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Optimising a *Fusarium solani* Biofilm Formation Protocol in Vitro

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Abstract Opportunistic fungi belonging to the *Fusarium solani* have become increasingly recognised as life-threatening pathogens causing keratitis and disseminated fusariosis among both healthy individuals and patients with haematological malignancies. These infections are associated with biofilm formation on different biotic and abiotic surfaces. Considering, a biofilm is a virulence factor for causing infections, the aim of this study optimising and illustrating a simple, cost-effective and highly reproducible 96 well microtitre-based method for *F. solani* biofilm formation via using crystal violet stain. The results revealed that the possibility of using either 570nm or 595nm as a wavelength for quantifying fungal biofilm formation. The best time for crystal violet de-staining was 10 min of incubation. This model can be used in-vitro to quantify and understand the virulence factor of fungal biofilm during infections, and for antifungal susceptibility testing.

Keywords: *Biofilm formation, Fusarium solani, fungi, crystal violet, 96 well microtitre plates*

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1. Introduction

Fusarium solani is responsible for more than 60% of Fusarium biofilm-related infections (fusariosis) among immunocompromised individuals [1,2]. This infection is challenging to treat due to the ability of pathogenic fungi for developing extensive biofilm leading to enhance drug resistance properties against common antifungal treatments, also, to absorb the stress of physical and biological [3]. Since fungal plankton cells require adhesion and colonisation on a synthetic surface such catheter or host tissue, biofilm initiates and expands allowing developing a highly structured community that microbial cells are integrated and enclosed within a protective extracellular matrix [4].

Conventionally, plating methods are considered the primary way for fungal biofilm quantification via inoculation on plates and counting recovered colony forming units in vitro [5]. Besides time and media wasting, these methods have a variable range of estimation and less accuracy to assess biofilm biomass [6]. Recently, quantification of microbial biofilm mass based on staining propagules matrix using 96 well-plate microtiter-based methods have been used widely for fungal biofilm production assays as can be used for screening large scale of samples at once with high accuracy resulting in reduction the estimation errors [7,8,9,10]. In addition, these techniques offer necessary data for investigating antifungal susceptibility [11,12].

In comparing with the high volume of bacterial biofilm studies, there is a little attention has been paid to medically biofilm-related fungi [13,14].

Based on our knowledge this study is the first work that focuses on optimising a model for biofilm production in vitro for human pathogenic *F. solani* based on forming biofilm in 96 well-microtiter plates and then staining with crystal violet. This model is efficient inaccuracy, cost-effective, easy to perform for many biological assays and can be used for any fungal species including filamentous and yeasts forms.

2. Material and Methods

2.1. Source of Strain

F. solani CBS 224.34 was isolated clinically from human infection (obtained from the CBS-KNAW culture collection, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands). This isolate was grown and maintained on PDA or in PDB (PDB; Difco, 254920) 2.4% containing 2% (w/v) agar). For liquid cultures, fungal spores at a concentration of 1 x 103 ml-1, or 3 x 5 mm agar plugs taken from the leading edge of PDA cultures, were inoculated into 250 ml conical flasks containing 100 ml of PDB. Flasks were incubated with shaking (New Brunswick Scientific, USA) at 125 rpm for 3 days at 37℃. Media was autoclaved at 121℃ for 15 min prior to use.

2.2. In Vitro Biofilm Formation Model Steps

2.2.1. Growing Biofilm

F. solani (CBS 224.34) biofilms were developed on pre-sterilized flat-bottomed, 96-well microtitre plates (442404; Nunc). After spores harvesting from 3-days old PDB flask by filtration through sterile two layers of Miracloth, spore suspension was washed three times with sterile MQ water, then spore concentration at (10^6 ml^{-1}) was prepared by using haemocytometer. For biofilm formation, 200 ul of spore suspension was added into each well of the microtitre plate, then incubated statically at 37°C for the different time interval (24 and 48 hr). A minimum of 32 replicates was performed for each assay, plus 8 replicates for controls which were inoculated with PDB only.

2.2.2. Staining the Biofilm

At each selected time point, the medium, plankton and died cells were removed by aspiration and the biofilms were rinsed three times with sterile PBS by repeated pipetting to remove non-adherent cells. The microtitre plates were left to drain in an inverted position on a paper towel at room temperature (RT). After adding a 200 µl aliquot of 0.5% crystal violate (CV) into each well including the control, the plates were sealed and incubated at RT for 30 mins. The plates were again washed three times with PBS, then left inverted to drain at RT.

2.2.3. Quantifying the Biofilm

To de-stain the fungal biofilm, a 200-µl of 95% ethanol was added to each well and then incubated at RT for either 1min or 10 min. The plates were then read at two different wavelengths (570nm and 595nm) using a MRX automated microplate reader (Dynex Technologies, Billingshurst, UK).

2.2.4. Statistical Analysis

The data was analysed using the statistical programme Minitab (Minitab Express, Minitab., Coventry, UK). Statistical differences between treatments and controls were established by means of one-way analysis of variance (ANOVA). Post hoc Tukey-Kramer analysis was then performed to distinguish which groups were significantly different from one another. Results of the test were considered significantly different when p-values were < 0.05.

3. Results

The *F. solani* biofilm formation morphology was observed visually, and spore germination for hyphal development was imaged using either a Leica Laser Microdissection Microscope and epifluorescence an Olympus microscope (IX81, Visitron System, GmbH). Following initial seeding, the spore-initiated adherence on the flat bottom of wells of the microtitre plate (~ 0 – 8 hr) (Figure 1), and then processed germination, elongation and hyphal networks formation as a primary step to make a monolayer matrix of hyphae within (~8 - 12 hr) (Figure 1). Forming extensive *F. solani* biofilm with abundant extracellular matrices was observed after (24 – 48 hr), which is the stage of forming mature biofilm and conidia dispersion (Figure 1). The dense of the biofilm biomass of *F. solani* after (24 and 48 hr) on flat-bottomed well before staining with CV was noticed as shown in Figure 2.

The biofilm biomass was quantified after the two-time points (24 and 48 hr) and measured at two different wavelengths (570nm and 595nm). The result shows that there were no significant differences for quantification fungal biofilm either at 570nm or 595nm ($p>0.05$) (Figure 3).

Details of measuring Absorbance (Ab) for the different de-staining time after (1 min and 10 min) at the same wavelength either 570 and 595 nm was recorded. The result indicates that the best time for destaining crystal violet was after incubation ethanol for 10 min (Figure 4).

Considering the measuring two wavelengths, there were no significant differences noticed for Ab values of *F. solani* biofilm formation ($p> 0.05$). However, the Abs of de-stained CV after (10 min) was significantly increased compared with (1 min) at the same wavelength ($p<0.01$).

Among the assays findings, CV is a sufficient assay to evaluate fungal biofilm formation and the best de-staining time for CV was (10 min) at either (570nm or 595nm).

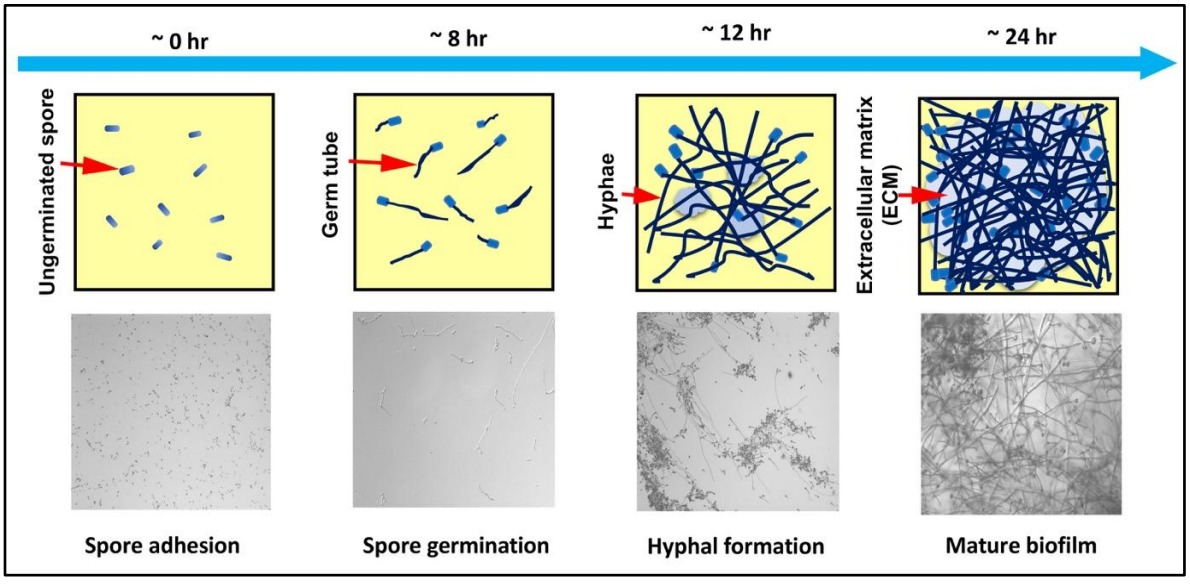

Figure 1. Schematic representation of different phases of *Fusarium solani* biofilm development from (0 to 24 hr)

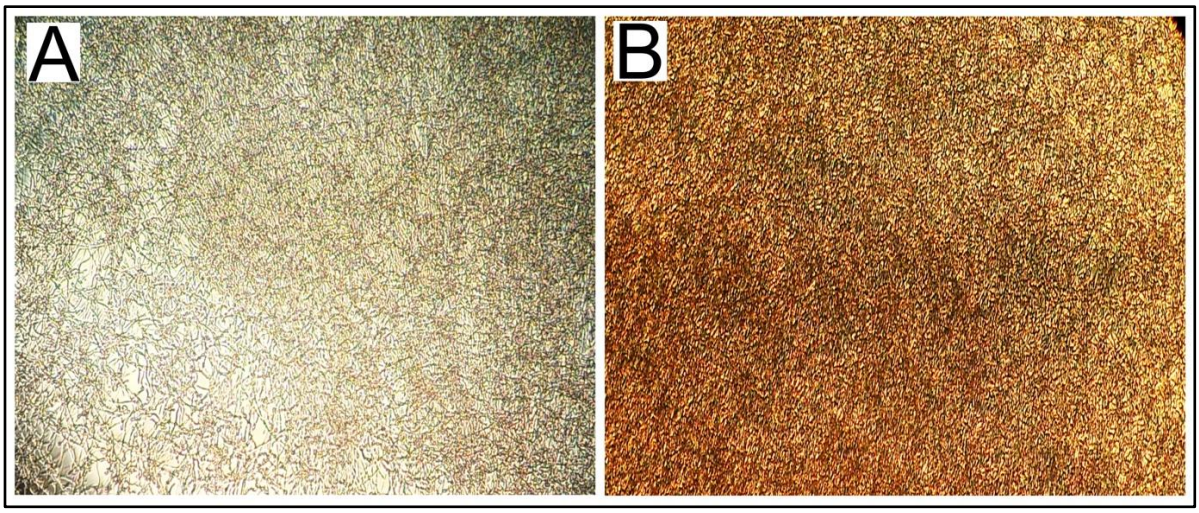

Figure 2. Development of *Fusarium solani* biofilm on a flat-bottomed well of microtitre plate before staining with crystal violet after (**A**) 24 hr, and (**B**) 48 hr

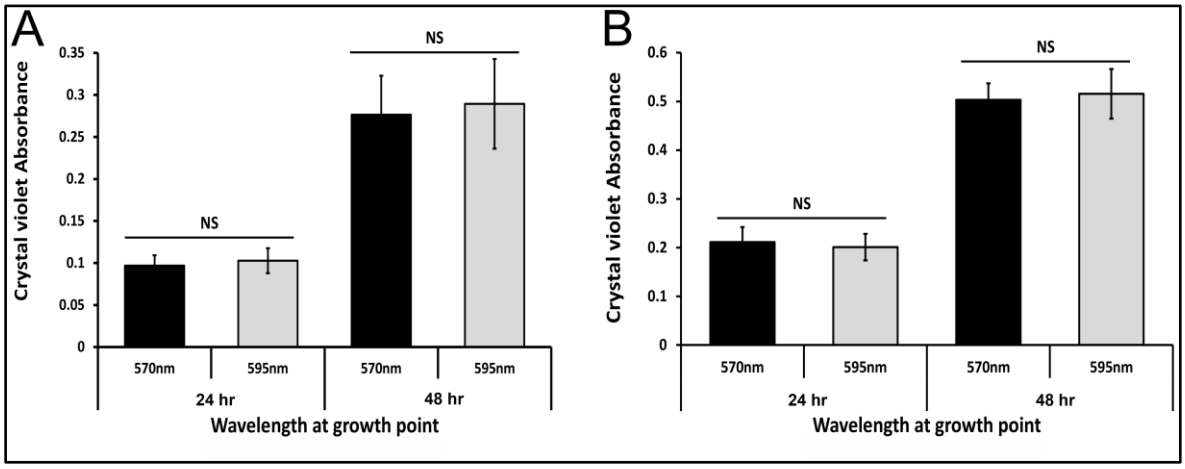

Figure 3. Comparison of two wavelengths for quantification biofilm formation. (**A**) absorbance after 1 min, (**B**) after 10 min. NS= non-significant

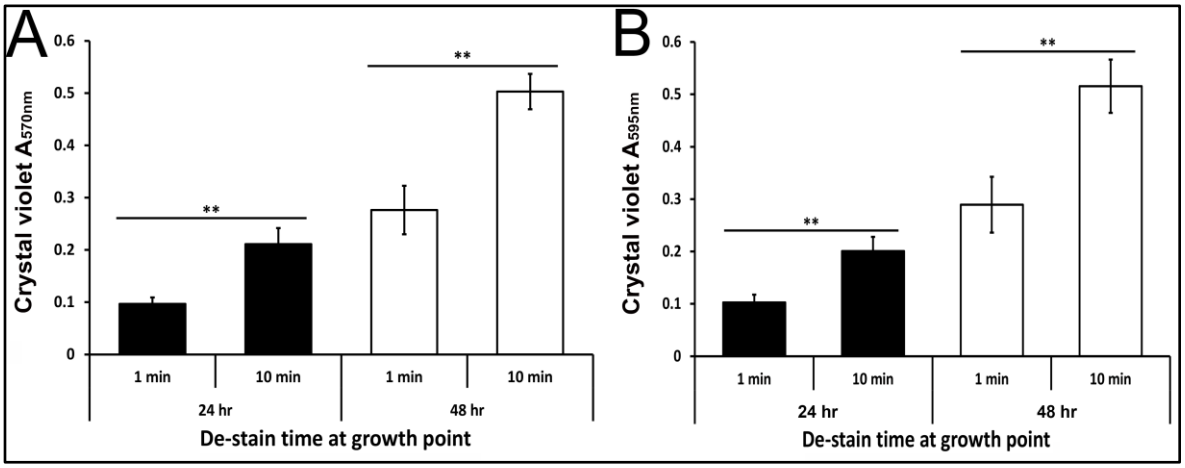

Figure 4. Comparison of destaining times (1 min and 10 min) quantification biofilm formation. (**A**) absorbance at 570nm, and (**B**) absorbance at 595nm. Significance indicate: **< 0.05

4. Discussion

To our knowledge, this is the first report of optimising a biofilm formation model invitro for the human pathogenic *F. solani* using 96-well microtitire plate and crystal violet. The fungal biofilm formation is a key step for developing fungal superficial, systemic or disseminated infections [13,15]. Biofilm architecture is considered a virulence factor that enhance the ability of pathogenicity via drug resistance particularly after developing multilayer biofilm [3,16].

Since dry weight method is unable to distinguish between dead and living propagules, our study showed CV staining method is a sufficient assay that can be used for detection only living cells after removing dead cells during washing steps. This dye binds non-specifically to negatively charged surface molecules including polysaccharides and eDNA

that located within the extracellular matrix components [17,18]. For this reason, the model of biofilm was used CV for total living biofilm quantification.

Collectively, the model that was developed in this study is reliably and can be used for quantifying *F. solani* biofilm formation with a chance to test large number of samples. The results of this study demonstrated that the *F. solani* biofilm biomass can be quantified at two different wavelengths with no differences.

The key observation of our developed biofilm model is the incubation period of ethanol for CV de-staining. The results of testing two different CV de-staining incubation times revealed that the Abs values of CV de-stained for 10 mins were more accurate and higher than short-time de-staining. The possible explanation is due to developing *F. solani* multilayers biofilm matrices which adsorbed large amount of CV stain.

5. Conclusion

In conclusion, *F. solani* is able to form biofilm through spore adherence and then germination on the flat-bottomed microtiter plate during the first 12 hr after incubation. The mature biofilm can be achieved after 24 hr. The best CV de-staining time is incubation for 10 mins. This model is simple to perform, accurate, easy to assess fungal biofilm and cost effective.

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