Biodegradation of polymers and adhesion properties of microorganism cells

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We demonstrate the possibility of using FTIR-spectroscopy to study the chemical composition of conidia films and to define surface hydrophobicity. It was possible to predict conidium hydrophobicity based on the data obtained for component ratios. This is the defining factor for microorganism adhesion to solid surfaces and for further processes of biodegradation.

INTRODUCTION

The biostability of polymeric materials depends on many factors, in particular the composition and properties of the interacting surfaces of the polymer and microorganisms which characterize adhesion—the initial stage of the complex biodegradation process. The reliability and character of the adhesion bond is defined by the nature of the chemical substances, separated by microorganism cells, which finally convert biodegradation to chemical degradation.

The cell walls of microscopic fungi perform many functions, from adhesion to cell growth and division. All this is important if the biostability of polymeric materials is considered to depend on the ability of the microstructural unit of microscopic fungi (conidia) to adhere to the material surface.

The conidium cell wall is considered to be a complex of several layers, whose amount and sizes depend on growth conditions. The following compounds are contained in a cell wall: polysaccharides (mainly chitin and glycans), lipids, proteins, and pigments.

Conidium cell wall composition is generally investigated by chemical analysis, a very labour-intensive activity which does not allow full indentification of chemical substances contained in the wall (in some cases because it involves the destruction of the wall itself). Recently, however, the method of attenuated total reflection (ATR) in the IR-range was used to identify substances contained in walls of yeast cells. This is the first time qualitative analysis of compounds in the wall has been performed by a nondestructive method.

The main aim of the present work was determination of the quantitative composition of substances contained in the conidium surface layer, and correlation between chemical composition and cell hydrophobicity by the NBRIG method the in IR-range.

MATERIALS AND METHODS

Conidia of the following microscopic fungi were used: Aspergillus niger, Aspergillus terreus, Penicillium chrysogenum, Penicillium funiculosum, Penicillium cyclopium, Paecilomyces variotii, Trichoderma viride, Chaetomucin globusum, and Aspergillus flavus. This list of microorganisms is provided by GOST (the Russian Standards Institution) for testing polymers for biostability.

Fungi were grown in test-tubes with agarized Chapek–Docks culture medium at 30°C for 14 days. Conidia were collected by washing with sterilized distilled water. They were then absorbed on Synpor membrane filters (conidium layer thickness ~0.01 mm), washed with distilled water, dried over CaCl₂ and used to obtain ATR spectra. The filter with the conidium layer was placed on the ATR crystal, then fixed by pressing with different forces, until the force value no
longer influenced the intensity of the IR bands. Spectra were obtained with a Fourier IR-spectrometer (Qualimatic, Digilab Co.), using prisms of KRS-5 or germanium.

The depth of IR penetration was determined by the formula:

$$1 = \frac{\tau_i/n}{2\pi[\sin^2 \theta - (n_i/n_o)^2]^{1/2}}$$  \hspace{1cm} (1)

where $\tau_i$ is the radiation wave length, $n_i$ is the crystal refractive index, $n_o$ is the sample material refractive index, and $\theta$ is the incident beam angle, equal to 45°.

To determine the penetration depth requires that the refractive index of the conidium wall is known. Refractive indexes vary a little for each group of substances contained in a cell wall. For this reason, we set $n_o$ equivalent to 1.5. For a KRS-5 crystal in the range of the main absorption bands of present substances, penetration depth varies from 0.5 $\mu$m to 0.8 $\mu$m (2000–1500 cm$^{-1}$). It increases sharply from 1500 cm$^{-1}$, and reaches 1.8 $\mu$m at 800 cm$^{-1}$. For germanium crystal this dependence is lower, giving values from 0.1 to 0.2 $\mu$m (2000–1500 cm$^{-1}$), and increasing to 0.3 $\mu$m at 800 cm$^{-1}$.

IR-spectra of the following substances in conidium cell walls were obtained on tablets (1–5 mg of substance per 300 mg of KBr): melanin, chitin (separated directly from Aspergillus niger), and glycans (Serva Co.). Spectra of phospholipids (lipid fraction, separated from Aspergillus niger) were obtained for their chloroformic solution in a NaCl cell, 0.03 cm thick.

Conidium surface hydrophobicity was estimated by measuring contact wetting angles with the help of a horizontal microscope, MH-1. For this purpose filters with a dense conidium layer were placed on the sample table, a water droplet 1–2 mm in diameter added by syringe and the contact angle measured. The degree of irregularity was calculated from:

$$\cos \theta = r \cdot \cos \theta_o$$  \hspace{1cm} (2)

where $\theta$ is the observed microscopic angle, $\theta_o$ is the real microscopic angle, and $r$ is the surface irregularity expressed as a ratio of the real area of irregular surface to the geometric surface. According to data previously recorded, the influence of irregularities of polymeric surface on contact angle $\theta$ was taken into account. Thus, surface irregularities increase $\theta$ if it exceeds 85° for the smooth surface, and decrease $\theta$ if it is lower than 60°. Taking into account these conditions, one can conclude that the irregularity contribution by rigid spheric spores should shift the contact angle in the sense of $\theta$ increase. However, for multiple change of contact angle we found no asymmetry of drop shapes, as would be expected in the case of significant half-sphere influence on irregularity. Thus, correction of irregularities of the surface layer of filtered conidia fungus has not been introduced.

**DISCUSSION**

Typical ATR spectra of the conidia of four types of fungus are shown in Fig. 1. Analysis of the spectra showed that the following absorption bands could be selected for identification of substances, contained in the conidium wall: 1639–1641, 1550–1560, 1033–1100 and 831–835 cm$^{-1}$. These bands possess the following properties:

(i) They do not overlap significantly i.e. they can be analyzed easily by their position and intensity.

(ii) They change their intensity sufficiently from one sort of fungus to another, i.e. they are applicable for quantitative analysis of substances contained in the conidium wall.

**Absorption at 1639 1641 cm$^{-1}$**

Proteins and glycans possess high absorption in this range. The protein absorption band, amide 1, includes vibrations of C—O bonds (~80%), C—N bonds (~10%) and N—H bonds (~10%) of the amide group. The conformation of a protein molecule can be estimated by the position of the amide 1 band. Absorption at 1656, 1650 and 1637 cm$^{-1}$ is due to irregular structures, $\alpha$-spiral and $\beta$-structure, respectively. Absorption in the range of 1639 cm$^{-1}$ is also characteristic for $\beta$-glycans, separated from the cell wall of the fungi.

**Absorption at 1275 cm$^{-1}$**

This band is related to vibrations of phospholipid P=O bonds. One can discern phosphate groups,
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linked with proteins by hydrogen bonds, and free groups. The first absorb at 1380–1220 cm⁻¹, the second at 1300–1250 cm⁻¹. It can be noticed that absorption at 1275 cm⁻¹ is characteristic of all types of fungi and testifies to the presence of free phosphate groups.

**Absorption at 1550–1540 cm⁻¹**

This is due to deformational vibrations of NH groups (amide II) present in both proteins and chitin.

**Absorption at 831 cm⁻¹**

This is caused by out-of-plane vibrations of C–H group, and is due to the presence of α-glycans. It has been shown that different α-glycans—mannans, nigerans—separated from cell wall of fungi, possess absorption maxima in the range 850–830 cm⁻¹.

**Absorption at 1070–1050 cm⁻¹**

This is due to vibrations of CO groups, which is characteristic of glycans and proteins. The base line was drawn through the minima at the peak bases for calculation of intensities. Extinction coefficients were obtained from absorption spectra of individual substances of various concentrations for each substance at the analysed frequencies. Pure substances were chitin, proteins, phospholipids and glycans.

Table 1 shows the values of optical density for nine microscopic fungi.

The following approximations were made in calculation of substance concentration in conidia walls:

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![Fig. 1. Typical IR spectra of outer fungal cell wall.](image-url)
Table 1. Optical densities of the main absorption bands of the wall of microscopic fungus conidia

<table>
<thead>
<tr>
<th>Microscopic fungus</th>
<th>Optical density (rel. units)</th>
</tr>
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</table>
|                                | 1639–1641      | 1550–1560      | 1275
|                                | v (cm⁻¹)       | v (cm⁻¹)       | v (cm⁻¹)       |
| Penicillium funiculosum         | 0.35 ± 0.02    | 0.01           | 0.36 ± 0.03    | 0.28 ± 0.02    | 0.39 ± 0.03 |
| Penicillium chrysogenum         | 0.26 ± 0.02    | 0.05 ± 0.01    | 0.13 ± 0.02    | 0.53 ± 0.03    | 0.13 ± 0.02 |
| Trichoderma viride              | 0.50 ± 0.03    | 0.06 ± 0.01    | 0.38 ± 0.02    | 0.50 ± 0.03    | 0.41 ± 0.03 |
| Aspergillus niger               | 0.30 ± 0.02    | 0.08 ± 0.01    | 0.20 ± 0.02    | 0.40 ± 0.03    | 0.14 ± 0.02 |
| Aspergillus terreus             | 0.64 ± 0.03    | 0.07 ± 0.01    | 0.56 ± 0.06    | 0.68 ± 0.04    | 0.50 ± 0.05 |
| Chaetomucor globisum            | 0.26 ± 0.02    | 0.05 ± 0.01    | 0.13 ± 0.02    | 0.63 ± 0.03    | 0.10 ± 0.02 |
| Aspergillus flavus              | 0.35 ± 0.02    | 0.05 ± 0.01    | 0.16 ± 0.02    | 0.33 ± 0.02    | 0.12 ± 0.02 |
| Penicillium cyclopium           | 0.28 ± 0.02    | 0.05 ± 0.01    | 0.17 ± 0.03    | 0.58 ± 0.03    | 0.17 ± 0.02 |
| Paecilomyces variotii           | 0.33 ± 0.02    | 0.08 ± 0.01    | 0.13 ± 0.01    | 0.55 ± 0.03    | 0.08 ± 0.02 |

The Beer–Lambert law is fulfilled for all substances

\[ C = \frac{D}{l\varepsilon} \]

where \( D \) is the optical density, \( \varepsilon \) is the extinction coefficient, and \( l \) is the beam penetration depth.

Extinction coefficients are similar for each sort of substance (chitin, protein, phospholipid, \( \alpha \)-glycan) for all investigated microscopic fungi.

Correlations of optical densities of different absorption bands for each microscopic fungus were used in order to avoid errors in obtaining reflection spectra.

The sum of the masses of the main substances in the conidium wall equals unity.

\[ C_a + C_1 + C_\alpha = 1 \]

where \( C_a \) are substances containing amide group (proteins, chitin), \( C_1 \) are phospholipids, and \( C_\alpha \) are glycans.

Concentrations of phospholipids, \( \alpha \)-glycans and substances containing amide groups were calculated from correlations of optical densities of absorption bands, characterizing these substances. We used 1275 cm⁻¹ for phospholipids, 831–835 cm⁻¹ for \( \alpha \)-glycans, and 1550–1560 cm⁻¹ for amides. We then have:

\[ \frac{D_a}{D_1} = AX, \quad \frac{D_a}{D_1} = BY \]

where

\[ A = \frac{l_a}{l_1}, \quad B = \frac{\varepsilon_a}{\varepsilon_1} \]

\[ X = C_a; \quad Y = C_\alpha \]

Using a balance equation, we obtain:

\[ C_1 = \frac{1}{X + Y + 1}; \quad C_\alpha = \frac{Y}{X + Y + 1}; \quad C_a = \frac{X}{X + Y + 1} \]

Experimentally, we estimate for the above-mentioned absorption bands: \( l_a/l_1 = 1.80; \ v_a/v_1 = 1.6 \pm 0.2; \ l_1/l_1 = 0.20; \ v_1/v_1 = 0.22 \pm 0.03 \).

Calculated concentrations of phospholipids, \( \alpha \)-glycans and substances containing amide groups (chitin, protein) are shown in Table 2.

The absorption bands at 1639–1641 cm⁻¹ and 1033–1100 cm⁻¹ are complex, i.e. they include absorption of several groups:

\[ D = \sum \varepsilon_i C_i \cdot l \]
Table 2. Concentrations of phospholipids, α-glycans and substances containing amide groups in conidia walls

<table>
<thead>
<tr>
<th>Microscopic fungus</th>
<th>Mass parts</th>
<th>Contact angle (grade)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amide</td>
<td>Phospholipid</td>
</tr>
<tr>
<td><em>Penicillium funiculosum</em></td>
<td>0.01</td>
<td>0.73</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>0.56</td>
<td>0.33</td>
</tr>
<tr>
<td><em>Trichoderma viride</em></td>
<td>0.28</td>
<td>0.54</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>0.58</td>
<td>0.34</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>0.30</td>
<td>0.54</td>
</tr>
<tr>
<td><em>Chaetomicin globusum</em></td>
<td>0.57</td>
<td>0.35</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>0.52</td>
<td>0.39</td>
</tr>
<tr>
<td><em>Penicillium cyclopium</em></td>
<td>0.49</td>
<td>0.38</td>
</tr>
<tr>
<td><em>Paecilomyces variotii</em></td>
<td>0.69</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Analysis of experimental data showed that these bands are consistent with absorption by both amides and α-glucans.

According to our data, the general content of the main components in the surface layer for nine sorts of microscopic fungus is: α-glycans, 0.05–0.25; phospholipids, 0.26–0.72; amides, 0.01–0.69.

Analysis of literature data on the composition of fungus walls also gives different correlations. According to data previously recorded, the ratio of glycans in the external and internal walls of *Cimitic conidia* was found to be 3:2, i.e. glycans are mostly concentrated in the external wall. According to the results of Ref. 12, the external wall of several sorts of fungus contains 50% protein, 25% phospholipid and up to ~15% glycans. Other studies give no information on lipid content in the analysis of fungus wall composition, but discuss glycans and chitin ratios (6:1). Different information obtained in the analysis of the conidia wall should be connected, first of all, with different methods of sample preparation: disintegration, dissolving, different biochemical reactions. Claims have been made, based on analysis by NMR-spectroscopy of five types of gram-positive bacteria, that peptidglycans are predominant compounds in the cell walls of bacteria.

As has been mentioned above, contact angle θ is a parameter which characterizes fungus wall wetting by water. To determine the connection between the θ value and wall composition for each fungus a correlational equation was postulated that reflects the dependence of θ on the sum of components with weighting coefficients for each component’s contribution (α, β, γ).

\[ \log \theta = \alpha C_\alpha + \beta C_\beta + \gamma C_\gamma \]  

Solving consistently the equations for nine sorts of fungus, we obtained coefficient values characterizing the contribution of each substance to the surface properties of fungus wall controlling water wetting, i.e. surface hydrophobicity:

\[ \alpha = 1.85 \pm 0.05; \quad \beta = 2.6 \pm 0.1; \quad \gamma = 0.75 \pm 0.05 \]

Thus, the external wall contribution to hydrophobicity is dominated by phospholipids via coefficient β, whose value is 1.5–3.5 times higher than other coefficients.

REFERENCES