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Research Article
Antifungal Activity and Physico-chemical Surface Properties of the Momentaneously Exposed *Penicillium expansum* Spores to Carvacrol

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Abstract

Objective: In this study, the antifungal activities, the minimum inhibitory (MIC) and fungicidal (MFC) concentrations, of carvacrol were determined vis-a-vis the growth of *Penicillium expansum* spore. **Methodology:** In addition, the surface properties of spores of the studied strain were also investigated in terms of hydrophobicity-hydrophilicity and electrons donor-acceptor properties by the contact angle method. **Results:** Thus, the obtained results allowed at first, to find a strong sensitivity of these spores relative to the studied essential oil component through a very low MIC and MFC. Moreover, the untreated spores of *P. expansum* exhibited hydrophilic characteristics both qualitatively and quantitatively with very pronounced electron donor properties. The momentaneous exposure of the spores to the carvacrol molecule has led to strengthening of the hydrophilic character of the spores surface with almost monopolar basic properties over treatment time. **Conclusion:** Although, the mode of action, by which the essential oil components inhibit the growth of microorganisms, is poorly defined in the scientific literature, this study contributes to elucidate the impact of these molecules and especially the carvacrol, on the surface properties of fungal spores. These physicochemical properties govern the approach and adhesion of microorganisms in biofilm formation on materials.

Key words: Antifungal activity, carvacrol, hydrophobicity, Lewis acid/base interactions, *Penicillium expansum* spores

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The *Penicillium* genus, with over 300 species[1], is one of the most known in the family of fungi, with *Aspergillus* and *Paecilomyces*[2], in particular thanks to certain strains for their positive roles in the food industry as well as in the medical field. Indeed, the species such as *P. roqueforti*, *P. nalgiovense* or *P. camemberti* are widely used in the manufacturing processes of certain cheeses, when *P. chrysogenum* and *P. notatum* produce the penicillin antibiotic. However, several other species of the *Penicillium* genus are known for their negative roles, notably *P. digitatum*, *P. italicum* or *P. expansum*[3-5]. This latter is responsible for huge post-harvest losses of fruits[6-8] and can produce, among other mycotoxins, the patulin which is one of most toxic for the human health[7,9].

Many studies are regularly reported, in recent decades, on the significant antibacterial and antifungal potential of essential oils. Indeed, they are frequently used as antimicrobial, antioxidant and insecticidal agents[10-12]. However, few studies are carried out with the main components of these essential oils. These latter are known to be very complex volatile compounds consisting of several tens or even hundreds of different molecules. Some of them, present in high proportion, confer to these essential oils their important antimicrobial activities.

Similarly, very few studies were reported in the scientific literature on the impact of antifungal molecules on the physicochemical surface properties of pathogens fungal that may present a major risk in the food processing industries, medical field and even in the preservation of the historical monuments. These studies on the physico-chemical properties would allow to better understand the adhesion phenomenon of microorganisms and the forces that govern their approaches[13-16] on the materials surfaces regularly used in these sectors. They would also help to better understand the effect of substances and molecules with antimicrobial effect, used in the treatment of materials, on the physico-chemical surface properties of these microorganisms in their vegetative and spore forms.

Thus, this study aimed to evaluate the antifungal activity of carvacrol, which is a main component of the *Thymus vulgaris* essential oil, on the growth of *Penicillium expansum* spores by determining the minimum inhibitory (MIC) and fungicidal (MFC) concentrations. In addition, the physico-chemical properties of the *Penicillium expansum* spores surface were also evaluated by the contact angle method before and after exposure of the spores to the MIC of carvacrol and their evolution as a function of time.

This study is the first in the recent scientific literature, which shows the effect of momentaneous exposure of fungal spores to an antifungal molecule on their surface properties in terms of hydrophobicity-hydrophilicity, electron donor-acceptor properties and interfacial free energy.

MATERIALS AND METHODS

***Penicillium expansum* strain, growth conditions and harvesting spores:** *Penicillium expansum* was isolated from cedar wood decay and identified in the laboratory[17]. Growth was obtained at 25°C using malt extract agar. After 7 days of incubation, the spores of *P. expansum* were then harvested by scraping the culture surface in sterile 0.1% tween 20. The spores suspensions was concentrated by centrifugation at 10,000 g for 15 min at 4°C until a concentration of 10^6 spores mL^{-1} (counted with a hemacytometer).

Antifungal activity evaluation: The antifungal activities, MIC and MFC, of the essential oil component (carvacrol) against *Penicillium expansum* spores were determined according to the micro-well dilution method[18]. Briefly, the carvacrol was diluted in sterile 1% tween 20 and was distributed in the microplate wells containing already 10 µL of the prepared spores suspension; the carvacrol was therefore diluted successively from well to well and the range of concentrations was 5-0.005%. In the control wells, the equivalent volume to that of the tested component was replaced by the solution of tween 20. Thus, the MIC, that is defined as the lowest concentration which inhibited the growth of the studied strain was determined after incubation for 48 h at 28°C. The determination of the CMF was conducted from wells that have not shown a visible growth by depositing spots (5 µL) on malt extract agar. The Petri dishes were then incubated at 28°C for 72 h and the lowest concentration that has not showed visible growth was considered as MFC.

Essential oil component: The essential oil component used in this study, for the antifungal activity and the physico-chemical surface properties of the *P. expansum* spores was carvacrol (99% pure) purchased from sigma-aldrich.

Momentaneous exposure treatment of the *Penicillium expansum* spore surface: The spore suspension concentrated to 10^6 spores mL^{-1}, prepared such as mentioned above was distributed in three volumes of 10 mL each. Then the tested compound was added, so as to obtain a final concentration of 0.625% for the 2 volumes. Finally, the spores were then incubated for the 2 studied time (1 and 3 h). After each incubation time, the corresponding erlenmeyer flask was removed and the spore suspension was filtered through a nitrocellulose membrane of porosity 0.45 µm. The membranes were then placed in sterile petri dishes before proceeding to the contact angles measures with the 3 liquids after 20-25 min.

Contact angle measurements and calculation of the interfacial free energy: The physico-chemical properties of the cedar wood surface were characterized by the contact angles measurements through the sessile drop technique using a goniometer apparatus[19-21]. The initial contact angle of each liquid was measured after drop stabilization on the solid sample surfaces. For the determination of the interfacial free energy of the solid surface (treated and untreated samples), three liquids are recommended[22]. They consist of two polar liquids (water and formamide) and one apolar liquid (diiodomethane) with known surface tension characteristics (Table 1). Therefore, contact angles measurments on each wood samples were made using these pure liquids. Then, all parameters of the surface physico-chemical characteristics (the Lifshitz-van der Waals component (γ^{LW}), the electron donor or Lewis base (γ^-) and the electron acceptor or Lewis acid (γ^+)) allowing to determine the surface free energy of each sample (ΔGiwi) were calculated by the Young's equation[23]:

$$\gamma_L(\text{Cos}\theta + 1) = 2(\gamma_S^{LW}\gamma_L^{LW})^{1/2} + 2(\gamma_S^+\gamma_L^-)^{1/2} + 2(\gamma_S^-\gamma_L^+)^{1/2} \quad (1)$$

where, the terms (S) and (L) denote solid surface and liquid phases, respectively.

Lewis acid-base component (γ_S^{AB}) is obtained by:

$$\gamma_S^{AB} = 2(\gamma_S^-\gamma_S^+)^{1/2} \quad (2)$$

Moreover, the degree of hydrophobicity of each sample surface was evaluated by applying the approach[23]. According to this study, the degree of hydrophobicity of a given material is expressed as the free energy of interaction between two entities of that material immersed in water (w): ΔGiwi. This parameter has been calculated through the surface tension components of the interacting entities, according to the following equation:

$$\Delta \text{Giwi} = -2\gamma_{iw} = -2\left[\begin{array}{l}((\gamma_i^{LW})^{1/2} - (\gamma_w^{LW})^{1/2})^2 + \\ 2\left((\gamma_i^+\gamma_i^-)^{\frac{1}{2}} + (\gamma_w^+\gamma_w^-)^{\frac{1}{2}} - (\gamma_i^+\gamma_w^-)^{\frac{1}{2}} - (\gamma_w^+\gamma_i^-)^{\frac{1}{2}}\right)\end{array}\right] \quad (3)$$

The values of the surface tension parameters for the three pure liquids used in this study are shown in Table 1.

Statistical analysis: Data are expressed as Mean±Standard Error (SE) and analyzed using SPSS Statistics software by one-way ANOVA followed by Tukey *post hoc* test with significance defined at $p<0.05$.

RESULTS

Effect of carvacrol on spores growth inhibition of *Penicillium expansum*: The antifungal activity results of the essential oil component, after an incubation period of 72 h at 28°C, were shown in Table 2.

As it can be seen in Table 2, the carvacrol had a very important antifungal effect on the growth of *P. expansum* spores. Indeed, the minimum inhibitory concentration of the tested component for this strain was 0.625% of carvacrol concentration. This reflects a strong sensitivity of this fungal strain to the carvacrol molecule.

The MFC was determined by depositing spots (5 µL) from wells 9-12 in petri dishes containing sterile malt extract agar, then incubated for 72 h. The results showed that there was growth of spores only for the spots taken from the well 9. Therefore, the concentration of the well 10 is considered as the MFC. Thus, the CMF was determined at 1.25% of carvacrol concentration.

Physicochemical surface characterization of *Penicillium expansum* spores: To study the effect of momentaneous

Table 1: Surface tension properties of pure liquids used to measure contact angles[24]

	Surface energy parameters (mJ m^{-2})		
Liquids	γ^{LW}	γ^+	γ^-
Water (H_2O)	21.8	25.5	25.5
Formamide (CH_3NO)	39.0	2.3	39.6
Diiodomethane(CH_2I_2)	50.5	0.0	0.0

Table 2: Effect of carvacrol on the growth of *Penicillium expansum* spores

Wells	1	2	3	4	5	6	7	8	9	10	11	12
Concentrations(%) (v/v)	Control (0)	0.0049	0.0098	0.0195	0.0391	0.078	0.156	0.3125	0.625	1.25	2.5	5
Penicillium expansum	+	+	+	+	+	+	+	+	-	-	-	

+: Presence of growth and -: Absence of growth exposure of *P. expansum* spores to carvacrol on their physico-chemical surface properties, the spore suspension was treated at a concentration of 0.625% (corresponding to the MIC determined in the previous step of the antifungal essay) for 1 and 3 h.

Table 3 presents, for the three used liquids, the mean values of contact angles obtained for the untreated spore surface (control) and those exposed to carvacrol as a function of time. In Table 3, the calculations results of the intermediate surface tensions properties (the Lifshit Van der Waals (γ^{LW}) and the Lewis acid (γ^{+}) and base (γ^{-}) parameters) and the interfacial free energy (ΔGiwi) obtained by using the Young's equations[23,24].

In Table 3, the water contact angle results showed an evolution of the wetting behavior of the spore surface before and after exposure to carvacrol. Indeed, the untreated *P. expansum* spore surface showed a very low water contact angle of the order of 26.5°. However, after 1 h of exposure to carvacrol, the spores wetting behavior evolved and given a water contact angle of 38 and 28.7° after 3 h of incubation. These obtained results, for the three samples (treated and untreated), are quite characteristics of hydrophilic surfaces.

In fact, when the value of the water contact angle exceeds 65°, the surfaces are characterized as hydrophobic and hydrophilic, when inversely the value of the water contact angle is less than 65°. Moreover, a positive value of the interfacial free energy (ΔGiwi) means that the surface is hydrophilic and a negative value indicates that it is hydrophobic[24,25].

There is also an important decrease of the contact angles values with the apolar solvent (diiodomethane) after the treatment.

Electron donor-acceptor properties and the interfacial free energy of untreated and treated *P. expansum* spores surfaces: The surface tension components of Lifshit Van der Waals, as well as those of acid and base of Lewis are essential intermediate parameters to determine the interfacial free energy of surfaces by the Young's equation.

The untreated surface of the *P. expansum* spores showed a very low electron acceptor character while the electron donor component were considerably high (Table 3). It is known that the surfaces which have very high electron donor components are characterized as hydrophilic.

On the other hand, although already very low for the untreated spores (γ^{+} = 1.63 mJ m^{-2}), the exposure to carvacrol has visibly the effect of significantly ($p<0.001$) reducing the surface tension, corresponding to the Lewis acid, in time. Indeed, after 3 h, it was almost zero (γ^{+} = 0.003 mJ m^{-2}) (Table 3).

However, there was an increase of the Lifshit Van der Waals component from γ^{LW} = 33.8 mJ m^{-2} (for control) to γ^{LW} = 40.8 mJ m^{-2} (after 1 h) and γ^{LW} = 41.6 mJ m^{-2} (after 3 h). The latter is inversely proportional to the diiodomethane contact angle value. In fact, the more the value of the diiodomethane contact angle is high and the more γ^{LW} is lower.

The hydrophilic or hydrophobic quantitative characterization of a material is defined by the calculated value of its surface free energy. Thus, according to the results presented in Table 3, the untreated surface of the *P. expansum* spores can be characterized as hydrophilic with a positive value of the interfacial free energy (ΔGiwi = 28.88 mJ m^{-2}>0).

The results also showed positive values for the interfacial free energies of the samples of both treatments after the momentaneous exposure of spores to the carvacrol molecules with ΔGiwi = 14.01 and 45.54 mJ m^{-2}, respectively for 1 and 3 h of exposure. This indicates quantitatively and in the both cases, the hydrophilic character of the treated spore surfaces, after 1 h, a decrease of the interfacial free energy. However, the latter was much more strengthened in the second time (3 h) compared to the control.

These results clearly demonstrate the impact of the *P. expansum* spores exposure to carvacrol on their surface tension parameters. Thus, in light of all these findings, it appears quite clearly that the untreated spores surface is both qualitatively and quantitatively hydrophilic with more electron donor than acceptor characters.

The treatment by momentaneous exposure to carvacrol allowed to strengthen the hydrophilicity of the spores surface from ΔGiwi = 28.88 mJ m^{-2} (for control) to ΔGiwi = 45.54 mJ m^{-2} (after 3 h).

Table 3: Physico-chemical surface properties of *Penicillium expansum* spores depending on the exposure time to carvacrol

	Liquids contact angles (°)			Surface energy parameters (mJ m^{-2})			
Exposure time (h)	θ_W	θ_F	θ_D	γ^{LW}	γ^{+}	γ^{-}	ΔGiwi
Control	26.50±1.70	26.90±1.75	53.20±0.50	33.80±0.30	1.630±0.17	50.98±0.45	28.88±0.96
1	38.00±0.20***	31.40±1.21**	38.80±0.33***	40.80±0.20***	0.410±0.05***	38.24±0.45***	14.01±0.89
3	28.70±0.81*	39.40±0.48***	35.50±0.35***	41.60±0.20***	0.003±0.00***	58.57±1.37***	45.54±1.40*

Statistically different from the control, *$p<0.05$, **$p<0.01$ and ***$p<0.001$

DISCUSSION

The very important antibacterial and antifungal potential of essential oils is regularly reported in the scientific literature of these last decades. At the same time, their antimicrobial activities is attributed to their major phenolic components, such as thymol, eugenol or carvacrol[26]. This latter is present in a very high proportion in particular in the thyme and oregon essential oils[27,28].

The important activity of carvacrol against the *P. expansum* strain, highlighted in this present study, is in agreement with the different works related to this molecule and reported in the literature.

Indeed, several studies have reported the inhibition of the spores of *Botrytis cinerea*[29] by carvacrol and its antifungal property against *Aspergillus* spp. and *Penicillium* spp.[30]. Similar results of the inhibition of *Aspergillus niger* (MIC = 50 and MFC = 75 μg mL^{-1}), *Aspergillus flavus* (MIC = 100 and MFC = 125 μg mL^{-1}), *Penicillium citrinum* (MIC = 150 and MFC = 175 μg mL^{-1}) and *Penicillium chrysogenum* (MIC = 125 and MFC = 150 μg mL^{-1}) by the cavracrol have also been reported by Abbaszadeh *et al.*[31] in their recent study.

However, very few studies have explained the mechanism of action of these essential oils and their volatile terpene derivatives in the inhibition of the growth of microorganisms. This moreover explains why the mechanism of action by which these compounds act against microorganisms is not very well defined in the literature. But, several hypotheses have been reported by researchers and would involve the perforation of the membrane, the destruction of the cell walls and the degradation of the spores of fungi.

In the case of carvacrol, its mechanism of action would consist in disrupting the membrane of microorganisms by affecting their membrane permeability; this action is enhanced by its hydrophobic nature[32,33].

In addition to its proved antifungal activity, it was also indicated by different studies that this phenolic compound presents the anti-inflammatory, antioxidant, insecticidal, antiparasitic and hepatoprotective properties[34-36].

However, no study has investigated on the effect of carvacrol on the physico-chemical surface properties of microorganisms in term of hydrophobicity-hydrophilicity and electron donor-acceptor characteristics.

The importance of these surface properties, both those of microorganisms as those of materials, is now well established in the literature and their implications in the mediation of the adhesion phenomenon of microorganisms in the biofilm formation on materials.

Thus, it is matter to show, in this study, how the surface properties of *P. expansum* spores could be affected when they are exposed to a substance with known antimicrobial property such as carvacrol.

The initially hydrophilic character of the surface of the *P. expansum* spores observed in the present study is in agreement with that reported in the study of El Abed *et al.*[37]. Indeed, the authors have reported the both qualitative ($\theta_w = 45.3\pm1.5°$) and quantitative ($\Delta Giwi = 15.29$ mJ m^{-2}) hydrophilic characteristics of the spore surface of this fungal strain.

Similar results have also been reported for several other species of the *Penicillium* genus with in particular *P. crustosum* ($\theta_w = 11.9\pm1.98°$, $\Delta Giwi = 20.2$ mJ m^{-2}), *P. granulatum* ($\theta_w = 36.5°$, $\Delta Giwi = 18.7$ mJ m^{-2}), *P. commune* ($\theta_w = 17.9\pm0.75°$, $\Delta Giwi = 8.5$ mJ m^{-2}) and *P. chrysogenum* ($\theta_w = 10.4\pm4.54°$, $\Delta Giwi = 6.3$ mJ m^{-2}) with electron donor properties much more marked than the electron acceptor properties[38].

Capizzi and Schwartzbrod[39] showed, using hydrocarbons, that the hydrophobic potential of *Ascaris suum* eggs was 95% (for fresh eggs) and 72% (for stored eggs at -20°C) obtained with octane as a solvent.

The results have also highlighted an electron donor characteristic reinforced over time further to the treatment. In fact, when the γ^- value is greater than 27.9 mJ m^{-2}, the material is characterized as hydrophilic and hydrophobic[23] when conversely the γ^- value is less than 27.9 mJ m^{-2}. Thus, with a value of $\gamma^- = 50.98\pm0.45>>27.9$ mJ m^{-2}, the untreated *P. expansum* spores could be characterized as hydrophilic. This confirms the qualitative results obtained above with the water contact angle.

The physico-chemical properties of microorganisms depend on the chemical composition and the morphology of their surfaces. But they are also influenced by the different physical parameters of their environments such as temperature[40] and pH[41], but also by the nature of the available nutrients[42,43] and even their growth phase[42].

Indeed, the Lewis base component (γ^-) of the surface is often due to the presence of different chemical groups to the surface of these microorganisms and which are mostly negatively charged, such as carboxylate (COO^-), amino (NH_2), phosphate (PO_4^{-2}) or SO_3 groups[44,45]. These chemical groups belong to the main macromolecules which constitute the external membranes of microorganisms (lipopolysaccharides, lipoproteins and phospholipids).

On the other hand, Jeffs *et al.*[46] found in their study that spores with rugose surfaces were hydrophobic whereas those with smooth surfaces were hydrophilic. Also, these studies have shown that the chemical treatment of the aerial conidia of the genera *Beauveria*, *Metarhizium*, *Paecilomyces*, *Tolypocladium* and *Verticillium* with Sodium Dodecyl Sulfate (SDS) and the Formic Acid (FA) reduced the surface hydrophobicity.

Azcurra *et al.*[47] also reported similar results on effect of the High Molecular Weight Chitosan (HMWC) and sodium alginate (NaAL) on *Candida albicans* hydrophobicity and adhesion to cells. Indeed, they found a decrease of 44% of the hydrophobicity in presence of HMWC and 82% in the presence of NaAL, when they used chloroform as organic medium. On the other side, they found a decrease of the hydrophobic character of the order of 30 and 19% in presence of HMWC and NaAL, respectively when they used xylene, what caused a significant decrease in the adhesion of *C. albicans* to epithelial cells and human fibroblasts.

However, contrary to these authors, the results showed that the hydrophilic character of the *P. expansum* spore surface was enhanced by their momentaneous exposure to carvacrol. This effect of carvacrol has also been reported recently on the surface of cedar wood[19]. Indeed, the initially hydrophobic surface of the wood has become hydrophilic after the treatment by cavacrol.

Thus, the strengthening of the hydrophilic character of the *P. expansum* spores surface could be due to the hydroxyl function of the carvacrol molecule. This hydroxyl group induces a stronger electron donor character of the surface through electron pairs of the oxygen atom.

Furthermore, Ultee *et al.*[28] have suggested that the hydroxyl function of molecules such as thymol and carvacrol play an important role in the antibacterial and antifungal activity of these derivatives terpene.

The antimicrobial activities and the physico-chemical properties of carvacrol could be very effective in surface treatments in both food industry and the medical field to prevent the adhesion and biofilm formation by pathogenic microorganisms.

CONCLUSION

In conclusion, the obtained results showed the important antifungal activity of the carvacrol against *P. expansum* in a lower concentration. Based on the results, this molecule, which is naturally present in medicinal and aromatic plants, has the ability to inhibit the growth of *P. expansum* spores and to eliminate them definitively at concentrations of 0.625 and 1.25% (v/v), respectively. On the other hand, the momentaneous exposure of these spores to carvacrol has significantly ($p<0.05$) modified their surface properties.

These findings allowed to notice the strengthening of the hydrophilicity of the spore surface which is revealed into an increase of the wetting behavior and the interfacial free energy. If the antifungal properties of phenolic compounds of the essential oils have repeatedly been reported. This present study is the first one to enable a better understanding of the effect of these compounds on the surface properties of fungi especially in terms of hydrophobicity-hydrophilicity and electron donor-acceptor properties. Besides the bioactive potential of carvacrol against the growth of *P. expansum* spores and its ability to influence their physico-chemical surface properties, this essential oil component could reduce and even completely inhibit their adhesion on materials.

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