dermatolog Treat. 2019; doi: 10.1080/09546634.2019.1708240.
8. Li Y, Liang S, Wang J, Ma D, Wen J. Enhancing the production of tacrolimus by engineering target genes identified in important primary and secondary metabolic pathways and feeding exogenous precursors. Bioprocess Biosystems. 2019; 42: 1081-1098.
9. Silva SCM, Ferrari WM, Moreira JV, Franco TT. *Streptomyces tsukubaensis* fermentation using Brazil nut oil to enhance tacrolimus production. Appl Biotechnol Rep. 2019; 6: 109-112.
10. Moreira JV, Silva SCM, Cremasco M. Evaluation of carbon:nitrogen ratio in semi-defined culture medium to tacrolimus biosynthesis by *Streptomyces tsukubaensis* and the effect on bacterial growth. Biotechnol Rep. 2020; 26: e00440.
11. Doran PM. Unit 0perations. Bioprocess engineering principles. Massachusetts, Academic Press, 2013: 926.
12. Mao N. Nonwoven fabric filters. In: Kellie G, ed. Advances in technical nonwovens. Sawston, Woodhead Publishing, 2016: 273-310.
13. Absher M. Evaluation of culture dynamics. In: Kruse Jr PF, Patterson Jr MK, eds. Tissue culture. Massachusetts, Academic Press, 1973: 896.
14. Zhang M, Gu L, Zheng P, Chen Z, Dou X, Qin Q, Cai X. Improvement of cell counting method for Neubauer counting chamber. J Clin Lab Anal. 2020; 34: e23024.
15. Sahin D. Atomic spectroscopy. In: Khan M, Nascimento GMD, El-Azazy M, eds. Modern spectroscopic techniques and applications. Massachusetts, Academic Press, 2020: 1-11.
16. Görög S. Ultraviolet-visible spectrophotometry in pharmaceutical analysis. Boca Raton, CRC Press, 2017.

17. Turlo J, Gajzlerska W, Klimaszewska M, Król M, Dawidowski M, Gutkowska B. Enhancement of tacrolimus productivity in Streptomyces tsukubaensis by the use of novel precursors for biosynthesis. Enzyme Microb Technol. 2012; 51: 388-395.
18. Jung S, Lee K, Park Y-J, Yoon S, Yoo YJ. Process development for purifying tacrolimus from *Streptomyces* sp. using adsorption. Biotechnol Bioprocess Engin. 2011; 16: 1208-1213.
19. Noble JE. Quantification of protein concentration using UV absorbance and coomassie dyes. Methods in Enzymology. Elsevier Inc, 2014: 17-26.
20. Hanafy NAN, El-Kemary M, Leporatti S. Micelles structure development as a strategy to improve smart cancer therapy. Cancers. 2018; 10(7): 238.
21. Butler M, Spearman M. Cell counting and viability measurements. In: Pörtner R, ed. Methods in Biotechnology: Animal Cell Biotechnology, Totowa, Humana Press, 2007: 205-222.
22. Murphy DB. Fundamentals of light microscopy and electronic imaging. USA, Wiley-Liss, 2001.
23. Wollman EW, Frisbie CD, Wrighton MS. Scanning electron microscopy for imaging photopatterned self-assemmbed monolayers on gold. Langmuir. 1993; 9: 1517-1520.