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# Automated image analysis to observe pellet morphology in liquid cultures of filamentous fungi such as the basidiomycete *Coprinopsis cinerea*

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**Abstract**

In this study, an image analysis system for fungal pellets was developed using the commercial software system analySIS® and the protocol was evaluated in morphological studies of pellet formation in submerged cultures of the basidiomycete *Coprinopsis cinerea*. Pellets were analysed on large scale (60 to 130 pellets per image, 225 to 400 pellets per culture). Morphologies of pellets were characterised by the parameters grey value, pellet area, convexity, shape factor, sphericity and pellet diameter. Threshold values were defined for all parameters for object filtering. By application of the parameter filter, aggregated hyphal fragments present in larger amounts particularly in cultures grown at higher temperature ($37^0$C) could be clearly distinguished in image analysis from compact pellets. At a lower growth temperature ($25^0$C), there was little background of loose hyphal material, fungal pellets were regularly shaped and the pellets remained constant in shape and size over a longer cultivation period.

**Key words**

Automated image analysis, filamentous organism, pellet morphology, hyphal aggregates, *Coprinopsis cinerea.*

## Introduction

Various filamentous fungi are used in biotechnology for production of biomass, secondary metabolites, polysaccharides and/or enzymes and other proteins. Usually, filamentous fungi are cultivated in liquid medium in suitable fermenters. Typically, the fungi will grow in a pelleted form (1, 2). Determination of pelleted growth of filamentous organisms in liquid cultures is mostly done by image analysis, which can be conducted manually (3, 4) or automatically (5, 6). Automatic image analysis implies that pictures of the pellet culture are taken and analysed by a computer based programme regarding specific parameters, which can be e.g. pellet concentration and pellet diameter. Studies exist where such an automatic image analysis was performed on liquid cultures of basidiomycetes (7 - 9).

In an early study, Michel et al. (3) analysed manually just 15 pellets obtained from *Phanerochaete chrysosporium* cultures with respect to pellet diameter. Later on, Márquez-Rocha et al. (6) determined by automated image analysis the diameter of *Pleurotus ostreatus* pellets but only 20 pellets per culture were surveyed. However, also in more recent studies of three *Phellinus* species and of *Ganoderma resinaceum* (8, 9) only the diameter of 50 pellets were analysed per fungal sample. In contrast, Gehrig et al. (7) gave a more complete picture of pellets of *Cyathus striatus* during a fermentation process. The authors analysed in total about 2000

pellets and a broad spectrum of parameters, such as pellet concentration, pellet diameters, total pellet volume (by using average diameter values) and pellet density (from the total biomass dry weight and the total pellet volume).

The studies have in common that all used a CCD camera to obtain photos either on the microscopic scale or on a larger scale with images of areas of up to a few $cm^2$ [e.g. 2.25 $cm^2$ in the study by Gehrig et al. (7)]. The photos were required for determination of pellet parameters. For evaluation of the images, different image analysis software was applied, some of which were commercially (6, 8) and some of which were especially written for the analysis of pellet morphology [programme by Defren (10) appointed in Gehrig et al. (7)].

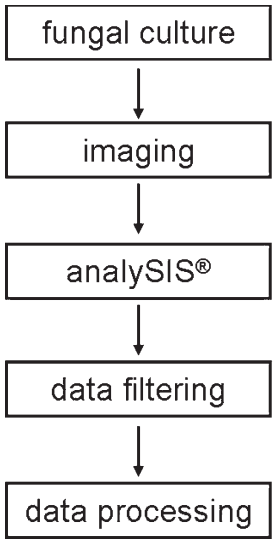



**Fig. 1** Flow chart of the steps to be performed during the process of pellet morphology determination.

In the following, using shake flask cultures of the basidiomycete *Coprinopsis cinerea*, a protocol is defined to be applied for morphological studies of fungal pellets and bioreactor cultures. For the analysis of the fungal morphology in liquid cultures, complete shaken flask cultures or samples taken from the bioreactor cultures are poured onto a bordered glass plate. Detailed images are taken with a CCD camera and the software analySIS® (Soft Imaging System GmbH, Münster, Germany) is used to analyse the fungal morphology from obtained images. The so obtained raw data are filtered by defined parameters that unequivocally distinguish pellets from other objects (small hyphal fragments and loosely aggregated hyphal filaments). Data processing obtains afterwards the final data describing the actual fungal morphology (Fig. 1).

## Material and Methods

### Fungal cultures

A monokaryotic *C. cinerea* strain (FA2222) transformed with the pYSK7 plasmid expressing the laccase gene *lcc1* from *C. cinerea* under control of the *gpdII*-promoter of *Agaricus bisporus* was used (11). The pYSK7-transformant (clone 26) was grown on YMG -agar (per litre: 4 g yeast; 10 g malt extract; 4 g glucose; 10 g agar) plates at $37^0$ C until the mycelium reached the edge of the petri dish. Sterile water ($ddH_2O$) was poured onto the plates and the mycelium with the asexual spores was scraped with a sterile spatula from the agar. Spore solutions were filtered using a sterile funnel filled with glass wool in order to hold back the fungal hyphae. A Thoma counting chamber was used to determine spore concentrations in the solutions.

For shake flask cultures, pre-cultures inoculated with $10^6$ spores/ml medium were prepared in 500 ml flasks filled with 50 ml of modified Kjalke medium [(12); per litre: 10 g yeast, 20 g glucose, 0.5 g $CaCl_2$ x 2 $H_2O$, 2 g $KH_2PO_4$, 50 mg $MgSO_4$ x 7 $H_2O$]. Inoculated flasks were incubated for 4 days at $37^0$C as stationary (standing) cultures. Pre-cultures were homogenised by an Ultra-Turrax® (IKA Werke GmbH & Co. KG, Staufen, Germany) for 30 sec at 8000 rpm and 30 sec at 9500 rpm (rotations per minute). For main-cultures, 500 ml flasks

with 100 ml of sterile modified Kjalke medium supplemented with 0.1 mM $CuSO_4$ were inoculated with each 5 ml of the homogenised pre-culture. Cultivation took place at 25°C and 37°C on a rotary shaker at 120 rpm for 4 and 10 days, respectively. Per culture day and cultivation temperature, two or three parallel cultures were analysed.

Fungal shaken cultures were poured onto a glass plate (28.5 cm x 38.5 cm, Fig. 2) whose edges were sealed by a silicone border in order to keep the liquid samples on the plate. Water (200 to 300 ml) was added to equally distribute the mycelium as a monolayer of pellets on the glass plate. When necessary, pellets lumped together were manually dispersed with the help of a spatula or forceps.

Total fungal biomass was determined after taking images by filtering complete cultures through a Büchner funnel containing a cellulose filter of known dry weight. The filters together with the wet biomass were dried at $80^0$C and the dry weights of the biomass were determined.

**Imaging**

To record pellet growth, the glass plate with a fungal sample was placed onto the illuminated translucent plate of a camera stand (Kaiser Copylizer eVision ini.tial HF, Kaiser Fototechnik GmbH & Co. KG, Buchen, Germany) with a digital CCD camera (Color View II, Soft Imaging System GmbH, Münster, Germany) installed at a distance of 28 cm to the glass plate. Images were recorded with the CCD camera to which a Lametar 2.8/25 objective (Jenoptik GmbH, Jena, Germany) was fixed. Three non-overlapping photographs (dashed lines in Fig. 2) were taken of each culture.

**Analysis of the images**

Pictures were evaluated using analySIS® (Soft Imaging System GmbH, Münster, Germany), a software tool for the analysis of microscopic images of biological materials. The magnification, defined by the specific objective at the CCD camera used at a distance of 28 cm to the specimen, was set to be 0.132 (equivalent to 0.0418 mm/pixel).

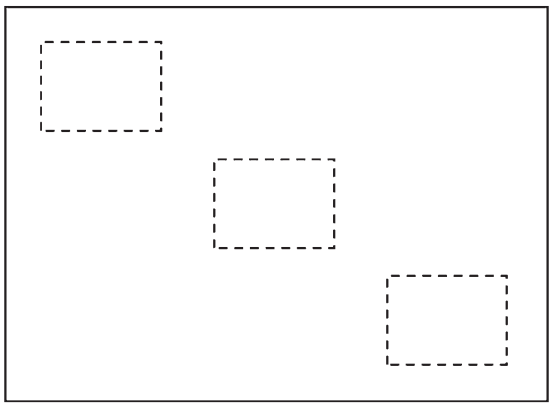


**Fig. 2** Scheme of a glass plate (solid line representing the silicone border; size 28.5 cm x 38.5 cm) onto which liquid fungal cultures are poured and the positions of fields (dashed lines; each field size 6.4 cm x 8.5 cm) of which photographs are routinely taken.

The subsequent steps for the process of automated image analysis using predefined functions of the software analySIS® are explained in the following:

- Function RGB reduction: The coloured photograph (Fig. 3A) were reduced to their RGB (red, green and blue) colours. Of the three pictures obtained, the green monochromatic images (Fig. 3B) gave the best contrast between pellets and background. The green monochromatic images were therefore used for further analysis in form of a grey value scale.
- Function Define ROI: A region of interest (ROI, Fig. 3C) had to be defined and loaded into a picture. This ROI defines the area in the image, where objects were analysed. With the magnification as defined above, a ROI of 40.66 $cm^2$ was used (Fig. 3C).

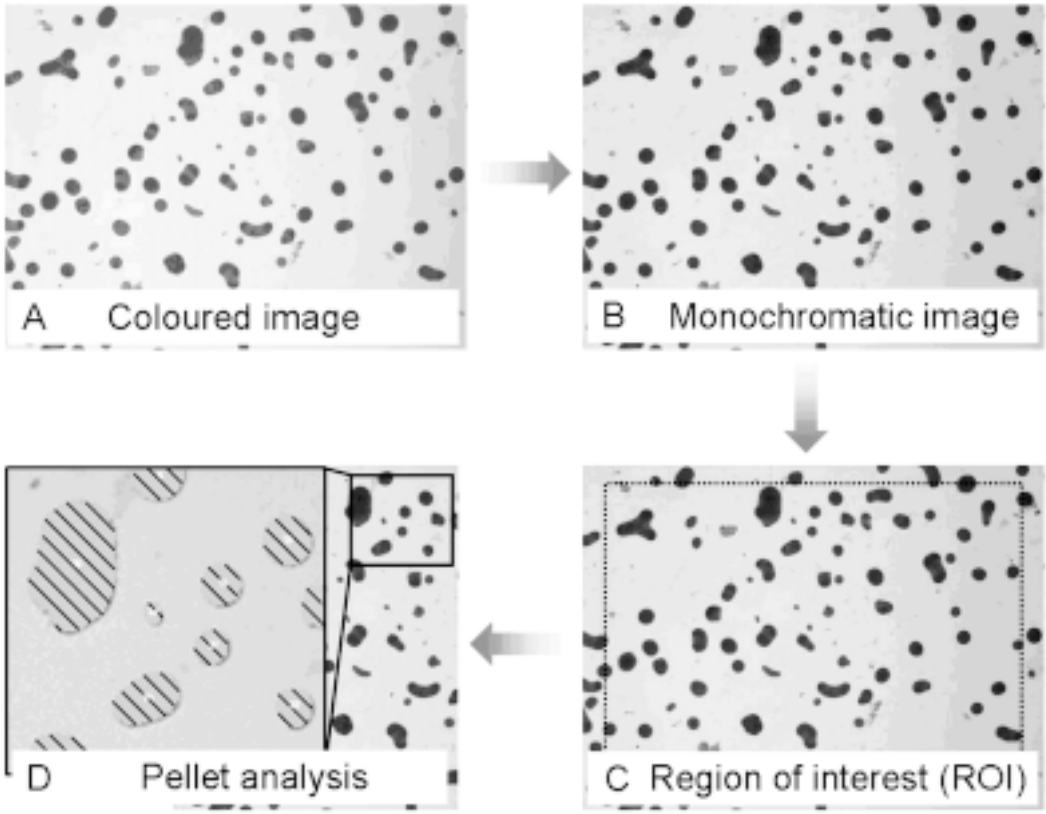



**Fig. 3** Processing of photographs of fungal cultures with the analySIS® software. A photograph of the fungal culture (A) is reduced to a monochromatic image (B). A region of interest (ROI) for pellet determination is defined (C) and, after setting a specific threshold for best distinction of pellets and background (see Fig. 4), the pellets are detected by analySIS® (D) as documented in Fig. 5.

- Function Define measurement: In analySIS®, a pre-defined set of object parameters can be chosen of which the following were selected for pellet detection and description (Fig. 3D): grey value mean, area, convexity, shape factor and sphericity. Moreover, the average diameters of the individual pellets were determined in analySIS® from 180 measurements of diameters per object, i.e. one at every angle.
- Function Set threshold: To distinguish the compact mycelial pellets from loose hyphal fragments as background, the settings of the analySIS® software had to be manually adjusted in the programme to a specific threshold determined by the background in a given monochromatic image. Usually, the threshold was set to 180 in order to reject the large peak of smaller objects (hyphal fragments) and other background within the bimodal distribution of greyness level as shown in Fig. 4.
- Function Define Detection: A projected area per object is determined by counting the pixels per object being connected within the object area due to a same grey value contrast. A minimum pixel amount of 10 per object was set to exclude any remaining

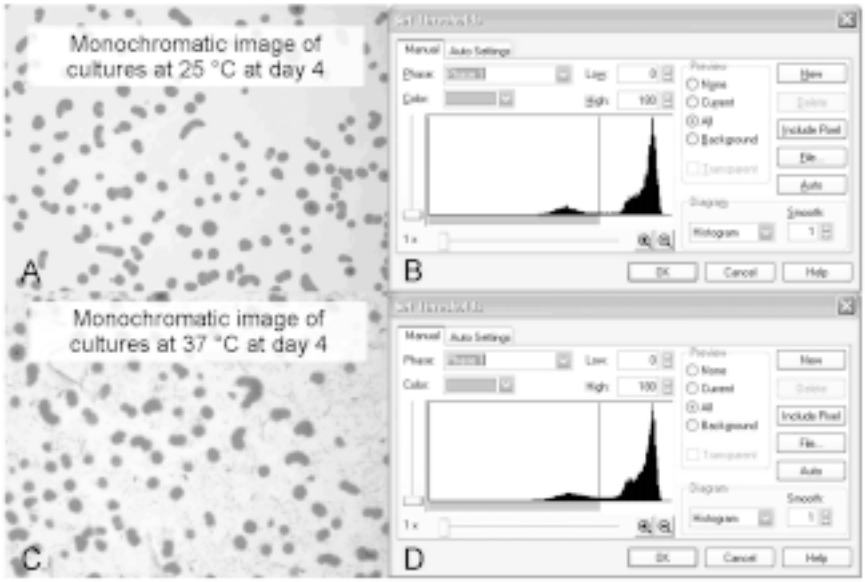



**Fig. 4** Differences in the background noise of culture images taken at day 4 of cultivation of *C. cinerea* in modified Kjalke medium at 25°C and 37°C, respectively (A and C). The bimodal distribution of greyness levels (on a scale from 0 = black to 255 = white; black is at the left and white is at the right side of the histograms shown) deduced from these images and the corresponding settings for defining the background threshold for the monochromatic images are presented in B and D, respectively.

smaller loosely aggregated filamentous fragments from further analysis. Detection of the fungal pellets covered by the ROI was done twice: Include and Exclude. In case of the Include function, all pellets inside the ROI plus those that cross the ROI borders were measured. In case of the Exclude function, only pellets localised fully within the ROI were detected (Fig. 5).

- Function Particle Results: Parameters for all pellets were calculated for the Include and for the Exclude setting, respectively, and obtained data were saved in separate spreadsheet files, where each row presents the information for one specific object and each column specific object parameters as defined above.

**Data processing**

All calculations were done for the raw data set and for filtered data sets. Object data from the three analysed images per culture were combined (Include and Exclude measurements separately) and the amount of objects, the average value of the projected object areas and the average object diameter were calculated with the help of a self-implemented programme constructed with pre-defined analysis objects (average with standard deviation and general histogram used to automatically cluster objects into 19 distinct groups of 0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8, 0.8-1.0, 1-2, 2-4, 4-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, 18-20, 21-25, 25-30, 30-50 and 50-500 $mm^2$ pellet area) of the programming language LabView™. The object data were saved automatically in individual spreadsheet files for

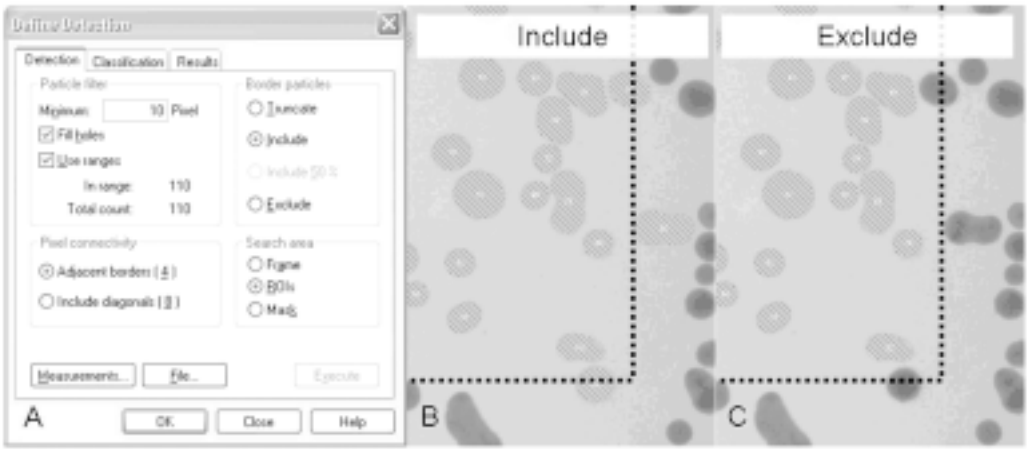



**Fig. 5** Each image was analysed twice by analySIS®. The settings for the determination (A) were used for an Include-recording (B) of the ROI (dashed line). For an Exclude-recording (C), the border particles were respectively set onto function Exclude.

further analysis of Include and Exclude data in Excel (Excel 2002, Microsoft). The average Include data and, respectively, the average Exclude data for the projected area and the object diameter from two or three parallel cultures per growth condition were combined and averaged. Next, values derived from Include and Exclude measurements were averaged in order to include a proportionate fraction of objects into the analysis that either touch or cross the ROI border lines so that the complete ROIs are best covered. For calculating the average amounts of objects per culture, the total amounts of objects in the three ROIs analysed per culture were determined and averaged between the cultures of a same growth condition separately for the Include and Exclude data set, respectively. Subsequently, the values derived from Include and Exclude measurements were averaged and this average value was multiplied by a factor of 9.0 resulting in the average number of pellets per 100 ml culture liquid.

## Results

Upon inoculation of fresh *C. cinerea* cultures in modified Kjalke medium, compact round to oval pellets dominated at day 4 of cultivation in the shaken cultures, both at $25^0$C and $37^0$C. In the monochromatic images of the cultures, pellets had a strong grey-shade. However, at the higher cultivation temperature the background including fine hyphal material was generally higher and, in addition, lighter shaded loosely aggregated hyphal fragments were also present (Fig. 6). In older cultures at day 10 of cultivation at $25^0$ C, there were no larger differences of pellet structure in the views of the cultures as compared to day 4 of cultivation. In contrast, in addition to dark-shaded compact pellets and loose aggregates of filamentous fragments, pellets of less dense structure and lighter grey shading accumulated in the aging cultures grown for 10 days at $37^0$C (Fig. 6).

Particularly in the $37^0$C cultures, the projected areas of some of the loosely aggregated filamentous fragments were larger than the minimum size of 10 pixel (projected area = $0.017mm^2$) per object as fixed for pellet recognition in the analysis software (see below and Fig. 7 for further details). Such filamentous fragments will thus interfere with pellet analysis.

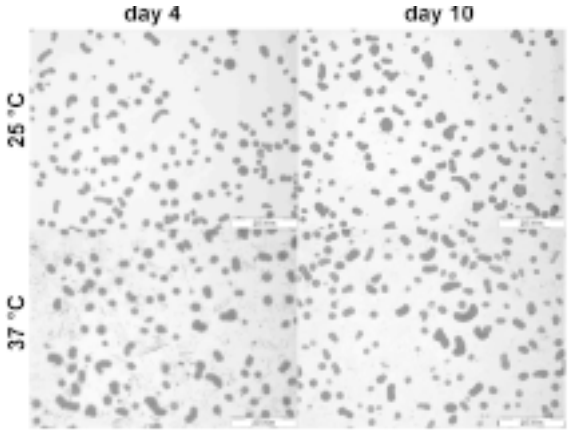
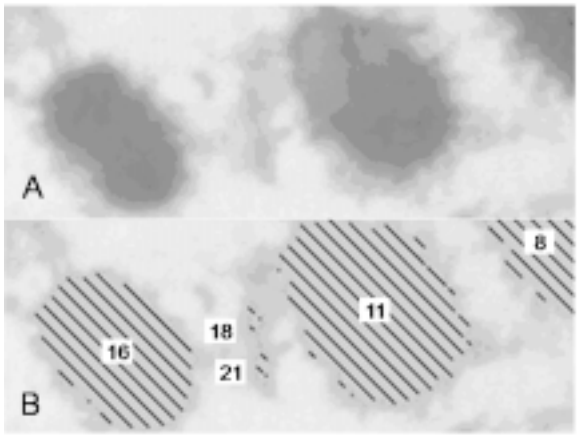



**Fig. 6** Monochromatic images of *C. cinerea* cultures grown for different times in modified Kjalke medium at 25°C and 37°C, respectively. The size bars in the images represent 20 mm.

In addition to the mean grey value and the projected area of an object, convexity, shape factor and sphericity (Table 1) were therefore chosen as other parameters in order to better describe pellet morphology and clearly distinguish between fungal pellets and loose filamentous aggregates. Threshold values for filtering as given in Table 1 were empirically defined in order to confidently exclude any loose filamentous aggregates from further pellet analysis.

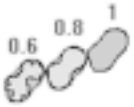
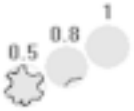
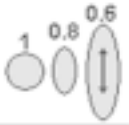
For processing the raw data sets in the spreadsheet files obtained from the function 'Particle Results', at least 3 of the measured values per row (each row defining one detected object) have to pass the defined threshold values for positive filtering of an object as a pellet. If not achieved, a row will automatically be deleted from the raw data set. Fig. 7 demonstrates examples of such positive and negative filtering. Each recognised object in Fig. 7A was identified by a number and overlaid with a shading representing the projected area of an object (Fig. 7B). When comparing the objects in the original monochromatic image (Fig. 7A) with the shades overlaid by the analySIS® programme (Fig. 7B), it becomes clear that the objects with the number 18 and 21 having a projected area above 10 pixel (0.06 and 0.09 $mm^2$, respectively) are not pellets but loosely aggregated filaments. The filter implemented for the pellet data however remove these objects from the raw data set, because the




| Object No | Grey value mean | Area [mm²] | Convexity | Shape factor | Sphericity | Pellets [yes/no] |
|---|---|---|---|---|---|---|
| 8 | 140 | 12.12 | 1.05 | 0.48 | 0.34 | yes |
| 11 | 149 | 9.57 | 0.89 | 0.51 | 0.51 | yes |
| 16 | 144 | 6.47 | 0.94 | 0.75 | 0.43 | yes |
| 18 | 179 | 0.06 | 0.75 | 0.48 | 0.87 | no |
| 21 | 178 | 0.09 | 0.83 | 0.44 | 0.43 | no |
| Threshold value | ≤162 | ≥0.1 | ≥0.7 | ≥0.5 | ≥0.3 | |

**Fig. 7** Monochromatic pellet images taken from a *C. cinerea* culture at day 4 of cultivation at 37 °C (A), shades overlaid by analySIS® on present objects defining object areas for calculation (B) and a data sheet of parameters measured for these objects. The data for each object obtained by the software were used for distinguishing pellets and loosely aggregated hyphal fragments, respectively. Values shaded in grey did not reach the defined limits.

**Table 1** Parameters for definition of pellet morphology

| Parameter provided by the analysis® software | | Description as given by the analysis® software | Threshold value for positive filtering as set in this study |
|---|---|---|---|
|  | Grey value mean | The arithmetic mean of all grey values of the particle | ≤ maximum object grey value in a given image minus 10 % of the maximum grey value * |
|  | Area | The area of a particle is (number of pixels of the particle) times (calibration factors in X and Y direction) | ≥ 0.1 mm$^2$ |
|   | Convexity | The fraction of the particle's area and the area of its convex hull | ≥ 0.7 |
|   | Shape factor | The shape factor provides information about the "roundness" of the particle. For a spherical particle the shape factor is 1, for all other particles it is smaller than 1 | ≥ 0.5 |
|   | Sphericity | Describes the sphericity or 'roundness' of the particle by using central moments | ≥ 0.3 |

* Note that for the grey value mean, the absolute values may vary between different images because of the different background values; e.g. for a maximum grey value of 180 measured in an image, the maximum value for positive filtering is 162 (= 180 - 10% of 180).

threshold values for parameters grey value, area and shape factor were not reached (see Fig. 7 data sheet). In other cases of loosely aggregated filaments, the threshold values e.g. for the combination grey value, shape factor and sphericity or for the combination grey value, convexity and shape factor were not reached (data not shown). In extensive empirical data analysis, a minimum of three parameters were found to be necessary for save pellet filtering from other objects in order not to eliminate very small pellets of low grey shading and/or low convexity from the raw data sets (not further shown).

The defined filter were applied to the raw data sets (Include and Exclude separately), the Include and Exclude data were combined and averaged as described in the methods in order to obtain the average number of pellets per culture. After applying the filter, the number of objects from the raw data sets reduced by 2-24%, respectively, depending on the type of cultures analysed (Table 2). In total, about 225 to 400 pellets (30 to 160 per individual image) were analysed per individual culture. The calculated total pellet numbers per culture varied from about 2300 to 3400 (Table 2).

With the time in 25$^0$C cultures, there was a strong increase in absolute numbers of detected pellets per culture (about 2400 pellets at day 4 to about 3400 pellets at day 10) and a little increase in average pellet area [about 4.8 mm$^2$ at day 4 to

about 5.0 mm$^2$ at day 10; both values corresponded well with the peak value in histograms of the individual pellet areas (not shown)], whereas the average pellet diameter decreased slightly from about 2.57 mm at day 4 to about 2.51 mm at day 10. The percentage of non-pellet objects (loose hyphal aggregates) increased from 2% at day 4 of cultivation to 9% at day 10 of cultivation. These data from automated image analysis confirm the impression of the former rough overview that pellets in cultures at 25$^0$C change little in shape and size within the 6 following days of incubation.

The situation in the 37$^0$C cultures was different. Absolute pellet numbers decreased from about 3000 pellets per flask at day 4 of cultivation to 2300 per flask at day 10 of cultivation, but the average pellet area [corresponded again well with the peak value in histograms of the individual pellet areas (not shown)] increased from 5.1 mm$^2$ at day 4 of cultivation to about 5.5 mm$^2$ at day 10 of cultivation along with the average pellet diameter (from 2.4 mm at day 4 to 2.7 mm at day 10). As documented in Fig. 8, the 4 day-old cultures at 37$^0$ C had generally a higher background of small hyphal fragments and a larger fraction of total detected objects (21%; Table 2) were identified in the raw data set as loose filamentous aggregates. At day 10 of cultivation, the amount of hyphal fragments in the background was lower (Fig. 8) and, in parallel, also the amount of loose aggregated filaments detected by the automated image analysis (9%; Table 2). In summary, the data imply that major morphological changes occur at 37$^0$C during the period of cultivation from day 4 to day 10.

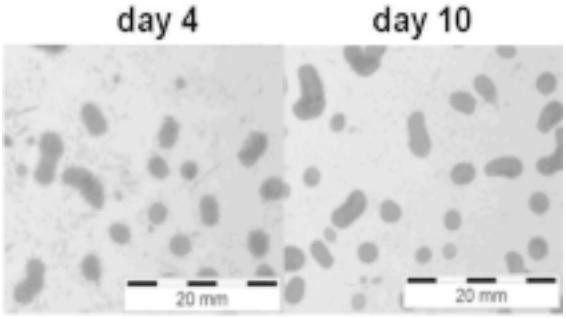



**Fig. 8** Enlargement of sectors of photographs from Fig. 6 showing fungal pellets, small hyphal fragments and loose filamentous aggregates in the culture broth of *C. cinerea* cultivated in modified Kjalke medium at 37 °C for 4 and 10 days, respectively.

At both growth temperatures, the differences in average pellet numbers and average pellet areas (Table 2) corresponded well with changes in biomass. The 25$^0$C cultures had a mycelial biomass of 5.6 ± 0.2 g/l and 8.3 ± 0.1 g/l at day 4 and day 10 of cultivation, respectively. The 37$^0$C cultures had a biomass of 8.3 ± 0.2 g/l and 5.5 g/l ± 0.3 g/l at day 4 and day 10 of cultivation, respectively. At 25$^0$C as the suboptimal temperature for growth of *C. cinerea* (14), 10 days are obviously required to achieve highest biomass yields whereas the reduction in total biomass at 37$^0$C implies that the fungus is at day 10 of cultivation already in a major phase of biomass degeneration.

**Discussion**

Pellet morphology is known to be influenced by culture conditions and, in turn, to affect biomass formation and production yields in submerged fungal fermentations (2, 13). Effects of temperature and age on submerged growth of *Ccinerea* used as an example in this study are obvious from the photographs shown in Fig. 6 and Fig. 8 and from the data presented in Table 2. This fungus has its growth optimum at 37$^0$C (14). Accordingly, we observe that cultures at this temperature have a faster growth rate, reach the highest possible biomass levels earlier and undergo faster biomass degeneration than cultures kept at the lower temperature of 25$^0$ C (Table 2 and Rühl et al. unpublished results). With the lower growth rate and the delay

**Table 2** Pellet distribution in *C. cinerea* cultures at day 4 and day 10 of cultivation in modified Kjalke medium

| Culture age | Cultivation temperature | Raw object data | | | | Filtered object data | | | | Reduction in object number per flask [%] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No of total analysed objects* | Object area [mm$^2$] | Object diameter [mm] | No of objects per flask | No of total analysed pellets* | Pellet area [mm$^2$] | Pellet diameter [mm] | No of pellets per flask | |
| Day 4 | 25 °C | 804 | 4.85 ± 4.24 | 2.57 ± 1.32 | 2411 ± 63 | 786 | 4.81 ± 3.20 | 2.57 ± 1.06 | 2355 ± 58 | 2 |
| | 37 °C | 860 | 4.13 ± 5.27 | 2.06 ± 1.88 | 3866 ± 283 | 677 | 5.10 ± 5.35 | 2.40 ± 1.80 | 3043 ± 86 | 21 |
| Day 10 | 25 °C | 1247 | 4.58 ± 3.89 | 2.36 ± 1.39 | 3739 ± 240 | 1139 | 4.95 ± 3.67 | 2.51 ± 1.24 | 3415 ± 267 | 9 |
| | 37 °C | 839 | 5.24 ± 5.51 | 2.66 ± 1.81 | 2516 ± 199 | 763 | 5.49 ± 4.72 | 2.70 ± 1.38 | 2286 ± 223 | 9 |

* For each culture condition, three flasks were analysed except for day 4 of cultivation at 37°C where only two flasks were used. Absolute numbers of objects analysed per culture condition were added from the two or three cultures, respectively.

in biomass degeneration, the overall pellet structures of the cultures grown at 25$^0$C were much more compact and uniformly than the structures of pellets grown at 37$^0$C. Moreover in the 25$^0$C cultures, there was much less background of small hyphal fragments and of loose aggregated hyphal filaments (Fig. 6 and Fig. 8). *C. cinerea* is a filamentous basidiomycete that can easily be manipulated by genetic transformation (15, 16). The fungus has thus found interest for production of glycosylated enzymes from higher basidiomycetes that will not or only poorly be expressed and most possible wrongly be glycosylated in established ascomycete systems for recombinant enzyme production (11, 17, 18). Laccases are for examples enzymes in focus for recombinant production with *C. cinerea* in efficient fermentation processes (11, 19, Rühl et al. unpublished results). Studying fungal pellet morphology will be beneficial for optimising the fermentation processes and enzyme production yields.

An efficient and easy to apply routine technique for observation and large scale pellet analysis for *C. cinerea* but also other filamentous fungi is presented in this work. By taking three images per culture, 225 to 400 pellets per culture are simultaneously and easily characterised by applying specific functions of the commercial analySIS® software. If higher pellet numbers of the same culture are required, further non-overlapping images might easily be taken of other areas of the glass plates onto which the pellets were poured (compare Fig. 2). Pellets for taking photographs are easily spread on the bordered glass plate from cultures with a pellet number of about 2000-2500 per 100 ml. With increasing pellet numbers, in some cases pellets were found packed in densely clusters on the glass plate and had to be separated manually with a spatula or forceps. When wanting most accurate numbers of pellets per culture, simply diluting the cultures is not as advisable since the fungal pellets sink quickly and are difficult to be kept evenly dispersed in solution. Evenly distributing of

pellets from dense cultures on the glass plate was therefore in our hands the most time consuming step in the procedure leading in the worst case to 20 min preparation time per culture until images could be taken. Subsequent usage of the analySIS[Æ] software helped in a fast and, especially, representative determination of pellet morphology, as up to 400 pellets (or more) can be analysed per culture in a few minutes. Considering the parameters grey value, pellet area, convexity, shape factor and sphericity with the implemented filter, fungal pellets and filamentous aggregates could clearly be recognised at the same time and distinguished as such both when relative low and when relative high amounts of these two growth forms were obtained in *C. cinerea* cultures. Such data will allow to quantify the nature of mycelial aggregates, fragmentation of pellets as well as pellet regrowth (20).

Most literature studies on fungal morphology during submerged cultivation consider only small samples of pellets (see examples for basidiomycetes in the introduction). Gehrig et al. (7), in contrast, observed approximately 2000 pellets by image analysis in a *C. striatus* culture by taking pictures of pellets (1-6 mm in diameter) present in a reference area of 15 mm x 15 mm. Total pellet volume was defined by the authors from determination of an average pellet diameter measured from the images of fungal pellets. Moreover, Kelly (21) studied the ascomycete *Aspergillus niger* and similarly determined the pellet diameter of 500 to 1000 pellets per *A. niger* culture. The description of the method used by Kelly (21) is not presented in much detail in the publication but it appears that also this author used a relatively small reference area with maximum 30 to 35 pellets when these were comparably small and laying quite crowded on the area, forcing the researcher to take many different images for obtaining high numbers of different pellets for large scale analysis. In other cases, pellets of *A.niger* were observed on 1 mm-deep cavity slides in case of very small sizes under a microscope or in case of larger pellets on an adjustable camera stand equipped with a CCD camera fitted with a macroscopic zoom lens. Since the size of the images are restricted by the width of the cavity slide, also in these studies numerous pictures have to be taken for analysing high pellet numbers (20, 22, 23).

The protocol presented here allows analysis of much higher numbers of pellets (60-130 pellets) per single image. Moreover, additional parameters of morphology were considered in this study. For simplification of analysis, Gehrig et al. (7) and Kelly (21) assumed a spherical shape of the fungal pellets. The photographs and images in Fig. 3 to 8 show that fungal pellets may be stretched in one dimension and that pellets can be of fringy shape. Therefore, a definition of the projected area calculated from all connected pixels of an object by the software analySIS® will give much more accurate pellet area values than is possible by only considering the pellet diameters. Although not analysed in more detail in this study, the independently collected individual values for the extra parameters convexity, shape factor and sphericity of the pellets are available from the same analysis if a more deep insight into such extra growth parameters for a more detailed description of pellet morphology in *C. cinerea* will be required. Furthermore, analysing the individual grey shades of pellets can serve to define a relative distribution of more compact and less dense pellets in a culture. The usefulness of such data for morphological classification of fungal pellets by automated image analysis has been demonstrated before for *A. niger* (22, 23).

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