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On the origin of the electrostatic surface potential of *Aspergillus niger* spores in acidic environments

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Abstract

The electrostatic surface potential of fungal spores is generally regarded as potentially influencing spore aggregation and pellet formation in submerged cultures of filamentous fungi. Spores of *Aspergillus niger* are typically characterized by negative zeta potentials over a wide range of pH values. In this study, this particular behavior is ascribed to the presence of an extensive melanin coating. It is proposed on the basis of zeta potential and pigment extraction experiments that this outermost layer affects the pH-dependent surface potential in two manners: (i) by the addition of negative charges to the spore surface and (ii) by the pH-dependent release of melanin pigment. Chemical analyses revealed that deprotonation of melanin-bound carboxyl groups is most probably responsible for pigment release under acidic conditions. These findings were incorporated into a simple model which has the ability to qualitatively explain the results of zeta potential experiments and, moreover, to provide the basis for quantitative investigations on the role of electrostatics in spore aggregation.

Keywords: *Aspergillus niger*; fungal spore; melanin; electrostatics

1 Introduction

The filamentous ascomycete *Aspergillus niger* is widely used in industrial processes. In general, there is a strong connection between productivity and morphology of growing filamentous fungi (Grimm et al., 2005b). Previous studies have shown that the early stage spore aggregation is an important step in the morphological development of *A. niger* (Elmayergi et al., 1973; Galbraith and Smith, 1969; Grimm et al., 2004).

A parameter often used to characterize the aggregation behavior of microbial cells is the zeta potential. The zeta potential is the electric potential at the plane of shear of a charged particle migrating in an electric field. It can be calculated directly from the electrophoretic mobility of the cell, for example, by the Smoluchowski equation (Lyklema, 2003). Even though the measurement of this quantity is straight forward, the quantitative interpretation in terms of repulsive forces acting between two biological surfaces is not necessarily an easy task. Nevertheless, the role of electrostatic repulsion in the inhibition of spore aggregation has been discussed by various authors (Dynesen and Nielsen, 2003; Gerin et al., 1993; Jones et al., 1988). In a former investigation the effect of pH and ionic strength on the adhesion force between single *A. niger* spores was studied by us (Wargenau and Kwade, 2010). The results suggested that also the deaggregation must be considered to be affected by the surface charge. Therefore, a closer view on the water–spore interface may be worthwhile in both respects, aggregation and deaggregation.

Unfortunately, there is little literature dealing with surface charge determining components of the fungal spore wall. Earlier trials in this area were made using microelectrophoresis (Fisher, 1973). In a study of *A. niger*, Hannan (1961) concluded from the observation of only negative mobilities that the spore surface contains no or only little protein. In fact, he attributed his results to the presence of polysaccharides. However, his assumptions are not in line with the chemical analyses of the outer spore wall of two different *A. niger* strains investigated by Cole and Pope (1981) and Cole et al. (1979).

The aim of this study was to determine the origin of the electrostatic surface charge under acidic conditions in order to get a deeper insight into the aggregation behavior of *A. niger* spores. For this purpose the melanin coating of the spores, which is extractable in aqueous solutions over a wide range of pH values, was focused upon, as investigations of an albino mutant have shown that the lack of melanin pigment is linked to positive zeta potentials at low pH values (A. Fleißner and A. Wargenau, unpublished observations). Furthermore, melanin from *A. niger* contains ionizable functional groups (Zavgorodnyaya et al., 2002), and it is therefore expected to affect the net surface charge. Consequently, the investigations were particularly aimed at characterizing the electrokinetic properties of the spores, the pH-dependent release of pigment and the chemical nature of extracted wall material.

2 Material and methods

2.1 Organism, cultivation and spore suspensions

A. niger AB1.13 (Mattern et al., 1992) was used as a model strain. The fungus was grown on a potato dextrose agar (Roth, Karlsruhe, Germany) with 0.01 M uridine for 4 days at 37 °C. The harvesting and storing of spores was conducted using a solution of 9 g l^{-1} NaCl adjusted to pH 3 with HCl. The spore concentration of the final suspensions was determined by counting in a hemocytometer. In case of extraction experiments and zeta potential determination, spores were allowed to settle on the bottom of a petri dish, and the spore concentration was calculated from at least 800 spores counted under an inverted microscope.

2.2 Extraction experiments

The extraction of melanin pigment was carried out in spore suspensions of different pH and spore concentration. In case of pH-dependent extraction, a spore concentration of 10^7 ml^{-1} was used. Extraction solutions were prepared from HCl (pH 1, 2, 3.5), McIlvaine citrate-phosphate buffer (pH 4.5, 5, 6, 6.5, 7), 0.125 M Tris-HCl buffer (pH 8.5) and NaOH (pH 10.5).

Spores were first washed in 0.01 M HCl and then resuspended in the extraction solutions by placing test tubes in an ultrasonic bath (Sonorex TK20, Bandelin, Berlin, Germany) for 1 min. After 30 min of shaking at 25 °C, the suspensions were centrifuged and the supernatants were separated for UV-vis investigation. A DU 720 spectrophotometer (Beckman Coulter, Krefeld, Germany) was used to measure the absorbance of suitable dilutions of the supernatants against the appropriate blanks. The pH-dependent degree of extraction was determined at 430 nm by comparison with the absorbance of maximum extraction. This value refers to the extraction in 0.1 M NaOH and was calculated from a dilution curve by linear regression. The relative release of pigment with varying spore concentration refers to a value of maximum extraction at a spore concentration of 10^7 ml^{-1}.

2.3 Transmission electron microscopy analysis

Before preparation for transmission electron microscopy (TEM) analysis, spores were washed in 0.01 M HCl, and the melanin was separated from the spore walls of a part of the sample. For this purpose, spores were resuspended four times in 0.1 M NaOH by sonication for at least 1 min.

Fixation was achieved in 1 × PBS (pH 7.4) containing 5% formaldehyde and 2% glutaraldyhde. After fixation, samples were washed in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose, pH 6.9) and thereafter osmificated with 1% aqueous osmium tetroxide for 1 h at room temperature. After washing again, pellets were embedded in 2% water agar and cut into small cubes. Dehydration was achieved with a graded series of acetone (10%, 20%, 50%, 70%, 90%, 100%) for 30 min on ice. In the last step, samples were allowed to reach room temperature and were infiltrated with an epoxy resin according to Spurr's formular for a hard resin (Spurr, 1969) (1 part acetone and 1 part resin for 24 h, 1 part acetone and 2 parts resin for 24 h, pure resin overnight and several changes of pure resin the following two days). Samples were then transferred to resin filled gelatine capsules and polymerized for 10 h at 70 °C. Ultrathin sections of about 80 nm were cut with a diamond knife and picked up with formvar-coated copper grids (300 mesh).

The sections were examined under a TEM910 transmission electron microscope (Zeiss, Oberkochen, Germany) at an acceleration voltage of 80 kV. Images were recorded digitally using a slow-scan CCD camera (Proscan, Lagerlechfeld, Germany) and iTEM software (Olympus Soft Imaging Solutions, Münster, Germany).

2.4 Zeta potential determination

The electrophoretic mobility of spores was measured using a Zetamaster S (Malvern Instruments, Malvern, UK). In order to do this, spores were dispersed in solutions of pH 2 to 6 at a concentration of 10^6 ml^{-1} by sonication for 1 min. The solutions contained 1 mM citric acid, 0.05 M NaCl and were adjusted with either HCl or NaOH to the desired pH. Determination of the electrophoretic mobility of spores after pigment extraction was achieved in 0.05 M NaCl containing 0.01 M HCl. Extraction was conducted using McIlvaine citrate-phosphate buffer solutions as described above.

The zeta potential was calculated from the mean of five measurements using the Smoluchowski equation (Lyklema, 2003).

2.5 Preparation of the alkali-soluble wall material

Spores were mixed in 0.1 M NaOH on a vortex mixer for a few seconds at ambient temperature in order to seperate the melanin coating from the spore walls. The suspension was centrifuged twice at 16100 × g (5 min), and the wall material was precipitated for 30 min by adjusting the pH of the supernatant to 1 with HCl. The precipitate was centrifuged at 16100 × g (10 min), washed with deionized and distilled water and centrifuged again. After drying to constant mass (48 h, 65 °C), the obtained material was stored in a desiccator.

2.6 Conductometric titration experiment

Conductometric titration of the alkali-soluble wall material was performed in a flask placed in a water bath for temperature stabilization. Temperature and specific conductivity was measured with a WTW LF 530 conductivity meter (Weilheim, Germany). Nitrogen was bubbled into the sample solutions before and during titration.

In order to prepare the sample solutions, 1 to 2 mg of the alkali-soluble wall material was weighed, dissolved in 0.5 ml 0.1 M NaOH and added to 100 ml of deionized and distilled water. Samples were then adjusted to pH 3.5 with HCl and titrated with 0.1 M NaOH. In addition to the titration of three sample solutions, a blank experiment was performed in the absence of wall material.

Temperature induced variations in the specific conductivity were corrected in the data analysis using a temperature coefficient of 0.02 K^{-1}. Additionally, the variable part of the Na^{+}-conductivity was subtracted, and resulting data points were interpolated with cubic splines. Curves obtained from the alkali-soluble wall material were compared with the blank curve by matching the minimum specific conductivities and the corresponding lines of the first five recorded data points (linear regression). The number of titratable groups between pH 3.5 and 7 was then determined from the mean difference in volume between the minima. Accuracy of the whole method was verified by titration of acetic acid solutions of known concentration.

2.7 Protein and carbohydrate determination

A weighed amount of the alkali-soluble wall material (1.81 mg) dissolved in 10 ml 0.1 M NaOH was used for carbohydrate determination and amino acid analysis after enzymatic or acid protein hydrolysis.

The determination of carbohydrates was conducted by the phenol-sulfuric acid method (Dubois et al., 1956) using an appropriate mixture of the sample solution and sulfuric acid as a blank. D-glucose was chosen as a standard.

For the purpose of hydrolysis, a part of the sample solution was neutralized with 1 M HCl and mixed with an equal volume of the respective double-concentrated hydrolysis reagent. Enzymatic hydrolysis was performed in 0.01 M Tris-HCl (pH 8.5) containing 0.5 g l^{-1} proteinase K (Sigma-Aldrich, Taufkirchen, Germany) and 1 mM $CaCl_2$. This mixture and a control sample without wall material were incubated at 37 °C for 24 h. Acid hydrolysis was carried out in 6 M HCl in a sealed tube under nitrogen at 110 °C for 24 h. The product was dried in a desiccator under reduced pressure over NaOH pellets and subsequently dissolved in 1.1 mM NaOH. Finally, all samples were centrifuged (20 min, 15000 × g) through a membrane with a molecular weight cut-off of 10 kDa (Vivaspin 500, Sartorius Stedim Biotech, Göttingen, Germany) in order to separate amino acids from high molecular weight compounds.

Amino acids were quantified by an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) using fluorescence detection, pre-column derivatization with ortho-phthalaldehyde and α-aminobutyrate as an internal standard as previously described (Krömer et al., 2005). In this study, a Gemini column (5 µm, 150 × 4.6 µm, Phenomenex, Aschaffenburg, Germany) was used and 3-mercaptopropionic acid was applied as a thiol reagent.

3 Results

3.1 Pigment extraction

A. niger melanin is generally regarded as water-soluble (Wheeler and Bell, 1988). Extraction of melanin pigment in aqueous solutions of different pH yielded brown colored but clear supernatants. All spectra obtained from UV-vis investigations of these solutions were of similar shape, with maxima at around 290 and 430 nm. The broader peak in the visible region, which has been previously ascribed to a particular chromophore associated with the native melanin pigment of *A. niger* (Ray and Eakin, 1975), was used to quantify pigment release.

Although melanin from *A. niger* is typically isolated by alkaline treatment (Koroleva et al., 2007; Ray and Eakin, 1975; Saiz-Jimenez et al., 1995), it was partially extracted even under acidic conditions. This can be taken from the absorption bands shown in Fig. 1a. The degree of extraction as calculated from the absorbance at 430 nm is depicted in Fig. 1b. The amount of released pigment increased with the pH value, but not continuously. A plateau between pH 6 and 7 indicated that extraction took place in at least two stages. Furthermore, the equilibrium concentration of melanin in the solution was achieved within a short time, suggesting that fast deprotonation of functional groups (e.g. carboxyl and phenol groups) was responsible for the step-by-step increase. In order to corroborate this assertion, a least square fit was performed on the experimental data up to pH 7. This was done under the simplified assumption of a proportional relation between the amount of released pigment and the degree of dissociation of the corresponding functional group. The best fit was achieved for a pK_a value of 4.3 (see Fig. 1b).

In addition to the investigation of the pH-dependent extraction behavior, experiments with varying spore concentration were performed. The results are illustrated in Fig. 2. The relative amount of pigment released at pH 6 increased linearly with the concentration of spores suspended in the extraction solution. The melanin content in buffer solutions of pH 4.5 was, as expected, lower but showed qualitatively the same dependence on the spore concentration. However, when spores were exposed to pH 6 buffer before measuring extraction at pH 4.5, almost no pigment was found in the second buffer solution. The slight increase of corresponding data points may even have originated from remaining pellet water after removal of the first buffer.

3.2 Ultrastructural aspects of the spore wall

In order to locate the melanin pigments, transmission electron micrographs of spores without alkaline pretreatment (Fig. 3a and b) were compared with micrographs of spores exposed to 0.1 M NaOH before preparing for TEM analysis (Fig. 3c and d). Note that the preparation procedure involved the fixation at pH 7.4 (see Material and Methods), and therefore, some pigment was lost even from untreated spores. Nevertheless, in contrast to the pretreated spores, the untreated ones showed an obvious surface coating of electron dense material (outermost layer) adjacent to the outer wall layer. The relatively smooth surface of the alkali treated spores suggested that a distinct boundary exists between this layer and the surface coating. Untreated spores appeared to exhibit a fluffier surface structure. Beyond that, they were associated with a higher amount of loose wall fragments. These fragments are possibly remnants of the initial wall (Tiedt, 1993) or parts of the coating itself, which may have arisen from the effect of simultaneous fixation and pigment release.

3.3 Zeta potential

The pH-dependent zeta potential was determined from the electrophoretic mobility of spores suspended in different buffer solutions of pH 2 to 6. The results are shown in Fig. 4. The potential of untreated spores decreased almost linearly with increasing pH. Moreover, all values obtained were negative, and therefore, no isoelectric point was apparent. The impact of the melanin on the zeta potential was examined from spores that were pretreated at pH values between 4 and 6 in order to partly remove the melanin from the spore walls. Mobility measurements, in this case, were performed at pH 2 to avoid the effect of further pigment release in the measurement solution. As a result of the pretreatment, the zeta potential was shifted to positive values. Although this shift was more pronounced the higher the pH, the difference in the potential between spores pretreated at pH 4 and those pretreated at pH 4.5 was more than three times greater than that which was obtained from spores pretreated at pH 5 and pH 6. Investigation of spores of completely removed coating was unfortunately not feasible because pellets obtained after centrifugation could not be resuspended sufficiently.

3.4 Analyses of the alkali-soluble wall material

The alkali-soluble wall material, which was isolated from the spore walls by a brief alkaline treatment (see Material and methods), was investigated by conductometric titration. Since it was not possible to completely dissolve the dried material in pure water, three weighed samples were dissolved in 0.1 M NaOH before dilution in distilled and deionized water. These mixtures were then adjusted to pH 3.5 with HCl and titrated with 0.1 M NaOH. A blank experiment without the addition of wall material was also performed. An exemplary titration curve is depicted together with the data from the blank experiment in Fig. 5. In the presence of wall material the minimum (approx. pH 7) was shifted to a larger titrant volume, indicating deprotonation of carboxylic acids. The total amount of acid groups titrated between pH 3.5 and 7 was calculated to be 1.83±0.08 mmol g^{-1} .

Melanin from *A. niger* is believed to be associated with proteins and possibly also with carbohydrates (Swan, 1974). However, the mass fraction of the carbohydrates as estimated from the phenol-sulfuric acid method based on a glucose standard was relatively small (approx. 4%). In order to examine whether side-chain carboxyl groups of proteins were responsible for the large amount of titratable groups, HPLC amino acid analysis was performed after hydrolysis of the extracted wall material. Concerning hydrolysis two strategies were followed. The first of which was a standard acid hydrolysis with 6 M HCl for 24 h at 110 °C. The second was an enzymatic hydrolysis with proteinase K. Comparison of the HPLC profiles of both samples with that of the control sample of the enzymatic hydrolysis did not reveal any significant difference. It was thus evident that no or only very little protein was associated with the precipitated wall material.

4 Discussion

One major application of the zeta potential is in the prediction of the stability of dispersions or suspensions. This is because prevention of aggregation of adjacent particles through electrostatic repulsion is more pronounced the higher the absolute value of the surface potential is. In the case of *Aspergillus* spores, however, several authors were not able to correlate the zeta potential with the tendency for spore aggregation or pellet formation in the culture broth on the basis of this assumption (Dynesen and Nielsen, 2003; Grimm et al. 2005a; Seviour and Read, 1985). An explanation for this may be found in the manifold of influencing factors on the electrostatics of the soft spore surface, especially in solutions of complex composition. Therefore, understanding the mechanism of how the surrounding solution affects the spore surface will help to reveal the impact of electrostatics on spore aggregation.

It was shown in this study that the surface coating on top of the spore wall plays a crucial part in the electrostatic nature of the spores of the investigated *A. niger* strain. It is clear from TEM analysis after pigment extraction that the outermost layer contains melanin, which is similar to other *Aspergillus* species, for instance, *A. nidulans* (Claverie-Martin et al., 1986, 1988) or *A. fumigatus* (Youngchim et al., 2004). Although melanin from *A. niger* is generally regarded as water- or alkali-soluble, the results obtained from extraction experiments with varying spore concentration under acidic conditions cannot be explained in terms of a pH-dependent pigment solubility. In this case, the equilibrium concentration would not change with the spore concentration as long as there is sufficient melanin on top of the spore surfaces. In fact, the linear increase in the amount of extracted pigment indicates that the pH-dependent equilibrium responsible for pigment release takes place at each individual spore surface independently of the melanin concentration in the solution. This assumption is confirmed by the strong diminution in pigment extraction at pH 4.5 due to pretreatment at pH 6. Upon consideration of a surface-controlled pigment release it is obvious that no further extraction appears, once the pH specific reduction of the melanin at the spore wall has been exceeded at higher pH. In this context it is interesting to note that the harvesting and storing of spores were performed in solutions of pH 3. Accordingly, it cannot be excluded that additional extraction steps at pH values smaller than 3 exist, even if not apparent from Fig. 1b. However, since all spore suspensions were prepared the same way, this fact does not constrain the interpretation of the results of this study.

The shape of the pH-dependent extraction curve suggests that deprotonation of carboxyl and phenol groups plays a decisive part in the mechanism of pigment release. Extraction data up to pH 7 could be described very well under the assumption that the amount of extracted pigment is proportional to the pH-dependent degree of dissociation of a carboxyl group with corresponding pK_a value of 4.3. Even if the exact relationship between pigment release and pH is still unclear, this finding is a strong indication of the presence of carboxyl groups somehow associated with the pigment. Moreover, the total amount of acid groups titrated between pH 3.5 and 7 is very similar to the amount of carboxyl groups observed in melanin preparations from *A. sydowi* and *A. glaucus* (Paim et al., 1990; Senesi et al., 1987). Although it cannot be presumed that the chemical composition of the titrated precipitate was fully representative of the surface coating, it is evident from chemical analyses that the large amount of acid groups found in the redissolved precipitate is not due to the presences of proteins or polysaccharides. It is therefore most likely that the melanin molecule itself contains carboxyl groups.

The determined zeta potentials of untreated spores reflect the typical electrokinetic behavior of *A. niger* spores under acidic conditions (Grimm et al. 2005a; Hannan, 1961; Seviour and Read, 1985). The shift in the potential at pH 2 due to pretreatment at higher pH values is in line with the pH-dependent release of pigment. Since it is obvious that washing the spores at certain pH levels reduces the alkali-soluble surface coating, it can be stated that the increase in the zeta potential at pH 2 is linked to a decrease in the thickness of this layer. Hannan (1961), who also investigated the effect of washing of *A. niger* spores, ascribed the mobility shift to the hydrolysis of esters at the spore surface. However, a hydrolyzation mechanism alone cannot explain a pH-dependent equilibrium concentration of melanin in the solution. Instead it appears more probable that the melanin pigments are physically aggregated to the outer wall layer, forming a net negatively charged coating. Deprotonation of acid groups would then entail deaggregation, and shift the total net charge at pH 2 in the positive direction.

A schematic representation of a simple model, which qualitatively explains measured zeta potentials, is given in Fig. 6. The underlying approach is based on the idea that the surface coating is net negatively charged even at low pH values, which may arise from partial ionization of the carboxyl groups. Thus, the coating has the ability, depending on its thickness, to more or less compensate for the charges of the spore wall, leading to surface potentials around zero. Here, the ionic strength of the solution and the accessibility of counter- and co-ions into the coating will be decisive for the actual surface potential (Makino et al., 1987). At higher pH values, where deprotonation of carboxylic acids is more pronounced, however, negative surface potentials will appear. Our hypothesis is that this is accompanied by a change in the net charge of the spore wall. It is even possible that the coating under certain conditions attenuates the decrease in the surface potential by the release of pigment. An increase of the surface potential, on the other hand, is to be expected if the pH is decreased again after reduction of the outermost layer. It is quite conceivable that such a procedure can lead to a positive surface potential as indicated on the right hand side of Fig. 6.

It can be summarized that the electrostatic spore properties are in numerous respects very sensitive against the composition of the surrounding medium. Accordingly, determination of the zeta potential under quite specific conditions alone will not necessarily lead to an adequate prediction of aggregation or pelleting behavior in the culture medium. The thickness of the surface coating, the ionic strength of the medium and also the factors attributed to the individual ion properties, for instance, as such as the accessibility into or a specific adsorption within the coating, will have a determining influence on the pH-dependent repulsion between the spores.

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Figure 1: (a) UV-vis spectra of equally diluted extraction solutions recorded in the region 350–700 nm. (b) pH-dependent degree of extraction determined from the absorbance at 430 nm. The fitted curve represents the extraction step around pH 4.3 (see text).

Figure 2: Relative amount of extracted pigment as a function of spore concentration. All the data refers to the value of maximum extraction at a spore concentration of 10^7 ml^{-1}. The diagram displays the relationship between pigment release and spore concentration at pH 6 and pH 4.5 and the reduction in pigment release at pH 4.5 after extraction at pH 6.

Figure 3: TEM images of *A. niger* spores with and without alkaline pretreatment showing the inner wall layer (IL), the outer wall layer (OL), a dark (electron dense) surface coating (SC) and wall fragments (WF). The micrographs reveal the absence of the surface coating on top of the alkali treated spores. The bars represent 0.1 μm (a, c) and 0.5 μm (b, d).

Figure 4: Zeta potential of *A. niger* spores. The pH-dependent potential of untreated spores is depicted together with the zeta potential at pH 2 obtained after pigment extraction.

Figure 5: Conductometric titration curves of HCl with and without alkali-soluble wall material. The diagram shows specific conductivities after data processing (see Material and methods). In the presence of alkali-soluble wall material (ASWM) the minimum of the titration curve is shifted to a larger volume of the titrant.

Figure 6: Impact of the alkali-soluble surface coating (SC) on the surface potential Ψ_S at the spore–solution interface. The potential arises from the spatial ion distribution across the outermost layer and the solution, the negative space charge density ρ in the surface coating and the pH-dependent net charge of the spore wall, represented by the surface charge density σ at the boundary to the outer layer (OL). Deprotonation of melanin molecules leads to an irreversible reduction in the thickness of the outermost layer δ through pigment release.