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# Antibiofilm activity of *Nigrospora sphaerica* CL-OP30 endophytic extract against *Streptococcus mutans* - the causative agent of dental caries

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## ABSTRACT

**Aims:** The antibiofilm activity of endophytic fungus *Nigrospora sphaerica* CL-OP30 isolated from *Swietenia macrophylla* King was investigated.
**Methodology and results:** The ability of the fungal endophytic crude extract to impede *Streptococcus mutans* biofilm formation was preliminarily screened with Congo red agar test. It was proven that *S. mutans* biofilm formation was hindered on the agar supplemented with the fungal endophytic crude extract. The antibiofilm activity of the fungal endophytic crude extract was evaluated using a microtiter plate method on both initially formed and preformed biofilm. Antibiofilm activity was recorded in a concentration-dependent pattern whereby higher concentrations reduced biofilm formation better than the lower concentrations of extract for both initially formed and preformed biofilm. The architecture of biofilm tested with fungal endophytic crude extract was also observed. Visualization under a light microscope and SEM revealed that the adherence of *S. mutans* biofilm treated with fungal endophytic crude extract was significantly reduced in both initially formed and preformed biofilm. In addition, observation under SEM showed that the matrices surrounding the bacterial cells were disintegrated and bacterial cells in biofilm completely lost their original shape. The overall data demonstrated that the ethyl acetate *N. spaherica* CL-OP30 crude extract showed good antibiofilm activity.
**Conclusion, significance and impact of study:** The antibiofilm study suggested the potential of *N. sphaerica* CL-OP30 crude extract against *S. mutans* biofilm by disrupting the biofilm formation, the disintegration of matrices surrounding the biofilm and responsible for the formation of irregular cell shape. This extract may have a promising potential to be developed as an antibiofilm agent.

*Keywords:* Antibiofilm, endophytic extract, *Nigrospora sphaerica*, *Streptococcus mutans*

## INTRODUCTION

The ability of an antibacterial agent with antibiofilm potential would be a better substitute as a therapeutic agent to combat dental caries. Therefore, exploring bioactive compounds with an antibacterial and antibiofilm properties is necessitated (Hussin *et al.*, 2018; Madani *et al.*, 2018). Endophytic fungi and bacteria reside uniquely in the plant tissue, either all or part of their life cycle, without causing any apparent damage to the host plant (Strobel and Daisy, 2003; Vaseeharan and Ramasamy, 2003). They serve as sources of novel bioactive metabolites (Shukla *et al.*, 2014; Tan *et al.*, 2016). In other words, endophytes act as a chemical synthesizer in nature to produce bioactive compounds with high pharmaceutical properties. Bioactive compounds such as terpenoids, lactones, phenols, alkaloids and many others are produced by endophytes (Deshmukh *et al.*, 2015).

The *S. mutans*, a gram-positive, alpha haemolytic streptococcus, is the resident of normal flora in the oral cavity (Vignesh and Geetha, 2018). This bacterium is known to be the major cariogenic agent in the oral cavity. It has diverse virulence factors associated with its carcinogenicity, such as acidogenesis, acid tolerance, biofilm formation and the ability to synthesize glucans from sucrose by glucosyltransferases. Having glycocalyx coating helps *S. mutans* to adhere to teeth surfaces easily (Klein *et al.*, 2015). Dental caries is a prevalent disease regardless the age and everyone is prone to dental caries all over their lifetime (Selwitz *et al.*, 2007). Bacteria are

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the primary etiological agent in the progression of periodontal disease (Kuang *et al*., 2018; Lau *et al.*, 2019). The multifactorial reasons contributing to dental caries are diet habits, consumption of food high in sugars, biofilm formation, mineralization of dentin from the *S. mutans* action and a few others (Selwitz *et al.*, 2007).

Biofilm is generally known as an established complex structure comprising an association of microorganisms enclosed in the exopolysaccharide matrix and attached to surfaces (Mah and O'Toole, 2001). Besides, biofilm acts as a diffusion barrier against antimicrobial compounds. Most bacteria exist in nature in the form of biofilm rather than planktonic cells and biofilm is the preferred form of life. Living in enclosed biofilm matrices is also a form of survival by bacteria (Krzyściak *et al.*, 2014). The looming threat is that the removal of biofilm is notoriously tricky due to the fact that the strong attachment of biofilm usually requires more than 1000 times antibacterial action compared to planktonic cells (Kalia, 2014; Wu *et al.*, 2015).

In the continual search for new antibiofilm compounds, the focus has been shifted to studying secondary metabolite production by the endophytes. Endophytes are an endosymbiotic group of microorganisms that are associated with plants (Jia *et al.*, 2016). They are prokaryotes (bacteria and archaea) and eukaryotes (fungi, algae and amoeba) that live invisibly inside the host plant without causing any apparent symptoms. The close association between endophytes and host plants is believed to be directly or indirectly involved in the production of secondary metabolites and other phytochemical constituents (Jia *et al.*, 2016). Endophytes are known to play an important role in the well-being of host plants, particularly in the interactions with plant pathogens and the production of important secondary metabolites (Tarnecki *et al.*, 2017; Sharma *et al*., 2020; Ahmad *et al.*, 2021).

According to a systematic review of the association between fungal endophytes and the host plant by Jia *et al.* (2016), the long period of co-evolution between fungal endophytes and the host plant has significantly influenced the production of secondary metabolites products. For instance, the production of billion dollars anticancer drug taxol by *Taxomyces andreanea* which is an endophytic fungus isolated from *Taxus breviflora* (Stierle *et al.*, 1993). In Malaysia, there are different fungal endophytes that have been isolated from various medicinal plants. Taufiq and Darah (2019) have isolated the endophytic fungi *Lasiodiploidia pseudotheobromae* from the medicinal herb *Ocimum sanctum* Linn. This endophyte is reported to have a plethora antibacterial and antifungal activities. In addition to that, the endophytic fungi *Ceratobasidium ramicola* was isolated from *Curcuma mangga* Valeton & Zijp reported to contain strong anti-candidal activity (Mu'azzam and Darah, 2019; Abdullahi *et al.*, 2020; Abdullahi *et al.*, 2021). Therefore, it is believed that nature has been the limitless source in the search for new healing compounds and there is very abundance in nature waiting to be explored.

Understanding the relationship and production of bioactive metabolites by fungal endophytes could benefit many fields. The usage of plant-associated antimicrobial agents could serve as an effective alternative treatment against oral infections. The ultimate intention has always been to isolate secondary metabolites from endophytic fungi that have favourable anti-biofilm activity without unnecessary side effects at the chosen concentration. By studying the fungal endophytes and culturing the endophytes on a lab scale, we could replace the traditional way of producing drugs depends on natural medicinal plants (Jia *et al.*, 2016).

Therefore, the purpose of this study was to discover highly effective antibiofilm extracts against the caries pathogen *S. mutans* and to establish whether such endophytic extracts could be used to compromise the pathogenic potential of *S. mutans* without jeopardizing human health. Findings from this study can provide a basis for the antibiofilm potential of fungal endophytic extract and the development of novel anti-caries agents.

## MATERIALS AND METHODS

### Culture and maintenance of endophytic fungus

The endophytic fungus *Nigrospora spaherica* CL-OP30 was previously isolated from the leaves of *Swietenia macrophylla*. A pure endophyte culture was then deposited at the Industrial Biotechnology and Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang. The endophytic fungus was cultured and maintained on Yeast Extract Sucrose (YES) agar and stored at the 4 °C prior to usage. Weekly routine culturing and maintaining the endophytic fungal culture was done to ensure the viability of the culture.

### Test microorganism

The pathogen of dental caries (*S. mutans* ATCC 25175) was provided by IBRL culture collection. The test bacterium was grown on Brain Heart Infusion (BHI) agar at 37 °C with 5% $CO_2$ for 24 h prior to inoculum preparation. The bacterial suspension was prepared by picking 4-5 colonies of bacteria and aseptically transferred into 5 mL of 0.85% sterile physiological saline (w/v). The turbidity of bacterial suspension was compared, corresponding to 0.5 McFarland standards (approximately $1 \times 10^8$ CFU/mL) (Zarkasi and Nazari, 2018).

### Inoculum preparation

Preparations of inoculum were carried out as described by Burmølle *et al.* (2006) with some modifications. The *S. mutans* was grown on BHI agar as described previously. Then, a few colonies were aseptically picked and transferred into a 250 mL Erlenmeyer flask containing 50 mL of BHI broth and incubated for 24 h at 37 °C in a

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shaker with 150 rpm agitation (Halim *et al.*, 2020). The culture was centrifuged at 2000× *g*, 4 °C for 30 min to separate the supernatant and cell pellets. The cell pellets were collected and gently mixed with 20 mL freshly prepared BHI broth. The bacterial culture was re-centrifuged and cell pellets were once again collected and re-suspended in 10 mL freshly prepared BHI broth. The final cell suspension was adjusted to 0.15 at 600 nm wavelength by diluting with sterile BHIB.

**Congo-red agar method**

A simple phenotypic characterization method to evaluate the ability of biofilm producer was done using the Congo-red agar method adopted and modified from Freeman *et al*. (1989) and Arciola *et al*. (2002). The differential medium, Congo-red agar medium contains BHI agar added with 36 g/L of sucrose and 0.8 g/L of Congo red dye. Test bacterium was streaked on the Congo-red agar and incubated at 37 °C for 24 h. The biofilm producer's colonies will appear as black/almost black crystalline colonial morphology whilst the non-biofilm producer will absorb the Congo-red dye colour and appeare red.

**Crude fungal extract preparation**

The preparation of the extract method was adopted from previous studies (Tong *et al*., 2011; Taufiq and Darah, 2019; Ito *et al.*, 2020). Firstly, two agar plugs with 1.0 cm diameter were excised from 7-day old pre-grown culture plate and transferred into 250 mL of Erlenmeyer flask containing 100 mL of YES broth. Cultures were incubated in dark conditions for 12 days at 30 °C. The YES broth was prepared by dissolving 20 g/L yeast powder (Difco), 40 g/L sucrose (Hi-media) and 0.50 g/L magnesium sulphate into 1000 L of distilled water. After 12 days of the incubation period, fungal fermented broth and biomass were separated using Whatman No. 1 filter paper. The fermented broth was collected and extracted thrice with an equal volume of ethyl acetate (1:1, v/v). The upper organic phase produce during extraction was collected and concentrated using a rotary evaporator to produce ethyl acetate crude extract. Ethyl acetate was used as the choice of solvent to extract the fermented broth. Extraction with ethyl acetate was considered the best choice because it can extract compounds with a wide range of polarities and is easy to be removed from the extract.

**Antibiofilm assay**

Antibiofilm assay was performed according to Burmølle *et al*. (2006) using crystal violet assay and read using a microtiter plate reader. The effect of crude extract was evaluated on initially formed biofilm which was grown together with the extract and preformed biofilm, which was grown 24 h prior to the inoculation of crude extract. A 100 µL of bacterial suspension was inoculated into a sterile 96-well microtiter plate and treated with extract of various concentrations ranging from 0.02 mg/mL to 10.00 mg/mL. For preformed biofilm, 100 µL of bacterial suspension was inoculated into the well containing 100 µL of sterile BHI broth and incubated statically for 24 h at 37 °C. After the incubation period, the suspension was gently aspirated out and washed using sterile Phosphate Buffer Saline (PBS) solution twice to thoroughly removed unattached biofilm. Then, 100 µL of freshly prepared BHI broth was inoculated into the well together with 100 µL of the extract. Controls were included as media control (200 µL of sterile BHI broth), growth control (100 µL of bacterial suspension and 100 µL of BHI broth) and background control (100 µL of extract of each concentration and 100 µL of BHI broth).

**Quantification of biofilm**

Biofilm was quantified using methods described by Xiao *et al*. (2007) and Merritt *et al*. (2011). Content in each well was gently sucked out using a micropipette to avoid disrupting the biofilm. Then, the 96-well plate was placed in a large beaker containing sterile PBS solution by leaning over a plate to the beaker at around 45° angle to wash biofilm. This step was done twice. The plate was then heating fix for 1 h in 60 °C oven. Next, 150 µL of 0.06% crystal violet solution was pipetted into each well and left for 15 min. Crystal violet solution was discarded from each well by gently pipetting out the content and the plate was rinsed thoroughly using sterile distilled water twice. The plate was left air dry with the lid open at room temperature (30 ± 2 °C) overnight to allow complete drying. Next, 200 µL of 30% acetic acid was pipetted into each well to solubilize stained cells and left at room temperature for 10 min. The plate was read using a microtiter plate reader (Thermo Scientific) at 570 nm wavelength and readings obtained were recorded. The experiment was performed in triplicates. The optical density (OD) was measured at 570 nm and the results were calculated based on the formula given below:

Percentage of inhibition = [(OD of growth control - OD of sample)/OD of growth control] × 100%

**Light microscopy (LM) and scanning electron microscopy (SEM)**

Qualitative analysis on biofilm was analysed using a light microscope adopting a method from Sayem *et al*. (2011) with some minor modifications. The stained biofilm structures were viewed and photographed. Then, the detrimental effects of ethyl acetate crude extract on the biofilm architecture were observed using SEM. Biofilm was previously grown and treated according to previously described methods. Samples for SEM observation were conducted following the method provided by the School of Biological Science USM. Samples were sputtered in gold using a sputter coater machine (Fison SC-515, UK) and viewed under scanning electron microscope (Leica Cambridge, S-360, UK).

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**Statistical analysis**

All the experiments were performed in triplicates (n=3) and the experimental results were expressed as mean ± standard deviation (SD). The antibiofilm activity of initial and preformed biofilm was analysed using one-way ANOVA using SPSS Version 18. The results were considered statistically significant if $p<0.05$.

## RESULTS

**Primary screening for biofilm production**

*Streptococcus mutans* biofilm formed on Congo-red agar media were showed as in Figure 1a. They produced very-black colonies (Figure 1a) in reference to the colourimetric scale by Arciola *et al*. (2002). This bacterium appeared to be a normal biofilm producer with a black and dry crystalline colony's structure. Meanwhile, the biofilm colony on the media containing crude extract (Figure 1b). *Streptococcus mutans* produced red-colour colonies, indicating the inability of bacterial cells to form a biofilm (Figure 1b). The ability to form a biofilm was hindered with the addition of crude extract into the culture media. Thus, it can be confirmed that fungal endophytic crude extract can potentially control biofilm formation.

**The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the fungal endophytic crude extract against *S. mutans***

The MIC value of the fungal extract against *S. mutans* is 1.25 mg/mL and the MBC value is 2.50 mg/mL. The extract is considered bactericidal against *S. mutans*.

**Antibiofilm assay of initial and preformed *S. mutans* biofilm**

The initial and preformed biofilm of *S. mutans* inhibition percentage versus different concentrations of fungal endophytic crude extract in a concentration-dependent pattern were shown in Figure 2. The inhibition pattern was observed to be slightly higher in initial biofilm as compared to preformed biofilm samples (Figure 2). Highest percentage of inhibition for both initial and preformed biofilm was recorded at the highest extract concentration, 10.00 mg/mL. At this concentration, the growth of initially grown biofilm was 88.81% inhibited, while in preformed biofilm, only 78.87% was reduced. At the lowest extract concentration tested (0.078 mg/mL), the extract exhibited poor inhibitory activity for both initial and preformed biofilm, which was only at 4.27% and 7.41% inhibition, respectively. The inhibition rate for 0.156 mg/mL to 2.5 mg/mL were almost similar for both initial and preformed biofilm ranging from 24% to 51%. It should also be noted that for initial biofilm when treated with 5.0 mg/mL extract, the inhibition percentage was doubled at 84.2% as compared to the concentration at 2.5 mg/mL. Meanwhile, the percentage of inhibition at 5.0 mg/mL and 10.0 mg/mL for preformed biofilm was steadily increased.

**Microscopic visualization of biofilm under light microscopy (LM) and scanning electron microscopy (SEM)**

The light microscope (LM) images only demonstrated the intensity of biofilms which were stained using crystal violet, while scanning electron microscopy (SEM) images showed a better resolution on biofilms architecture. The light microscopy images of *S. mutans* biofilm on the glass coverslip were shown in Figure 3. The control biofilm for initial and preformed biofilm sequentially also showed in Figure 3a and Figure 3c. Meanwhile, the image of treated initial biofilm on the coverslip and image of treated preformed biofilm were visualized in Figure 3b and Figure 3d respectively. Both images of control biofilm show the abundance of microcolonies forming on the coverslip that are densely stained with purple crystal violet. For initial biofilm, biofilm formation was drastically reduced when treated with the fungal endophytic crude extract. Similarly, the growth of preformed biofilm was also reduced when biofilm was exposed to extract. As distinctly shown in Figure 3, the preformed biofilm was less likely to be disturbed in comparison to the initial biofilm. Overall, the

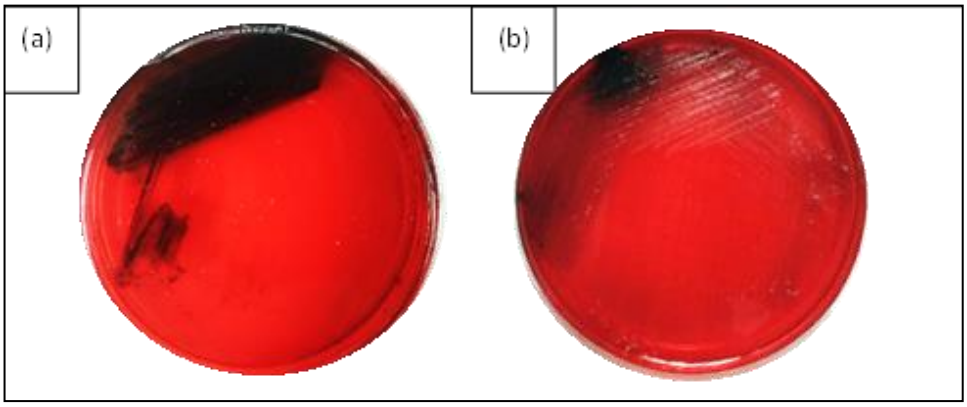


**Figure 1:** a) Colony of *S. mutans* biofilm on Congo-red agar media; b) The biofilm of *S. mutans* on Congo-red agar media with the addition of fungal endophytic crude extract.

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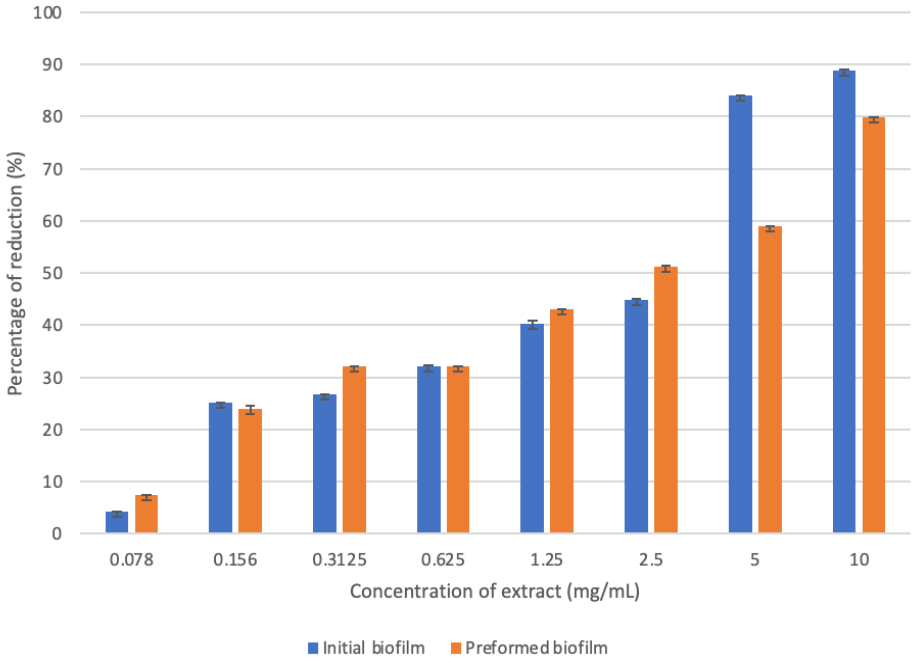


**Figure 2:** The anti-biofilm activity of the *N. sphaerica* CL-OP30 ethyl acetate crude extract against initial and preformed biofilms of *S. mutans*. The vertical axis indicates the percentage of reduction (%), while the horizontal axis shows the concentration of extract (mg/mL) used for the treatment of both initial and preformed biofilm.

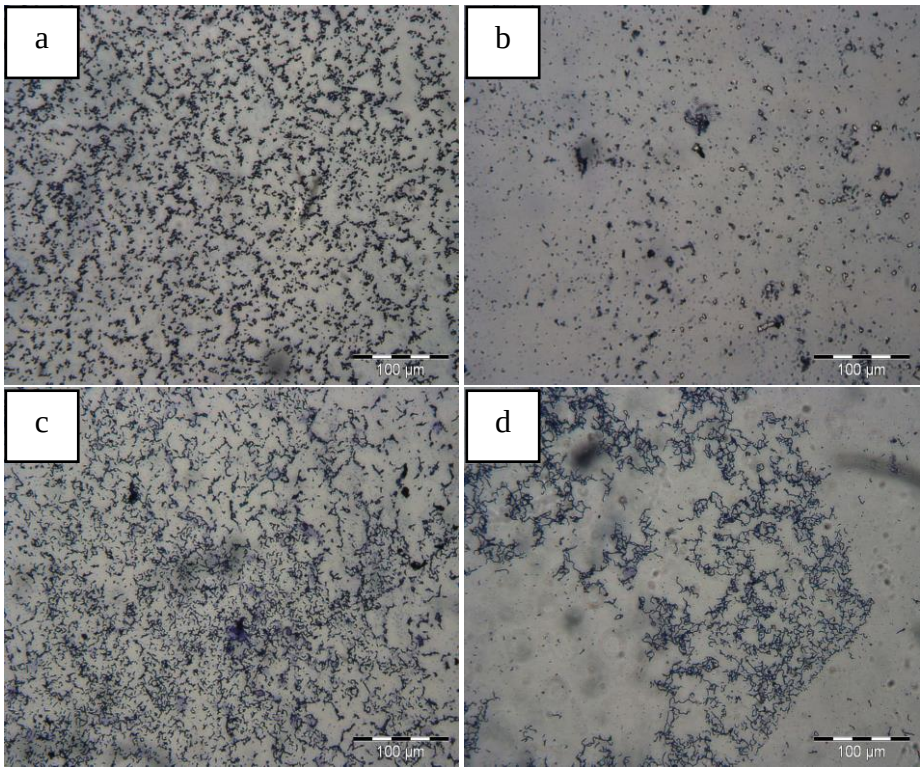


**Figure 3:** Light microscopic images of *Streptococcus mutans* biofilm on coverslip treated with ethyl acetate crude extract of *N. sphaerica* CL-OP30, under 200× magnification. a) control initial; b) initial treatment; c) control preformed; and d) preformed treatment.

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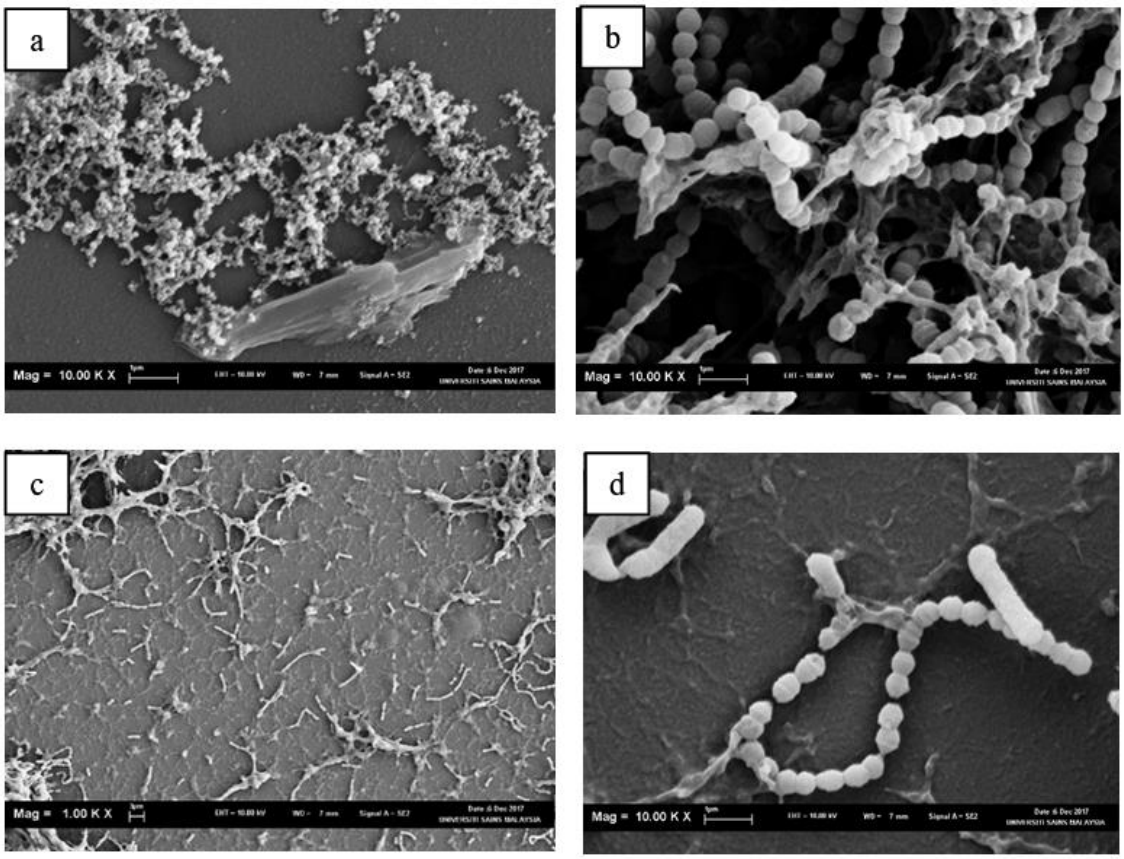


**Figure 4:** Scanning electron microscopy images of *S. mutans* initial biofilm. (a) control initial biofilm at 1000×; (b) control initial at 10000×; (c) treated initial biofilm at 1000×; (d) treated initial biofilm at 10000×.

light microscopic observation of biofilm inhibition correlated with the results obtained in the antibiofilm assay using microtiter plate, showing that the crude extract of *N. sphaerica* CL-OP30 was able to inhibit the biofilm formation by reducing the adhesion of the biofilm to the coverslip.

The SEM images allowed visualization of bacterial cells in three-dimensional (3D) structure, as shown in Figure 4a and Figure 4d under 1000× magnification and 10000× magnification, respectively. The result shows the abundant of biofilm was surrounded by matrices (Figure 4a) and in higher magnification, it was observed that cells in the biofilm were densely arranged in the chain and in normal cocci shape glued by matrices (Figure 4b). Cells were flawlessly undamaged and shows significant reduction of bacterial biofilm (Figure 4c). At higher magnification, some cells were deformed and enlarged as the result of the addition of extract (Figure 4d). This deformative bacterial shape showed that crude extract has probably affected the cell division process with the cell.

Robust biofilm formation with multilayers of cells was observed on the control biofilm under lower magnification (Figure 5a). Control biofilm at higher magnification shows clumping of complex bacterial cells in biofilm surrounded by matrices (Figure 5b). After treatment with fungal endophytic crude extract, lower magnification biofilm shows that *S. mutans* biofilm were dispersed (Figure 5c) and cells were loosely attached to slide compared to the control (Figure 5a). Meanwhile, at higher resolution, cells surface becomes remarkably disintegrated (Figure 5d). Irregular boundaries were observed, the margin of the cell wall was unclear, and cells were observed to be fused into one another resulting in a long chain of cells. The treated cell has completely lost its original cocci shape. Results obtained also concluded that the initial biofilm was greatly affected by the addition of fungal endophytic crude extract in comparison to the preformed biofilm. Overall, the microscopy images validated the significant inhibitory antibiofilm effect of fungal endophytic crude extract on the *S. mutans* biofilm. The results were in accordance with antibiofilm assay and light microscopy micrographs.

## DISCUSSION

The ability of the bacterial strains to produce biofilm was directly assessed using Congo-red agar method. It is a straightforward and simple method to detect biofilm formation. This method was adopted from Freeman *et al*.

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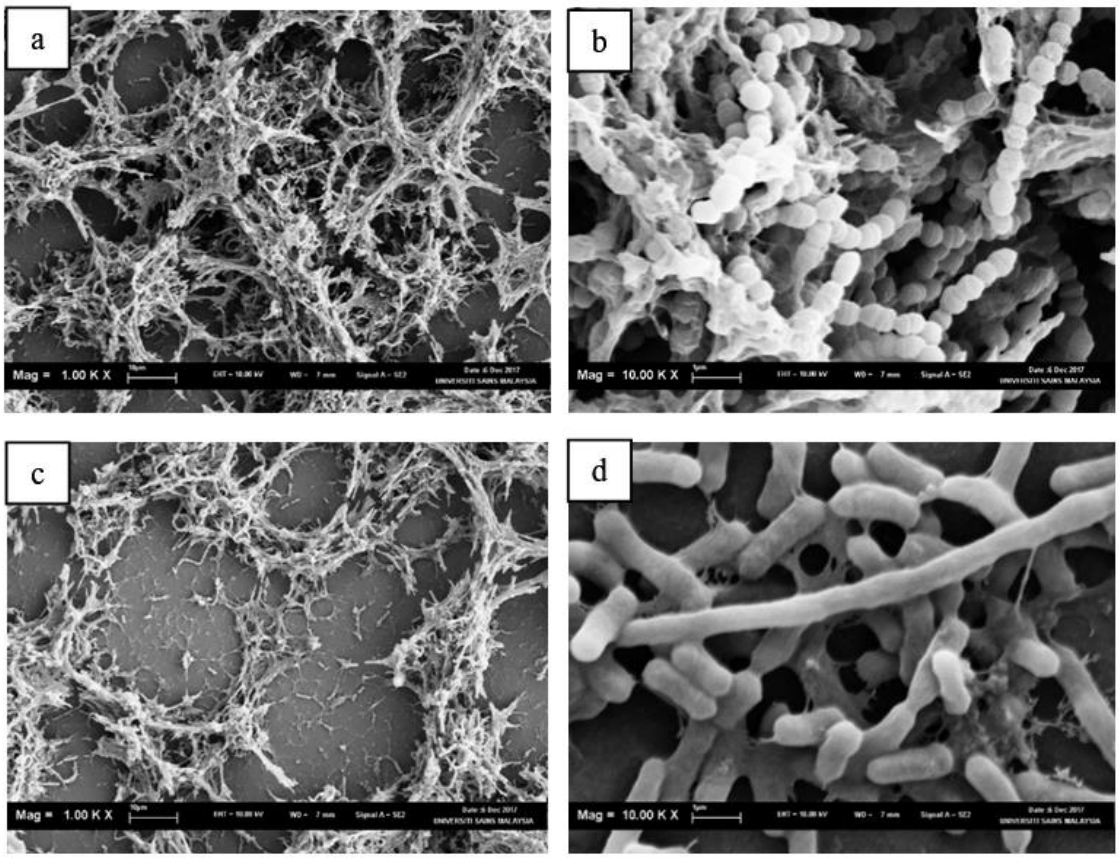


**Figure 5:** Scanning electron microscopy images of *S. mutans* preformed biofilm. (a) control preformed biofilm at 1000×; (b) control preformed at 10000×; (c) treated preformed biofilm at 1000×; (d) treated preformed biofilm at 10000×.

(1989) and modified to suit the present study. If the bacterial strain is capable to form biofilm, they will form black and dry crystalline colonies on the agar (Soesilo *et al.*, 2021). Unfortunately, if biofilm formation is impeded, bacterial strain will produce red-pinkish colonies. This is due to the interaction between polysaccharides produced by the bacteria and Congo Red dye in the agar (Kumar *et al*., 2012).

Based on this study, *S. mutans* is considered a normal biofilm producer as it can grow and produce black colonies on the control agar. Findings by Ambulkar (2019) also confirmed that *S. mutans* and other *Streptococcus* species have shown moderate to a strong ability to form biofilm. Unfortunately, *S. mutans* strain was not able to produce black colonies on the agar containing the crude extract. Therefore, this fungal extract is considered to contain antibiofilm properties as it can impede the production of exopolysaccharides in the *S. mutans* which was indicated by the absence of black and dry crystalline colonies. Similarly, Kalishwaralal *et al.* (2010) found the antibiofilm effect of silver nanoparticles. *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* grown on CRA with silver nanoparticles in the medium appeared as red, non-crystalline, whereas on CRA without silver nanoparticles appeared as black colonies.

Besides that, Kim and Park (2013) also adopted the method by Freeman *et al*. (1989) when studying the effect of ginger extracts on *P. aeruginosa* biofilm. Addition of this ginger extract somehow correlated with the inhibition of the production of exopolysaccharides. Biofilm was reported to reduce by 39%-56% when the ginger extract was added. Decrease in exopolysaccharide reduction was also shown by an electron microscopy study. Overall, it is concluded that the CRA method works as a good primary screening method in detecting biofilm producers (Rewatkar and Wadher, 2013).

The inhibition pattern of the pre-formed biofilm was less effective compared to the initial biofilm. As expected, the adherence of pre-formed biofilm to the surface is stronger considering that pre-formed biofilm (Dosler and Kaaraslan, 2014). The pre-formed biofilm was given ample time to multiply in the gooey protective sheaths and adhere tightly to the surface before fungal endophytic crude extract was inoculated (Khan and Ahmad, 2012). Once the biofilm is tightly attached to surfaces, it is tough to remove. It was also observed that biofilm removal was considerable in a dose-dependent manner (Abraham *et al.*, 2011). A study by Kim *et al.* (2020) also suggests the inhibition of *S. mutans* biofilm by positing a dose-dependent pattern. Also, they showed that the amount of

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pre-formed biofilm was less likely to be reduced compared to the initially formed biofilm.

In this antibiofilm study using microtiter plate assay, it is observed that the fungal endophytic crude extract is proved to have dual actions in preventing biofilm formation and reducing the growth of preformed biofilm. Similar dual actions potential was also observed by Teanpaisan *et al.* (2017) in studying the anti-biofilm activity from *Piper beetle* extracts. According to Kuang *et al.* (2018), the typical biofilm life cycle consists of a few stages. It started with attachment, growth/maturation and ended with biofilm being dispersed. Similarly, Kaur *et al*. (2020) also observed that in general preformed biofilm was less likely to be reduced compared to initial biofilm. This phenomenon shows that biofilm polysaccharide restricts the diffusion of antibiofilm drugs into the biofilm matrices (Mah and O'Toole, 2001).

To corroborate the qualitative and quantitative data of fungal endophytic crude extract on the *S. mutans* biofilm, the antibiofilm study was validated using a microscopy approach (Salta *et al.*, 2013). The light microscope (LM) and scanning electron microscopy (SEM) images help to observe how severe the damages of cells in biofilm and extra polymeric (EPS) substances with the extract treatment as well as to study the ability of extract in inhibiting adherence of bacterial biofilm on a glass surface.

There are many strategies to combat bacterial biofilm thru various modes of action (Roy *et al.*, 2018). Based on this finding, the fungal endophytic crude extract was shown to be able to promote the disintegration of *S. mutans* biofilm by reducing the attachment to the surface, which can act as a crucial preventive measure to eradicate biofilm formation. When viewed under a light microscope, the fungal endophytic crude extract has greatly reduced the biofilm attachment, especially in the initial biofilm. However, it is obvious that preformed biofilm is less likely to be inhibited by the fungal endophytic crude extract. It was observed that following treatment with extract, the dispersal of biofilm was increased and adherence of biofilm to the glass coverslip was decreased.

The recalcitrance of biofilm is indeed a multifactorial phenomenon. Preformed biofilm was less likely to be disrupted compared to initial biofilm because of the surface conditioning that occurs in the initial stage (Kumar and Anand, 1998, Zarkasi *et al.*, 2018; Zarkasi *et al.*, 2019). Given the ample time to develop 'conditioning film', this creates a favourable environment for biofilm attachment of the preformed biofilm. Bacterial cells in the biofilm use biofilm as a defence mechanism against antibacterial agents. Hence, the reason why preformed biofilm was more likely to be reduced by the fungal endophytic crude extract. The lesser amount of crystal violet stain in the initial biofilm indicated that fungal endophytic crude extract has the potential antibiofilm properties by promoting detachment of biofilm community on the substrate.

Under SEM, the control biofilm images showed that the biofilm was in good undisturbed shape and tightly secured with extracellular matrices. The matrices act as a scaffold to hold the biofilm and are responsible for the adhesion and cohesion of biofilm to the substrate (Flemming and Wingender, 2010; Kuthoose *et al.*, 2021). In this study, the fungal endophytic crude extract was found to clearly disintegrate the extracellular polymeric matrices from the biofilm structure in both initial and preformed biofilm. A less compact structure of cells was observed when matrices were removed by the action of crude extract. Extract is assumed to prevent the formation of biofilm on glass coverslip by attacking the biofilm before they reach glass coverslip or probably act by modifying the surface tension on glass coverslip hence preventing attachment of bacterial biofilm (Kim *et al.*, 2020).

Furthermore, it can be reasonably assumed that adding extract did not kill bacterial cells entirely but has been confirmed to reduce one of the virulence properties of *S. mutans* which is the production of exopolysaccharides (EPS). Similarly, Fu *et al.* (2017) also observed that the extract of *H. patriniae* clearly inhibits the biofilm formation of *P. aeruginosa* by reducing the exopolysaccharide production as well as increasing the swarming motility of bacterial cells. According to Lin *et al.* (2021), targeting the EPS is one of the effective ways to remove biofilm. The SEM images show that the production of EPS (which consists mostly of glucans) was disrupted. The extract could act in a way that it disrupts formation of EPS entirely or could possibly mimic the enzyme glucosyltransferases that are responsible in converting sucrose into extracellular glucans. Hence, the conversion will not occur. Targeting the enzymatic activity could disrupt the biofilm scaffold because a wide range of enzyme activities in the biofilm matrices influence the structural integrity and stability of biofilm (Sutherland, 2001).

Although there are two types of adhesion to the surfaces, which are sucrose-independent and sucrose-dependent, it is always the latter that is responsible for the persistent colonization of surfaces (Banas, 2004). Besides that, the extract was also observed to affect the cell division process in *S. mutans* biofilm, whereby cells underwent incomplete separation. The SEM images of the treated biofilm indicate that bacterial cells in the biofilm were damaged functionally despite the absence of bacteriolysis. A similar study was also presented by Ito *et al.* (2020) on the effect of abietic acid on *S. mutans* morphology. This condition was also observed by Si *et al*. (2006) on *S. typhimurium* cells when treated with Chinese green tea extract. A previous study reported that biofilm could be prevented in a few ways, which are by inhibiting cell attachment (at the early stage), disrupting the development of extracellular polymeric substances in the middle stage or induce in disengagement of biofilm from the substrate (Li *et al*., 2017). According to Nostro *et al*. (2012), interference at adhesion properties is caused by the reduction of surface tension with the action of the extract. The complex biological interactions between the extract tested, bacterial cells and substrate may vary the outcome.

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Finding an antibiofilm agent that can be impaired bacterial biofilm without inducing cell death is remarkable (Da Silva Trentin *et al*., 2011; Suhaimi *et al.*, 2019). The rapid development of bacterial resistance can be reduced and controlled. Considering all reasons mentioned above, it can be concluded that *N. sphaerica* CL-OP30 crude extract works excellently as a potential anti-caries agent in such a way that it probably kills the integrity of cell walls therefore, cells lose its original shape and by inhibiting attachment of cells to surfaces but not killing the cells entirely. The fungal endophytic crude extract also acts as an inhibitor for exopolysaccharide production. Therefore, it can be concluded that fungal endophytic crude extract acted on *S. mutans* biofilm in many ways.

Besides, the performance of crude extract on the preformed biofilm was lesser compared to the initially formed biofilm. This is because the preformed biofilm was given enough time to occupy the surface and conditioned the surface before being treated with the crude extract. When a crude extract is inoculated into the preformed biofilm, the crude extract is only able to eliminate bacterial cells that were loosely attached to the biofilm. Unlike the preformed biofilm, the initial biofilm was incorporated together with the crude extract; therefore, the biofilm does not have ample time to build the biofilm structure and hence will be easily removed by the crude extract.

In the future, optimization of the fungal endophytic crude extract could be done in order to maximize the production of antibiofilm activity. Many parameters could be optimized, such as temperature, pH value, agitation, incubation period and nutritional sources. Each parameter can be optimized one at a time, and the selection of each parameter can be done on the basis of the highest antibiofilm production. In addition, the combination of fungal endophytic crude extract with other synthetic or natural products can be studied to produce an alternative treatment for dental caries. As for the beginning, fungal endophytic crude extract can be incorporated into the commercial topical agent, for example, toothpaste, and study their effectivity at different concentrations. Then, the modified topical agent can be applied in the animal model study.

## CONCLUSION

When *S. mutans* assume the biofilm phenotype, the pathogenicity increases, making it difficult to eradicate. In this study, *N. spaherica* CL-OP30 fungal endophytic crude extract showed good antibiofilm activity. The efficiency of this crude extract to reduce biofilm formation and disrupt the growth of the primary causative agent of dental caries - *S. mutans* is well achieved. Further research is needed to evaluate and develop anticaries agents in the near future. The focus should be on understanding the metabolic pathway of bioactive metabolites in suppressing the growth of *S. mutans* biofilm.

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