Experimental and theoretical investigations of the adhesion time of *Penicillium* spores to cedar wood surface

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ABSTRACT

In this study, the adhesion of 4 *Penicillium* strains (*Penicillium granulatum*, *Penicillium crustosum*, *Penicillium commune* and *Penicillium chrysogenum*) on cedar wood was examined qualitatively and quantitatively by using the extended DLVO (XDLVO) approach and the environmental scanning electronic microscopy (ESEM) technique. A comparison between the XDLVO theories and the ESEM technique was also investigated. The adhesion tests revealed that *P. chrysogenum* was not able to adhere on the cedar wood substrata, as predicted by the XDLVO approach. We have also found by ESEM that the three *Penicillium* strains (*P. granulatum*, *P. crustosum*, *P. commune*) adhered on wood, as not predicted theoretically.

Moreover, the time of adhesion (3 h and 24 h) was used not only to compare the capacity of adhesion according to contact time but also to explain the discrepancies between the XDLVO approach prediction and the adhesion experiments. A positive relationship between the XDLVO approach and adhesion experiments has been observed after 3 h of adhesion. In contrast, a contradiction between the XDLVO predictions and the adhesion test results has been noted after 24 h of adhesion of *Penicillium* strains to the wood surface.

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1. Introduction

A considerable amount of research has been done to understand how microorganisms adhere to surfaces. The initial adhesion step is a critical point in the process of biofilm formation. This step is governed by van der Waals, electrostatic and acid–base interactions. These interactions depend on the physicochemical properties of the substratum and the microbial surface especially hydrophobicity, surface charge, and electron donor–electron acceptor properties [1–3].

The microbial adhesion to solid surfaces could be predictable by surface thermodynamics [4] Derjaguin–Landau–Verwey–Overbeek (DLVO) [5] or extended DLVO (XDLVO) theories. The DLVO theory has been widely used as a theoretical model to calculate qualitatively and quantitatively the actual adhesion energy variations involved in microbial adhesion and aggregation [6–10]. Although microbial adhesion is a multi-factorial process, much research has focused on the importance of hydrophobicity and hydrophilicity. Recently, the XDLVO theory was proposed. Compared to the 'classical DLVO theory', the XDLVO approach takes into account the polar interactions in addition to the other interactions (Lifshitz van der Waals "LW" and the electrostatic double layer "EL") [11,12]. In addition, it was claimed that the XDLVO approach may be the promising model to explain the

experimental results of microbial adhesion since it combines both the thermodynamic approach and the DLVO theory [13]. However, the validation of this approach as a predictive physicochemical model to study the microbial adhesion is still under investigation. Furthermore, the adhesion of microorganisms to surfaces is a very complex phenomenon that might restrict the applicability of surface thermodynamics, the DLVO and XDLVO theories. Thus, a dramatic advancement of optical, spectroscopic and microscopic methods for biofilm examination has been developed that include confocal laser scanning microscopy, light fluorescence, atomic force microscopy [14–18].

Many studies have compared the XDLVO approach with the abovementioned methods. For instance, the AFM force measurement curves are similar to the interaction energy profiles predicted by the XDLVO approach [19]. Sharma and Hanumantha Rao [20] have compared the predictions of XDLVO and the surface thermodynamics approaches, when studying the bacterial adhesion to minerals for different physicochemical conditions (ionic strength and pH). Their results showed that the extended DLVO approach is more effective in predicting the adhesion behavior than the expectations from the thermodynamic approach.

Taken together, the discrepancies between the XDLVO predictions and the experimental observations have been attributed to various surface properties and additional interactions including surface roughness, chemical and morphologic heterogeneities, and short-range non-DLVO forces [19,21–26]. However, the contact time of adhesion has not been investigated as a parameter to explain the discrepancies between the

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XDLVO predictions and the experimental observations. Thus, the aim of this study is to examine the adhesion of *Penicillium* spores on cedar wood and to compare the results obtained by the XDLVO approach and ESEM experiments depending on the contact time (3 h and 24 h).

2. Materials and methods

2.1. Isolation, identification and growth conditions

Penicillium strains were isolated from cedar wood decay from several sites of an old house built 450 years ago located in the former Derb lamté in the Medina of the Fez, Morocco. Fungi were also seeded on malt extract agar plates and incubated for 7 days at 30 °C.

In brief, polymerase chain reaction amplification was performed by using ITS1 and ITS2 primers as previously described by Gardes and Bruns [27] with the following protocol: denaturing at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min followed by a final extension step at 72 °C for 5 min. Amplified rDNA fragments were then sequenced by using ABI 3130 (Applied Biosystems, France) according to the manufacturer's instructions. The GenBank BLAST (Basic Local Alignment Search) tools were used for sequence analysis.

2.2. Substrate preparation

The substrate used was cedar wood. The cedar wood was cut in coupons of length 20 mm, thickness 1 mm, and height 10 mm. The cedar was cleaned for 15 min in ultrapure water and then autoclaved.

2.3. Surface tension components and hydrophobicity

Hydrophobicity was evaluated through contact angle measurements and by the approach of van Oss et al. [28–30]. In this approach, the degree of hydrophobicity of a given material (l) is expressed as the free energy of interaction between two entities of that material when immersed in water (w): $\Delta Giwi$. If the interaction between the two entities is stronger than the interaction of each entity with water, the material is considered hydrophobic ($\Delta Giwi < 0$); conversely, for a hydrophilic material ($\Delta Giwi > 0$). $\Delta Giwi$ is calculated through the surface tension components of the interacting entities, according to the following formula:

$$\Delta Giwi = -2\gamma_{iw} = -2\left[\left(\left(\gamma_i^{LW}\right)^{1/2} - \left(\gamma_w^{LW}\right)^{1/2} \right)^2 + 2\left(\left(\gamma_i^+ \gamma_i^-\right)^{1/2} \right. \right.$$
$$\left. \left. + \left(\gamma_w^+ \gamma_w^-\right)^{1/2} - \left(\gamma_i^+ \gamma_w^-\right)^{1/2} - \left(\gamma_w^+ \gamma_i^-\right)^{1/2} \right) \right]$$

where γ^{LW} accounts for the Lifshitz–van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid–base component (γ^{AB}), with $\gamma_S^{AB} = 2(\gamma_S^- \cdot \gamma_S^+)^{1/2}$.

The surface tension components of a solid material are obtained by measuring the contact angles of three pure liquids (one apolar and two polar) with well-known surface tension components [31], followed by the simultaneous resolution of three equations of the following form:

$$\gamma_L(\cos\theta + 1) = 2\left[\left(\gamma_S^{LW}\gamma_L^{LW}\right)^{1/2} + \left(\gamma_S^+ \gamma_L^-\right)^{1/2} + \left(\gamma_S^- \gamma_L^+\right)^{1/2} \right]$$

2.4. Contact angle measurements

Before angle contact measurements, strains were grown at 25 °C in malt extract agar, harvested by scraping the surface of sporulated cultures in a suspension of KNO_3 (0.1 M) and filtered through sterile glass wool to remove mycelium fragments and large spore clumps.

The conidia were pelleted by centrifugation (10,000 g, 15 min, 4 °C), washed twice with sterile KNO_3, suspended in the same solution, and counted with a hemacytometer (typically 10^7–10^8 spores ml^{-1}).

Contact angle measurements (for wood substrata and *Penicillium* spores) were performed by using a goniometer (GBX Instruments, France) by the sessile drop method. Three liquids with different polarities were used: water, formamide, and diiodomethane (Table. 1). For *Penicillium* spores, the measurements were performed on a cell lawn according to the method described by Busscher et al. [32]. Briefly, a suspension of *Penicillium* spores in KNO_3 solution was suspended in Millipore water and deposited on a cellulose acetate membrane filter (pore diameter: 0.45 μm) by filtration of the suspension using negative pressure. Filters containing the spores were placed on a metal sample disc with double sided sticky tape and allowed to air dry for 30–60 min in order to obtain stable, so-called "plateau" water contact angles. For each strain, three independently grown cultures were used, from which three filters of each were prepared and measured.

2.5. Extended DLVO theory

As described in the classical DLVO theory, the net interaction energy (G^{DLVO}) needed to bring a microorganism (m) into contact with a flat substratum surface (s) immersed in aqueous medium (l) is the balance between two additive interaction energies: the attractive Lifshitz van der Waals energy (G^{LW}) and the repulsive or attractive electrostatic double layer energy (G^{EL}). The total interaction or adhesion energy as a function of the separation distance (d) between a bacterium (sphere) and a substratum (flat plane) surface therefore can be written as:

$$G^{DLVO}(d) = G^{LW}(d) + G^{EL}(d). \tag{A.1}$$

Later, Van Oss et al. [11] suggested that the acid–base energy (G^{AB}), arising from hydrogen bonding between two surfaces immersed in a polar solvent (e.g., water), must be considered in addition to LW and EL interaction energies. The inclusion of the polar interaction energy (G^{AB}) resulted in the XDLVO approach, in which the total interaction energy (G^{XDLVO}) can be written as:

$$G^{XDLVO}(d) = G^{LW}(d) + G^{EL}(d) + G^{AB}(d). \tag{A.2}$$

From a thermodynamic point of view, adhesion or attraction between two interacting surfaces occurs when the total energy G^{XDLVO} is negative, and repulsion occurs when G^{XDLVO} is positive. Since the total interaction energy is evaluated as a function of the separation distance (d) between the interacting surfaces, therefore the interaction energy profile illustrates the type of interaction (attraction or repulsion) as the microbial particle approaches a substrate surface.

2.6. Adhesion experiments

Five cubic millimeters of fungal spore suspension containing 10^8 CFU ml^{-1} was incubated in a Petri dish containing cedar wood coupons for 3 h and 24 h at 25 °C. After the contact period, non-adherent cells were eliminated by three consecutive rinses with sterile distilled water, by moving in a small Petri dish [33–35].

Table 1
Energy characteristics (mj m^{-2}) of pure liquid used to measure contact angles.

Liquid	γ^{LW} (mj m^{-2})	γ^+ (mj m^{-2})	γ^- (mj m^{-2})
Water (H_2O)	21.6	25.4	25.4
Formamide (CH_3NO)	38.7	2.3	39.4
Diiodomethane (CH_2I_2)	50.5	0.7	0.0