

Improvement of XTT Assay Performance for Studies Involving *Candida albicans* Biofilms

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2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay has been used to study *Candida* biofilm formation. However, considering that the XTT reduction assay is dependent on cell activity, its use for evaluating mature biofilms may lead to inaccuracies since biofilm bottom cell layers tend to be relatively quiescent at later stages of biofilm formation. The aim of this study was to improve XTT reduction assay by adding glucose supplements to the standard XTT formulation. *Candida albicans* ATCC 90028 was used to form 24-, 48- and 72-h biofilms. The oxidative activity at 90, 180 and 270 min of incubation was evaluated. The control consisted of standard XTT formulation without glucose supplements, and was modified by the addition of 50, 100 and 200 mM of glucose. The XTT assay with 200 mM glucose showed more accurate and consistent readings correlating with biofilm development at 24, 48 and 72 h. Biofilm growth yield after 180 min incubation, when evaluated with the 200 mM glucose supplemented XTT, produced the most consistent readings on repetitive testing. It may be concluded that glucose supplementation of XTT could minimize variation and produce more accurate data for the XTT assay.

Key Words: *Candida albicans*, biofilm, XTT.

INTRODUCTION

Candida is a commensal fungus that inhabits half of human oral cavities. However under certain circumstances, particularly in compromised host populations such as HIV/AIDS patients and organ transplant recipients, *Candida* can cause opportunistic infection ranging from superficial mucous membrane infection to life-threatening systemic diseases (1).

A major virulent attribute of *Candida* is its ability to adhere and form surface attached microbial communities known as biofilms. *Candida* biofilms are especially widespread and have been observed in most medical devices, such as stents, shunts, implants,

endotracheal tubes, pacemakers, and various types of catheters (2). Denture wearers can also be named as surface substrata. Biofilm development can be divided in four sequential steps: first, adhesion of microorganisms to a surface; second, discrete colony formation and organization of cells; third, secretion of extracellular polysaccharides (EPS) and maturation into a three-dimensional structure; and fourth, dissemination of progeny biofilm cells (3). Therefore, it is important to evaluate the dynamic process of formation and development of *Candida* biofilms in order to understand the pathogenicity and treatment of this medically important fungus.

Development of biofilm on a surface can be

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better studied by quantifying the biofilm mass along different time points. Methods such as counting of colony forming units (cfu), spectrophotometric analysis, and colorimetric assays, such as 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay, have been employed to quantify the *Candida* biofilms. However, each method carries its own limitations and drawbacks. For example, cfu count has been shown to under-represent the cell number. In spectrophotometric analyses of cell density, all cells, both live or dead, contribute to the readings, which produces an over-estimation of cell number.

XTT reduction assay has been used as a routine tool for the quantification of *Candida* biofilms (4-7) because it measures cell activity. The intracellular reduction of XTT releases a formozan compound that can be quantified by colorimetric estimation (4). At present, XTT reduction assay formulation contains three components namely XTT, menadione and phosphate buffer saline (PBS). XTT reduction depends on cell activity, instead of cell mass, but some authors have questioned its accuracy in biofilm quantification (4,6). The development of biofilms into complex spatial structures results in uneven distribution of nutrients across their multiple layers, which can lead the cell population to a heterogeneous spectrum of metabolic activity. While nutrient-rich upper layers exhibit a relatively higher metabolic activity, nutrient-deficient basal layers have been shown to have lower metabolic activity (3,5,8,9). Therefore, XTT reduction assay in mature biofilms can underestimate the cell activity of the biofilm.

Previous studies have attempted to improve the XTT assay by supplementing various ingredients to compensate for the inequalities of the metabolic activity, increasing the accuracy of biofilm quantification. Carbohydrates are the main source of energy in *Candida* metabolism. Glucose is easily metabolized by *Candida* (1) and its reasonable low cost suggests its use as a first choice. Therefore, the purpose of this study was to evaluate whether the addition of glucose supplements to the standard XTT formulation can improve the XTT reduction assay for quantification of *Candida* biofilms. The tested null hypothesis was that the supplementation of XTT formulation with glucose can improve the reliability and reproducibility of the test by providing the sufficient nutrient to the *Candida* cells across the

different biofilm layers.

MATERIAL AND METHODS

Candida albicans Strain

A reference *C. albicans* strain (ATCC 90028) was selected for this study. The identity of the isolate was confirmed by carbohydrate assimilation tests using a commercially available API 32C identification system (bioMérieux, Marcy l'Etoile, France).

Preparation of Candida albicans Suspension

Prior to each experiment, the yeast strain was cultured aerobically at 37°C for 18 h on Sabouraud Dextrose Agar (SDA) and a loopful of growth was inoculated in Yeast Nitrogen Base (YNB) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 50 mM glucose. After 18 h of incubation, the cells, which were in the late exponential growth phase, were harvested, washed twice with PBS (pH 7.2) and resuspended in YNB supplemented with 100 mM glucose. Standard *Candida* suspensions were prepared to a concentration of 10^7 cells/mL by adjusting the optical density (OD) according to the McFarland standards using a Beckman Du 530 UV/visible spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). It has been demonstrated that this cell concentration is optimal for *C. albicans* biofilm development (1).

Biofilm Development

C. albicans biofilm formation was performed as described by Jin et al. (1) with some modifications. Briefly, biofilms were grown in commercially available pre-sterilized, polystyrene, flat-bottomed 96-well microtiter plates (Iwaki, Tokyo, Japan). Aliquots of 100 μ L of standard cell suspensions of yeasts (10^7 cells/mL) were transferred into each well and incubated for 1.5 h (*adhesion phase*) at 37°C at 75 rpm in an orbital shaker (Lab-line Incubator Shaker; Elliott Bay Laboratory Services Inc., Seattle, WA, USA). After the adhesion phase, the cell suspensions were gently aspirated and each well was washed twice with PBS to remove any remaining planktonic cells, taking care not to disturb the adhered cells. In order to allow the growth of biofilm (*biofilm phase*), 200 μ L of freshly prepared YNB with

supplemented with 100 mM glucose was added to each well. The plates were incubated for 24, 48 or 72 h at 37°C at 75 rpm in an orbital shaker. At 24 h of incubation, the medium was aspirated and, biofilms were washed twice with PBS followed by addition of 200 µL of fresh medium. At different time points, biofilms were quantified using the XTT reduction assay. All assays were repeated 6 times in two separate occasions.

Oxidative Activity Assay

In order to prepare the modified standard XTT formulation, anhydrous D (+) glucose (Sigma-Aldrich Corp., St Louis., MO, USA) was dissolved in sterile PBS to obtain final concentrations of 200, 100 or 50 mM. PBS without glucose was used to prepare the standard XTT formulation.

XTT reduction assay was performed as previously described (1). XTT (Sigma-Aldrich Corp.) was dissolved in PBS at a final concentration of 1 mg/mL. The solution was filter-sterilized and stored frozen at -70°C until use. Menadione solution (0.4 mM; Sigma-Aldrich Corp.) was prepared immediately before each assay. For each assay, XTT solution was thawed on ice and mixed with menadione solution at a volume ratio of 20:1.

The biofilms were washed twice with 200 µL of PBS to remove no adherent cells. Next, 158 µL of PBS with or without glucose at different concentrations, 40 µL of XTT and 2 µL of menadione were transferred to each well of 96-well plates. The plates were covered with aluminum foil and incubated in dark at 37°C for 1.5 h, 3 h or 4.5 h. Thereafter, 100 µL of the solution was transferred to each well of new 96-well plates. The colorimetric changes were measured at 492 nm using a microtiter plate reader (Spectra-MAX 340; Molecular Devices Ltd., Sunnyvale, CA, USA).

Statistical Analysis

The statistical analysis was carried out using the SAS 9.0 software (SAS Institute Inc., Cary, NC, USA) with the significance level at $\alpha = 0.05$. The dependent variables (time of incubation, glucose concentration and biofilm development) and the independent variable (oxidative activity) were analyzed statistically. The assumptions of homogeneity of variances and normal distribution of errors were tested. As the data were not normally distributed, they were transformed by

exponentiation ($\chi^{2.4}$). ANOVA was used to test the null hypothesis of no differences among the treatments. Tukey's HSD test was then used for post-ANOVA comparisons.

RESULTS

Initial analysis for the different XTT incubation times proposed in this study (90, 180 and 270 min) showed that the longest incubation time had the highest OD values ($p < 0.001$). However, the use of 270-min incubation showed a high metabolic activity in 24-h biofilm, overestimating the OD value for this initial period. An incubation period of 180 min showed OD values consistent with increased biomass and metabolic activity. Results of 90-min incubation showed a high variance of absorbance values, indicating its lack of accuracy (Fig. 1). The incubation periods of 90 and 270 min were discontinued from further analysis due to the inaccuracy of values for reduced XTT absorbance. Thus, 180 min of XTT treatment was considered for all subsequent analyses.

Glucose concentration ($p < 0.001$) and biofilm development ($p < 0.001$) were able to interfere in the oxidative activity of *Candida* biofilms. The 180-min readings of XTT-incubated biofilms at each glucose concentration showed a statistically significant difference from control ($p < 0.001$). The addition of glucose was responsible for oxidative activity, indicating interference in the metabolic activity and resulting in

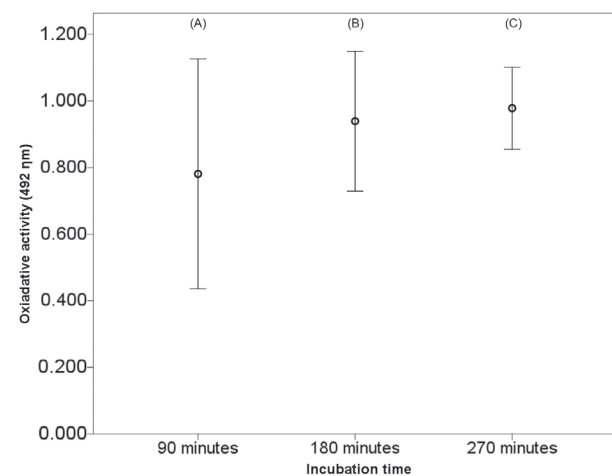


Figure 1. Biofilm oxidative activity at different incubation times.

lower coefficients of variation (control = 11.39%; 50 mM = 8.81%; 100 mM = 12.19%; and 200 mM = 6.56%) (Fig. 2).

DISCUSSION

The present study was carried out in an attempt to improve the reliability and reproducibility of the XTT reduction assay.

C. albicans biofilm can be considered in three distinct developmental layers. The basal layer of the *Candida* biofilm acts as founder cells, anchoring the developing biofilm on the substrate. The middle layer is composed of hyphae and pseudohyphae, and the most superficial layer of the biofilm consists predominantly of a thicker and porous hyphal layer with abundant extracellular matrix (3,5,8,9). During the growth phase, approximately 48 h after its formation, biofilm develops into metabolically active communities of cells that get interspersed with water channels. Biofilm structural complexity may create a gradient of environmental conditions in which the *C. albicans* cells enter distinct physiological states that differ from the planktonic counterparts (10).

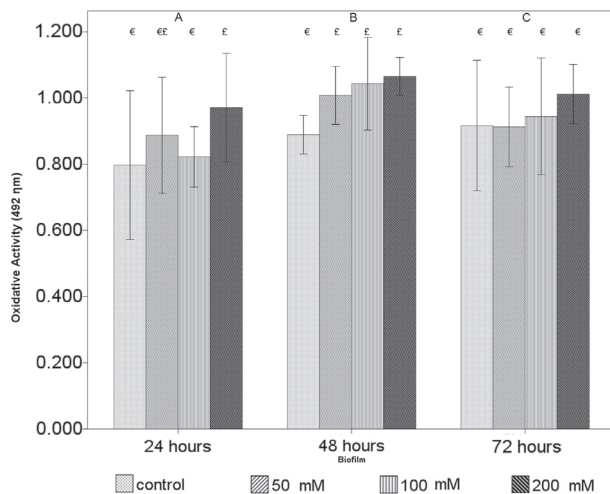


Figure 2. Oxidative activity of biofilms for different glucose concentrations. Distinct uppercase letters indicate statistically significant differences for oxidative activity among the biofilms developed at 24, 48 and 72 h ($p < 0.0001$). Distinct symbols indicate statistically significant differences for metabolic activity and glucose concentration among the biofilms developed at 24, 48 and 72 h ($p < 0.0001$).

The idea of using glucose to stimulate the mitochondrial activity of *C. albicans* biofilms derived from the hypothesis that cells in different layers undergo drastic differences concerning to oxygen and nutrient accessibility or oxidative/reductive stress. The tested hypothesis was that when not using any mitochondrial stimulus only the top layer cells, that remained exposed to outer environment, is in a position to reduce the XTT. Thus, an underestimation of the true cell content may occur. As it is well known that this yeast has a great affinity for carbohydrates (1) and that low-molar masses solutes may promptly achieve the bottom layers of biofilms (11,12) flowing through the interstitial spaces (13) and diffusing from these points, it is reasonable to suppose that more cells may consume the carbohydrate and use the enzymatic mitochondrial machinery.

None of the XTT-reduction based protocols currently used for biomass evaluation, proposes the use of glucose to enhance formazan detection. Knight and Dancis (14) proposed that the reduction of some tetrazolium salts by *C. albicans* in unbuffered conditions may be due to reductases and they used an assay buffer containing 277 mM glucose to assess the enzymatic activities. The results of the present study showed that the addition of glucose increased the detection of XTT-reduced products, the formazans, compared to the controls without any addition. Moreover, significant differences were not observed when different glucose amounts were added, though 200 mM glucose was proven to reduce the coefficient of variation, which what is desirable to improve the accuracy of the assay. It may be suggested that by adding glucose the examiner can detect simultaneously the cytosolic reductases (14) and the mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases (6).

Two questions arose during the elaboration of this idea: Is it possible that after the addition of XTT/glucose the cells in top layers started growing faster than those in basal layers, which could lead to an erroneous result? If the cells entrapped into the extrapolymeric matrix are under absence of oxygen, how could they active the mitochondrial enzymes that work in an aerobic pathway?

The first question sounds reasonable for the cells evaluated during the *adhesion phase*. However, it is believed that even those washed cells when received the XTT-glucose revealing solutions required a considerable time to pass through the "lag phase" and to enter in "log

phase". This assumption is based on the statement of Chen et al. (15) that this "lag phase" is usually attributed to the requirement for auto-stimulatory compounds that are released into the medium as the cells proliferate, but fall below some critical concentration when the cells are diluted into fresh medium. Moreover, it is opportune to consider that the XTT-glucose solutions are not *strictu sensu* culture broths.

In order to answer the second question, a previous review article (16) was revisited. The relative effective diffusion coefficient (D_e/D_{aq}) of oxygen in biofilms is near to $0.65 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, which is much higher than the $0.30 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for glucose. If it is assumed that glucose may achieve the bottom layers, it is reasonable to propose that the oxygen also achieves those microenvironments. In addition, both conditions of incubation (presence or absence of glucose) were conducted in opened-air systems, making it possible the diffusion of oxygen, whose diffusion coefficient in water at 35.5°C is $25.2 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (17). On the other hand, it is possible that aerobic respiration compromises the entire XTT reduction. It has been recently demonstrated (14) that the metalloenzymes superoxide dismutases (SODs), when present at $30 \mu\text{g mL}^{-1}$ may inhibit up to 36% of XTT reduction, although most XTT reduction is SOD-independent. Such enzymes are constitutively produced by planktonic *C. albicans* cells (18,19) and at least one isozymic form has its production increased upon the entry and during the stationary phase (20). To the best of our knowledge, there are no available data concerning to the production of SODs in *C. albicans* cells living in biofilm.

In conclusion, XTT supplementation with 200 mM of glucose and a 180-min incubation time were proven a promising modification to the existing XTT reduction assay. This XTT assay can be readily used to increase the reproducibility and reliability of the results for up to 72-h-developed *Candida* biofilms. Since XTT reduction assay is widely used for various applications in *Candida* biofilms, the suggested novel XTT formulation can be useful for further studies in this research line.

RESUMO

O teste de redução do 2,3-bis (2-metoxi-4-nitro-5-sulfopfenil)-5-[(phenylamino) carbonyl]-2H-tetrazolium hidróxido (XTT) tem sido utilizado para mensurar o desenvolvimento de biofilmes de *Candida*. Contudo, a reação de XTT é dependente da atividade celular e o seu uso para biofilmes maduros pode ser questionado,

considerando que diferentes camadas celulares têm atividade metabólica diferenciadas. O objetivo deste estudo foi avaliar se a adição de glicose à fórmula de XTT diminuiria a variabilidade na mensuração da atividade metabólica. Biofilmes de *Candida albicans* ATCC 90028 com tempos de crescimento de 24, 48 e 72 h foram utilizados. Para avaliar o melhor tempo de incubação do XTT, este foi mantido a temperatura de 37°C , em tempos de 90, 180 e 270 min. A fórmula padrão do teste XTT (controle) foi modificada com a adição de 50, 100 e 200 mM de glicose para os grupos experimentais. Os melhores resultados para a incubação foi observado com tempo de 180 min e para a suplementação de glicose à concentração de 200 mM ($p < 0.001$). Concluiu-se que a incubação de 180 min utilizando a suplementação de 200 mM de glicose apresenta resultados de atividade metabólica celular com a menor variação para o estudo de biofilmes de *Candida albicans*.

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