

Aspergillus japonicus Biofilms on Lab Fermenter: Development, Temperature, pH Survival Studies, and Drug Resistance

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Abstract

Background: Biofilms are described as a microbial population anchored to a surface and entrenched in a self-produced matrix of polysaccharides. The biofilms forming capacity of *Aspergillus japonicus* on glass fermenter and its susceptibility over temperature, pH, and drug resistance was evaluated in this study. **Materials and Methods:** *A. japonicus* was screened from an annual herb at Pattambi, Kerala. It was identified using fungal universal primers ITS4 and ITS5 at the ITS region of rDNA (NCBI Accession numbers MG833001, MG833000). *A. japonicus* was cultivated by submerged culture using Potato Dextrose Broth and potato dextrose agar. The formation of biofilms was observed on laboratory fermenter and confirmed by the crystal violet method, followed by a scanning electron microscope (SEM) analysis. The effects of temperature and pH on *A. japonicus* biofilms formation were studied. The antimicrobial resistance of *A. japonicus* against fluconazole and clotrimazole was evaluated. **Results:** The growth curve was obtained from plant infected isolate of the *A. japonicus* biofilms formation. Biofilms were confirmed by *in vitro* crystal violet method. SEM analysis showed the massive growth of *A. japonicus* biofilms. The inocula of 1×10^6 conidia/mL were incubated at 8°C, 20°C, 37°C, 50°C, and 70°C for 180 days. This proves that *A. japonicus* survives in all temperature conditions. The inocula of 1×10^6 conidia/mL were then incubated at different pH (3, 4, 5, 6, 7, 8, 9, and 10) for 12 days. This shows that biofilms are rapidly formed at pH 5, 6, and 7. *A. japonicus* exhibits resistant against fluconazole and clotrimazole. **Conclusions:** The study concludes that biofilms are the most stable, survive for a long time at different temperatures and pH, and also show resistance against fluconazole and clotrimazole. This is the first report of *A. japonicus* biofilms formation in India.

Key words: *Aspergillus japonicus*, biofilms, clotrimazole, fluconazole, pH

INTRODUCTION

Fungal biofilms are communities in which fungal cells fix in a matrix of extracellular polymeric compounds attached to a supporting surface. Biofilms help to protect fungi from the inimical conditions, and the development of fungal biofilms is an important factor in the infections of humans and animals.^[1] *Aspergillus* spp. is opportunistic and saprophytic fungi which cause several systemic and superficial infections. Aspergillosis is the secondary source of nosocomial infection, and these fungi cause pulmonary aspergillosis. *Aspergillus* spp. is widely used in industry for the production of various biotechnological products.^[2] Fungal pathogenic microorganisms cause severe diseases (meningitis and pneumonia) and chronic respiratory conditions (asthma and chronic obstructive pulmonary

disease).^[3] A fungus causes various diseases in the animal and plant kingdoms as well as in humans. Due to this, the use of the antifungal compound is widespread in both farming and clinical conditions.^[4] The problem of increasing antifungal resistance is exacerbated by the lack of new antifungal agents in development that has a unique mode of action and biofilms formation.^[5] Fungal spores are present in the soil and spread through the air. These spores may be inhaled or may come in contact with the surface of the human and animal skin.

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Resistance in *Aspergillus* spp. has turned into a worldwide health problem since the introduction of antifungal agents and extensive use of fungal antibiotics in the early 1990s.^[6] The formation and development of biofilms vary depending on the nature of the component, in which the microorganism comes in contact. The major components are water, microbial cells, exopolysaccharides, and lipopolysaccharides of the matrix. These components provide a physical wall against the diffusion of defense substances, antibiotics, and also protect against environmental stress factors, such as temperature, pH, ultraviolet radiation, osmotic strain, and dehydration.^[7,8] Biofilms development on the filtration membrane and the succeeding congestion of membrane pores is one of the most constant problems in membrane bioreactors for wastewater treatment.^[9]

MATERIALS AND METHODS

Fungal isolates

Aspergillus japonicus fungal strain was isolated from the leaves of *Phyllanthus niruri*. The leaves of *P. niruri* were collected, air-dried, and finely powdered using a blender. The fine powder of the leaf weighing 10 g was transferred into a 500 ml beaker. To this 100 ml of sterile water was added and the beaker was incubated aseptically 24°C for 48 h. The growth of different types of colored fungi was observed on the surface of the water. Five different types of colored fungi colonies were picked and purified by subculture technique using potato dextrose agar (PDA) medium. Purified five different fungal strains were individually grown at 24°C for 15 days on PDA medium, and growth profile was observed regularly. Among the five fungal strains, *A. japonicus* had shown the massive biofilms formation on the surface of

the glass lab fermenter. *A. japonicus* was taken for further phenotypic and genotypic analysis [Figure 1].

Morphotaxonomic and molecular identification of fungi

Fungal isolates were observed using a scanning electron microscope (SEM), and the morphotaxonomy was noted with respected colonies, conidial heads, chlamydozoospores, conidiophores, sterigmata, and vesicles.

Molecular identification

A. japonicus was isolated from the leaves of *P. niruri* collected from Palakkad, Kerala. Fungal culture was separated and purified by the streaking method, and it was identified by fungal universal primers ITS4 and ITS5 at the ITS region of rDNA.

Fermentation and observation of fungal biofilms

A. japonicus mycelia were obtained from a slant culture. The inoculum was developed in the following manner: The inocula of 1×10^6 conidia/mL were transferred into a 250 ml conical flask, which contained 150 ml potato dextrose broth (PDB). The flask was incubated for 8 days at 27°C at pH 6.0.

In vitro determination of biofilms formation

The ability of the *A. japonicus* biofilms formation was determined by the polystyrene microtiter plate method using crystal violet as described previously.^[10] Spore suspension of strain was prepared at the concentration of

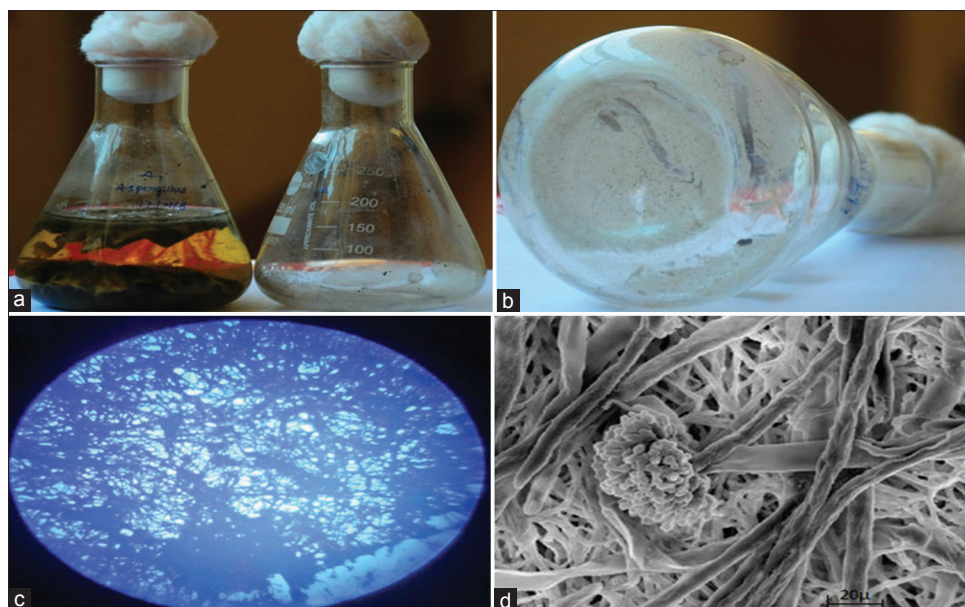


Figure 1: Growth of biofilms surface on glass fermenter (a) *Aspergillus japonicus* growth, (b) biofilm observed on the conical flask, (c) light microscope image, (d) scanning electron microscope image

1×10^6 spores/mL in distilled water. Fifty microliters of the spore suspension were placed in the containing 2 mL of PDB with 0.25% of glucose. The tubes were incubated at 37°C for 1 day and the suspension was diluted with freshly prepared PDB with glucose in the ratio 1:20. Two hundred microliters aliquot of the suspension were transferred to each well of a 96-well polystyrene microtiter plate and incubated for 24 h at 37°C. The microplate was rinsed 3 times with PDB and inverted to remove free-floating fungi. Two hundred microliters of 0.4% crystal violet were added in each well. The microplate was incubated for 15 min at room temperature. After incubation, the microplate was rinsed again 3 times with PDB. Two hundred microliters of ethanol: acetone mixture (70:30 V/V) were added to each well and the absorbance was measured at 490 nm. The transmittance percentage value (%T) of the sample was subtracted from the value %T of the reagent blank to obtain a measure of the amount of light absorbed by the sample (%T Absorbance).

SEM

The architecture of *A. japonicus* biofilms was analyzed using SEM (field emission-SEM). Image processing was done with the Scandium software for image acquiring and auto storage. Electron microscopic work was done at the Core Facility for Integrated Microscopy, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamil Nadu.

pH

The pH effect on biofilms production by *A. japonicus* was studied by preparing the standardized spore inoculum of 1×10^6 conidia/ml of fungal culture and inoculated at different pH (3, 4, 5, 6, 7, 8, 9, and 10) for 16 days.^[11]

Temperature

A biofilm temperature survival study was carried out for *A. japonicus* at different parameters such as inoculum concentration, different temperature, and incubation time. For this study, *A. japonicus*, an inoculum of 1×10^6 conidia/ml was incubated for 180 days at temperatures 8°C, 20°C, 37°C, 50°C, and 70°C.

Antifungal susceptibility testing

Antifungal susceptibility of *A. japonicus* was studied using the disk diffusion method. The inoculum was prepared using PDB. PDA media were prepared by autoclaving 3.9 g of media in 100 mL water. The test microorganisms were inoculated on the PDA plates using sterile cotton swabs. Antifungal disk fluconazole and clotrimazole were placed on the surface of culture inoculated PDA plates, and plates were

incubated at room temperature for 48 h. Antifungal activity was evaluated using the HiMedia zone reader.

Statistical analysis

Experiments were performed in triplicate. The results were given as mean \pm standard deviation. Student's *t*-test was used for comparison between two means and a one-way analysis of variance was used for comparison of more than two means. The difference was considered statistically significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

Morphotaxonomic and molecular identification of fungi

Colonies, on CZA at 25°C grows faster, velvety, mid floccose, blackish, and margin regular, reverse olivaceous buff. Chlamydospores globose to subglobose, solitary or in pairs, hyaline, variable in shape and size $18 \mu\text{m} \times 17.68 \mu\text{m}$. Conidial heads radiating, up to $50 \mu\text{m}$ wide, carbon black, spitted into definite columns. Conidiophores were colorless, smooth-walled $1795 \text{ nm} \times 15.75 \mu\text{m}$ long. Conidia globose, olivaceous form, rough walled $2.5\text{--}4.75 \mu\text{m}$. Vesicles globose to subglobose, olivaceous form, fertile all over, $81.15 \mu\text{m} \times 82.3 \mu\text{m}$.

Molecular identification

Identification was done by fungal universal primers ITS4 and ITS5 at the ITS region of rDNA (NCBI Accession numbers MG833001). Fungal culture was deposited at the National Fungal Culture Collection of India (NFCCI accession no: 4232). Phylogenetic evolutionary tree constructed using online Phylogeny.fr software. rDNA sequence data were deposited at NCBI in the FASTA format. BLAST analysis was done using NCBI web tools [Figure 2].

Fermentation and observation of fungal biofilms

The growth of the *A. japonicus* in PDA media was observed for 8 days at room temperature. The mass growth rate was observed in PDA medium with a mycelial dry weight of 16 g/L. *A. japonicus* robust growth of biofilms was observed at the surface of the glass fermenter [Figure 1].

Biofilms formation

A. japonicus strains which attained absorbance at 490 nm showed the capability to form biofilms. *A. japonicus* fungal isolates adhered to the surface of the glass fermenter and formed biofilms (n=3).

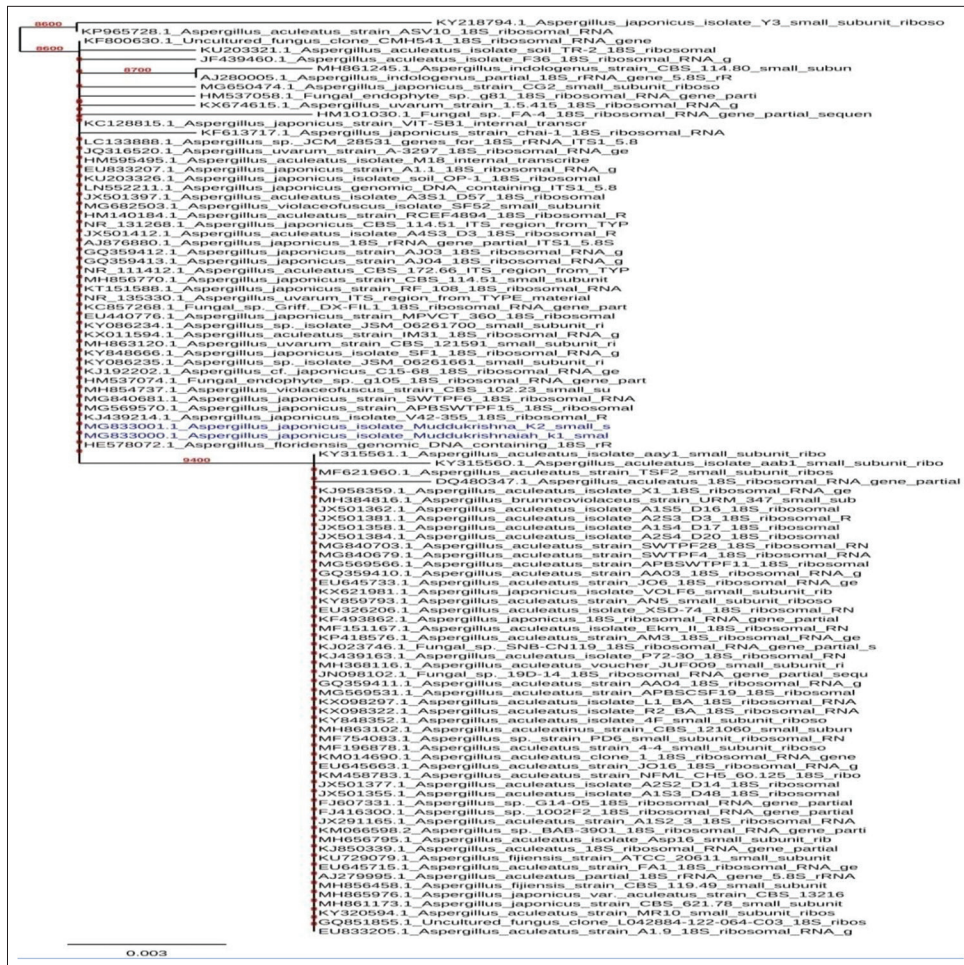


Figure 2: Phylogenetic evaluation of *Apostichopus japonicus*

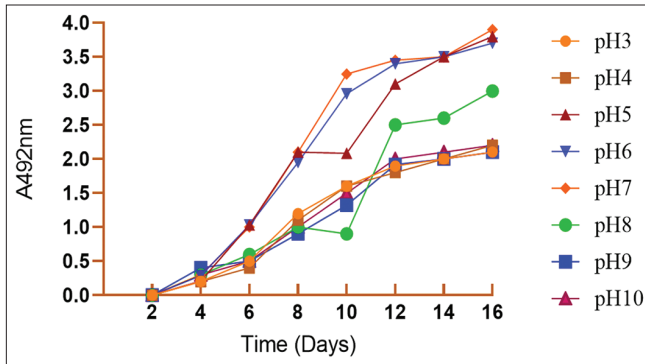


Figure 3: pH effects on biofilms development

Table 1: Study of biofilm formation at different temperature

Temperature (°C)	Holding time (days)	Active growth
8	180	+
27	180	++
37	180	+
50	180	+
70	180	-

(+) growth, (++) massive growth, (-) negative growth

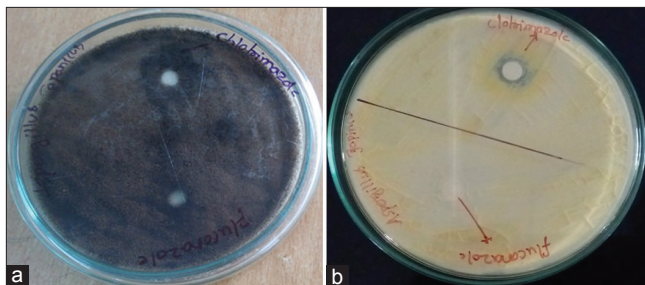


Figure 4: *In vitro* antifungal activities (a and b): *A. japonicus* existing resistance against fluconazole and clotrimazole

Table 2: *In vitro* antifungal activity

Antifungal disk	Zone of inhibition in mm (n=3)
Clotrimazole	R
Fluconazole	R

pH effect on biofilms development

Fungal growth and pH were the most important factor. The fast growth of *A. japonicus* was observed at pH 5, 6, and 7. *A. japonicus* biofilms were formed at different pH conditions (pH=3, 4, 5, 6, 7, 8, 9, and 10) and stable for a long time. The

initial phase of biofilm development (extracellular matrix substances) was increased with increasing incubation time and finally reached the saturation phase [Figure 3].

Temperature effect on biofilms development

A biofilm temperature survival study was carried out for *A. japonicus* at different temperatures. This showed high metabolic activity and fast growth at 27°C for 72 h. *A. japonicus* biofilms are stable for a long period of time (180 days) at various temperature conditions [Table 1].

In vitro antifungal activity by disk diffusion method

Antifungal susceptibility was studied using the disk diffusion method. *A. japonicus* exhibits resistance to clotrimazole and fluconazole [Table 2 and Figure 4].

DISCUSSION AND CONCLUSIONS

The development of biofilms by fungal pathogens has not been proved earlier. This is the first report in India regarding the biofilms formation by *A. japonicus*. Biofilms are community of microbes growing within an own produced extracellular matrix having lipids, mono and polysaccharides, proteins, and extracellular DNA.^[12] Extracellular polysaccharides play a significant structural and functional role in the growth and protection of biofilms.^[12,13] Fungal cells signal communication acting a significant role in the association and differentiation of the fungal cell in biofilms depending on the surrounding conditions.^[14,15] Fungal biofilms were confirmed using SEM.^[16] Environmental conditional factors affect the fungal biofilms formation such as pH, temperature change, metabolite accumulation, starvation, and oxygen deficiency. The temperature and pH level of the medium can have a marked influence on *A. japonicus* biofilms formation. *A. japonicus* fungal strain was isolated from the leaves of *P. niruri*. Molecular identification was done by fungal universal primers ITS4 and ITS5 at ITS region of rDNA (NCBI Accession numbers MG833001) and it has shown 100% similarity with an existing sequence. This study explains that the pH and temperature can have an influence on *A. japonicus* formation. SEM was used to observe the biofilms structure. SEM analysis showed that biofilms were the formation of own secreting extracellular matrix along with hyphal elements. *A. japonicus* has the ability to develop biofilms under unfavorable conditions such as high temperature and different pH. The survival studies suggest that *A. japonicus* biofilms have survival in cruel environmental conditions (pH and temperature) for long duration and that it also shows resistance to clotrimazole and fluconazole.

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