Coordinated Development of Yeast Colonies: An Experimental Analysis of the Adaptation to Different Nutrient Concentrations – Part 1

By *T. Walther**, *H. Reinsch*, *K. Ostermann*, *A. Deutsch*, and *T. Bley*

The development of yeast colonies on solid agar substrates served as a model system to investigate the growth of higher fungi in a heterogeneous environment. Applying a new analytical technique – which was based on the estimation of the intensity of transmitted light from microscopic images taken along the colony radius – cell-density distributions inside fungal mycelia were measured at an extremely high spatial resolution. Using this method, the adaptation of yeast colonies to the limitation of different nutrients was investigated. Under conditions of carbon or nitrogen limitation, populations of the dimorphic model yeasts *Yarrowia lipolytica* and *Candida boidinii* underwent a transition in their morphology from solid colony to mycelial colony patterns. When grown under conditions that induced the mycelial morphology, colonies extended linearly at a constant rate irrespective of the initial nutrient concentration. In general, the cell density within the population declined at higher degrees of limitation. Nitrogen-limited colonies of both model yeasts, as well as carbon-limited *Y. lipolytica* colonies proceeded to extend until the growth field was finally covered by the population. Under these conditions, areas of fairly constant cell densities were formed during the growth process. Only carbon-limited *C. boidinii* colonies stopped extending at a final diameter which was small when compared to the size of the growth field, and formed a cell-density profile which was monotonically declining. The observed differences in the final colony diameter, and in the cell-density profile morphology indicated the presence of different regulatory mechanisms that ruled the colony development of the model yeasts. The presented monitoring technique for the biomass distribution inside fungal populations provided the basis for a quantitative and non-invasive description of mycelial development.

1 Introduction

The design of most biotechnological applications ensures the homogenous distribution of all relevant state variables. This provides a maximum of process control since environmental changes take effect uniformly throughout the system and reproducible process conditions are guaranteed. However, in some technical processes this homogeneous distribution is restricted due to unfavorable properties imposed by the system. In dense cellular aggregates, such as biofilms [1] and fungal pellets [2], the biological phase itself creates a microenvironment that impedes nutrient transport which causes a heterogeneous distribution of nutrients. In solid-state fermentations, i.e., in fermentations of substrates that lack a free aqueous phase, slow transport processes in the solid cause the establishment of concentration gradients due to local nutrient deprivation [3]. In search of the further optimization of bioprocesses characterized by a heterogeneous distribution of nutrients, a detailed knowledge of the interplay of non-uniform environmental changes and the biological phase is essential.

Fungi are known to form interconnected mycelial networks of considerable spatial extension. When these organisms are employed in heterogeneous technical systems, various parts of the same organism may be exposed to different nutrient concentrations. Depending on the species and process conditions, fungi may respond to these non-uniform environmental conditions by the differentiation of their mycelium into distinct cell types [4], translocation of nutrients [5], and a transition from an exploitive to an explorative growth mode [6]. Since the excretion of metabolites and enzymes is often associated with mycelial compartments of a particular physiological state [4, 7], these phenomena certainly influence process performance and possibly the quality of the formed product.

However, despite the obvious economical relevance of adaptation and differentiation processes in fungal mycelia, only little is known about the factors that regulate the growth of fungi in a heterogeneous environment. Even the mycelial development in a comparatively simple model system, as represented by the growth of a fungus in an agar-substrate-filled Petri dish, is far from being fully understood. This has two major reasons: Firstly, the interplay between the extension of the fungal population, the differentiation of the mycelium into different physiological states, the nutrient translocation inside the hyphal network and the dynamically changing environmental conditions make this growth process extremely complex [8, 9]. Secondly, on the population scale, mycelial development is only insufficiently characterized experimentally, e.g., quantitative data about the biomass and nutrient distributions inside fungal mycelia, as well

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as estimates for the translocation of nutrients through the hyphal network are widely missing.

The described difficulties in the investigation of mycelial development directly point to the need for a further simplification of the experimental system and the refinement of analytical methods to investigate mycelial development. In this context, yeasts can serve as model organisms to study the behavior of fungal mycelia at a level of lower complexity. Dimorphic yeasts are able to form filamentous colony patterns similar to higher fungi [10]. In a large number of yeast species these filaments are built up of individual – *pseudo*hyphal – cells. Therefore, the translocation of nutrients inside such *pseudo*mycelia is negligibly small which facilitates the experimental and mathematical [11] characterization of population development based on exclusively extracellular factors. Furthermore, analytical methods are required that allow for the *quantitative* monitoring of cell-density distributions inside fungal mycelia. Only on the basis of such techniques, biomass distributions can be accurately balanced. As was argued in [11], only the quantitative experimental and mathematical description of mycelial development will provide deeper insight into the regulatory phenomena acting during colony development.

In this study, a new monitoring technique that facilitates the quantitative estimation of cell-density profiles in growing mycelia with an extremely high spatial resolution is presented. Applying this technique, the question is addressed, how do yeast populations adapt to nutrient availability in their environment. In particular, the response of *mycelial* colonies of *Yarrowia lipolytica* and *Candida boidinii* to the shortage of an essential nutrient (carbon or nitrogen source) was investigated.

2 Materials and Methods

2.1 Microorganisms and Media

Yarrowia lipolytica H222 was kindly provided by Prof. Gerold Barth from the Institute of Microbiology, TU Dresden, Germany. *Candida boidinii* DSM70034 was acquired from the DSMZ. The microorganisms were kept on YGC (Merk) agar plates at 4 °C and were subcultured every 4 weeks.

Growth media were derived from a standard minimal medium [12] containing 20 g/L glucose (Merck) as the only carbon source, 5 g/L $(NH_4)_2SO_4$ (Merck) as the only nitrogen source, and 1.7 g/L YNB (Sigma) as a defined vitamin and trace-element base. For the preparation of solid growth substrates 2 % [w/v] Bacto agar (Merck) was supplemented to the media. In order to impose various degrees of carbon or nitrogen limitation, the concentration of glucose or ammonium sulfate in the medium was decreased while the content of all other medium components was kept constant. Furthermore, **c**asamino **a**cids (**CA**, acid hydrolyzate of casein, Merck) were used to substitute the standard carbon and nitrogen sources, i.e., glucose and ammonium sulfate. In Tab. 1 the compositions of all growth substrates applied in this study are summarized.

All media were autoclaved for 20 minutes at 120 °C.

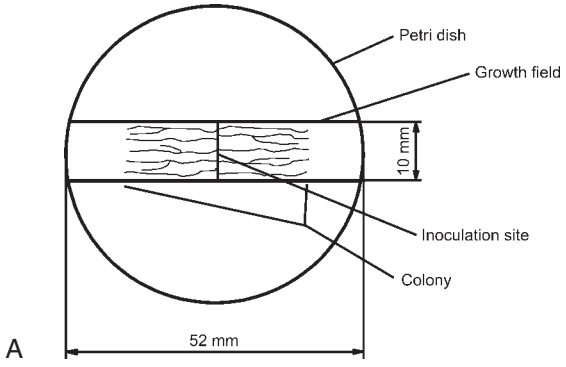
Table 1. Compositions of media applied in this study*.

Notation	Limitation	Carbon source	Nitrogen source
C-G-20	Carbon	20 g/L glucose	5 g/L $(NH_4)_2SO_4$
C-G-4	Carbon	4 g/L glucose	5 g/L $(NH_4)_2SO_4$
C-G-2	Carbon	2 g/L glucose	5 g/L $(NH_4)_2SO_4$
C-G-0.2	Carbon	0.2 g/L glucose	5 g/L $(NH_4)_2SO_4$
C-G-0.02	Carbon	0.02 g/L glucose	5 g/L $(NH_4)_2SO_4$
C-CA-20	Carbon	20 g/L casamino acids	5 g/L $(NH_4)_2SO_4$
C-CA-10	Carbon	10 g/L casamino acids	5 g/L $(NH_4)_2SO_4$
C-CA-2	Carbon	2 g/L casamino acids	5 g/L $(NH_4)_2SO_4$
C-CA-0.2	Carbon	0.2 g/L casamino acids	5 g/L $(NH_4)_2SO_4$
C-CA-0.02	Carbon	0.02 g/L casamino acids	5 g/L $(NH_4)_2SO_4$
N-A-0.5	Nitrogen	20 g/L glucose	0.5 g/L $(NH_4)_2SO_4$
N-A-0.1	Nitrogen	20 g/L glucose	0.1 g/L $(NH_4)_2SO_4$
N-A-0.05	Nitrogen	20 g/L glucose	0.05 g/L $(NH_4)_2SO_4$
N-A-0.005	Nitrogen	20 g/L glucose	0.005 g/L $(NH_4)_2SO_4$
N-CA-20	Carbon	20 g/L glucose	20 g/L casamino acids
N-CA-2	Nitrogen	20 g/L glucose	2 g/L casamino acids
N-CA-0.2	Nitrogen	20 g/L glucose	0.2 g/L casamino acids
N-CA-0.02	Nitrogen	20 g/L glucose	0.02 g/L casamino acids

*) Concentration of YNB was 1.7 g/L in all experiments. In solid substrates the agar content was 20 g/L.

2.2 Cultivation Conditions

The one-dimensional development of *Y. lipolytica* and *C. boidinii* mycelia, respectively, was investigated on various combinations of carbon and nitrogen sources at two different degrees of limitation (applied media: C-G-4 and C-G-2, C-CA-20 and C-CA-10, N-A-0.1 and N-A-0.05, N-CA-20 and N-CA-2). Thin layers of solid growth substrate were created by pipetting 2 mL of autoclaved medium into standard plastic Petri dishes (outer diameter: 60 mm, inner diameter: 52 mm). After solidification of the substrate, growth fields of 10 mm width were cut out from the agar using a sterile scalpel. Yeasts precultured on C-G-20 agar substrate (see Tab. 1) were transferred to the growth fields with a sterile tooth pick. In order to impose one-dimensional mycelial growth, colonies were inoculated as a thin line in the center of the growth field. Petri dishes were sealed using parafilm to prevent drying and incubated at 25 °C in a climate chamber (Friocell, MMM). Details of the experimental setup are illustrated in Figs. 1A and B.



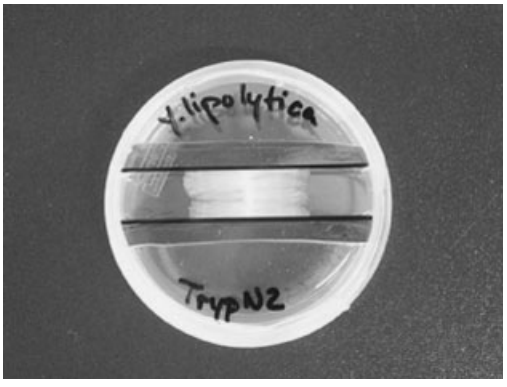


Figure 1A. Schematic view of the experimental setup for the investigation of one-dimensional colony development.
The figure shows the bottom of a Petri dish and the dimensions of the rectangular growth field which was cut out from the agar.

Figure 1B. Experimental setup showing a *Y. lipolytica* colony growing on 2 g/L tryptone as the only nitrogen source.

2.3 Cell-Density Profile Estimations

For every combination of medium composition and yeast, 5 replicate runs were started at a time. Colony diameters were measured at least weekly to monitor the progression of the growing perimeter. Cell-density profiles of the colonies were estimated after 5 weeks of incubation according to the following protocol: Microscopic images were taken along the longitudinal axis of the colonies (microscope: Axioscope 2 FS mot, Zeiss; camera: Hamamatsu C5810; magnification: 12.5x; transmitted light mode (lamp voltage: 1.6 V)) and assembled to obtain a single photograph. Since growth of the colonies was symmetrical, these photographs comprised only one moiety of the colony, i.e., the area between inoculation site and outer edge of the population. Local intensity of the transmitted light ($I_S(x)$) was estimated using the line morphology routine of the OPTIMAS 6.1 image-analysis-software package running in the luminescence mode. (The coordinate (x) denotes the position on the longitudinal colony axis.) The distance between the sampling points was 69 μm. $I_S(x)$ was corrected for background shading by the addition of a correction summand ($g(x)$) yielding the normalized intensity of the transmitted light ($I_{S,n}$)[1)].

$$I_{S,n}(x) = I_S(x) + g(x) \tag{1}$$

The correction summand ($g(x)$) was calculated from the difference between the global maximum intensity of transmitted light ($I_{B,max}$) and the local intensity of transmitted light ($I_B(x)$) estimated from microscopic images of blank growth fields.

$$g(x) = I_{B,max} - I_B(x) \tag{2}$$

The optical density (OD) profile of the colonies was estimated according to the following equation

$$OD(x) = -\log\frac{I_{S,n}(x)}{I_{0,n}} \tag{3}$$

where $I_{0,n}$ represents the normalized intensity of transmitted light measured in the absence of cells.

For every colony, six OD profiles were estimated and averaged. The distance between these profiles was 0.45 mm covering a growth field section of 3.2 mm in width and of a length which was chosen according to the extension of the colonies. OD measurements of at least two different colonies were averaged to yield the final profile ready for analysis.

2.4 Calibration of OD for Local Cell Density

In order to calibrate the colony OD for the local cell density, OD profiles (average of 10 individual profiles, distance

1) List of symbols at the end of the paper.

between profiles: 0.85 mm, distance between sampling points: 36 μm) of glucose-limited *Y. lipolytica* and *C. boidinii* colonies incubated on C-G-2 medium were estimated at different times within the first 36 h of cultivation. Immediately after the OD measurements, the colonies were cut out from the agar, transferred to 1.5 mL plastic tubes (Eppendorf), and incubated at 50 °C for 15 min in 250 μL gel solubilization buffer (Gibco). After the agar gel was completely dissolved, the samples were centrifuged at 15244 × g for 10 min. The supernatant was discharged and the cell pellet was resuspended in 0.5 % Tween 20 to obtain well separated single cells. Samples were diluted to allow for a convenient quantification of cells using an Abbe-Zeiss cell counting chamber.

Since the growth fields were cut out by hand, small deviations in the width of the agar tiles occurred. In order to increase the accuracy of the calibration, the cell number (n_S) estimated on a growth field of width (r) was normalized for the standard width ($w = 10$ mm) according to the relation

$$n_{S,n} = \frac{n_S \cdot w}{r} \tag{4}$$

yielding the number of cells ($n_{S,n}$) on a standard growth field.

Furthermore, the dimensions of suspended cells (obtained as described above) and pseudohyphal cells in colonies that were incubated for up to 28 days were estimated using the line morphology routine of the Optimas 6.1 image analysis software package. In yeast colonies round-shaped cells and pseudohyphal cells coexist throughout the whole cultivation. Since colony morphology and extension rate of the colony diameter were mainly dependent on the behavior of pseudohyphal cells, all cell-density estimations were normalized to the average size of the filamentous cell type. Thus, the total cell number of cells in the calibration experiments ($n_{S,n}$) was corrected for the total number of unit cells (n_C), i.e., pseudohyphal cells, using the relation

$$n_C = \frac{V_{C,s} \cdot n_{S,n}}{V_C} \tag{5}$$

Here, V_C denotes the volume of the unit cells and $V_{C,s}$ represents the average volume of suspended cells.

The number of unit cells in a colony equals the integral of the local cell density ($c_C(x) = [\mu m^{-2}]$) over the area covered by the OD measurements (A).

$$n_C = \int_A c_C(x) \cdot dA \tag{6}$$

Since the calibration factor (K) relates OD to the local cell density according to Eq. (7)

$$c_C(x) = K \cdot OD(x) \tag{7}$$

the combination of Eqs. 6 and 7 yields

$$n_C = K \cdot \int_A OD(x) \cdot dA \tag{8}$$

The distance between the sampling points (u) was very small ($u = 36$ μm). Therefore, the integral in Eq. (8) could be conveniently discretized obtaining

$$n_C = K \cdot \sum_{i=1}^{N} [OD(i) \cdot \Delta A] \tag{9}$$

with N being the number of sampling points

$$N = \left[\frac{A}{\Delta A}\right] \tag{10}$$

and ΔA representing the area between the adjacent sampling points.

$$\Delta A = w \cdot u \tag{11}$$

K was estimated after a linear regression from the ascent of the cell density ($n_C \cdot \Delta A^{-1}$) *vs.* sum of colony OD plot (Eq. (12))

$$\frac{n_C}{\Delta A} = K \cdot \sum_{i=1}^{N} OD(i) \tag{12}$$

For cells grown under different conditions, the calibration factor (K) determined under glucose limitation was corrected for different cell sizes using Eq. (13)

$$K_2 \cdot V_{C2} = K_1 \cdot V_{C1} \tag{13}$$

where K_1 is the known calibration factor measured for cells with a volume V_{C1} and K_2 is the new calibration factor for cells with a volume of V_{C2}.

2.5 Estimation of the Biomass Yield

Biomass yields of both yeasts on different nutrients were determined in shake flask experiments. 250 mL Erlenmeyer flask were filled with 100 mL medium and incubated at room temperature on a rotary shaker at 300 rpm.

In order to ensure the limitation of a particular nutrient, its concentration was strongly decreased when compared to the standard minimal medium (the applied media were (limiting nutrient/medium): glucose/C-G-2, ammonium sulfate/N-A-0.5, casamino acids/C-CA-10, N-CA-1.5). For each combination of nutrient and yeast, two replicate runs were started at a time. Flasks were inoculated with 0.5 mL of a washed yeast suspension obtained from an overnight culture grown on C-G-20 medium (Tab. 1) under otherwise identical cultivation conditions. Biomass growth was followed by OD measurements at 570 nm using a spectrophotometer (Genesis 10UV, Thermospectronic Rochester). Cultivations were aborted when OD remained constant for 2 days. 2 mL of the yeast suspension were transferred to 2 mL plastic tubes of known weight.

Cells were separated from the cultivation medium by centrifugation at 15244 × g for 10 min and washed once with distilled water. The plastic tubes containing the cell pellets were

dried in a climate chamber at 80 °C until weight consistency was reached. Dry biomass content of the tubes was calculated from the weight difference with and without the cell pellet. For each flask, six tubes were filled and dried. The biomass yield (Y) (listed in Tab. 2) was calculated from the average dry biomass content of the flasks at the end of the cultivation and the initial content of limiting nutrient. The inoculum of the flasks was negligibly small.

3 Results and Discussion

3.1 Induction of Mycelial Colony Morphologies

In order to investigate the growth strategies of yeast colonies at different degrees of nutrient limitation, the model organisms *Candida boidinii* and *Yarrowia lipolytica* were cultivated in Petri dishes on solid agar (20 g/L) substrates containing a defined reservoir of food resources. The compositions of the growth media were derived from a minimal medium containing 20 g/L glucose, serving as the only carbon source, 5 g/L ammonium sulfate, representing the only nitrogen source, and 1.7 g/L yeast nitrogen base (YNB), containing all essential vitamins and trace elements [12]. Various degrees of nutrient limitation were imposed by the selective dilution of one essential nutrient (carbon or nitrogen source) in the medium. Furthermore, glucose or ammonium sulfate was substituted by casamino acids to study the influence of different carbon and nitrogen sources on colony development. A medium was considered to impose carbon limitation when (according to the estimated biomass yields on the applied carbon and nitrogen sources (see Tab. 2)) after the complete depletion of the carbon source ample nitrogen could be expected to be present in the medium. Nitrogen-limited conditions were defined analogously.

Since the yeasts were cultivated on a comparatively large number of different media, a short notation was introduced to provide an easy reference to the medium composition. The first letter in the notation refers to the limiting nutrient resource (**C**arbon or **N**itrogen). The second part denotes the particular nutrient which served as the carbon or nitrogen source (**G**lucose, **A**mmonium sulfate, **C**asamino **A**cids). The number provides the concentration of the limiting nutrient in grams per liter, e.g., the notation C-CA-0.2 denotes a medium which imposes carbon limitation with 0.2 g/L casamino acids serving as the only carbon source. The concentrations of the other medium components (in the above described example nitrogen source (ammonium sulfate), trace element base (YNB), and agar) remained constant when compared to the standard minimal medium. The complete medium composition for each notation is provided in Tab. 1.

In contrast to the yeast *S. cerevisiae*, which only formed filamentous colony patterns under severe nitrogen limitation [13], both model yeasts developed mycelial morphologies irrespective of the limiting nutrient. Images of *Y. lipolytica* and *C. boidinii* colonies cultivated on different food resources at various degrees of limitation are provided in Figs. 2–5. Images were taken after 21 days of incubation. Please note that the presented colony patterns do not show the final state of the cultivations but rather correspond to intermediate stages in colony development.

In general, at high nutrient concentrations colony morphologies of both model yeasts were solid and exhibited a smooth boundary. Under strong nutrient limitation, mycelial colony patterns were formed and the colony boundaries became frayed. In particular, the morphology of *Y. lipolytica* colonies was very similar to fungal mycelia. The cell densities within the colonies declined with an increasing degree of nutrient limitation. However, even under severe nutrient limitation, colony patterns of *C. boidinii* were fairly dense

Table 2. Summary of growth parameters for *C. boidinii* and *Y. lipolytica* colonies cultivated on different carbon and nitrogen sources under two different degrees of nutrient limitation.

Medium	Extension rate (v) [mm/d]	Cell length (l_C) [µm]	Cell Diameter (d_C) [µm]	Replicat. time (Δt_p) [h]	Final colony diameter [mm]	Biomass yield (Y) [g/g]
Y. lipolytica						
C-G-4 (C-G-2)*	0.9 (1.0)*	30	2.3	1.7 (1.4)*	52*	0.33
C-CA-20 (C-CA-10)*	1.0	27	3.0	1.3 (1.4)*	52*	0.14
N-A-0.1 (N-A-0.05)*	0.7	27	3.0	1.83	52*	7.2
N-CA-20 (N-CA-2)*	1.0 (0.9)*	21	3.0	1.0	52*	2.5
C. boidinii						
C-G-4 (C-G-2)*	0.5	19	2.7	1.7	12 ± 2	0.42
C-CA-20 (C-CA-10)*	0.2	14	2.1	3.3	6 ± 1	0.04
N-A-0.1 (N-A-0.05)*	0.6 (0.5)*	24	2.7	2.3 (2.1)*	52**	5.2
N-CA-20 (N-CA-2)*	0.5 (0.7)*	21	2.7	2.1 (1.6)*	20 ± 2 (52**)*	2.3

* Numbers in parentheses refer to the second nutrient concentration (see left column in parentheses) when the results for the two concentrations were different.

** A maximum colony diameter of 52 mm indicates that the outer edge of the growth field was reached by the colony boundary.

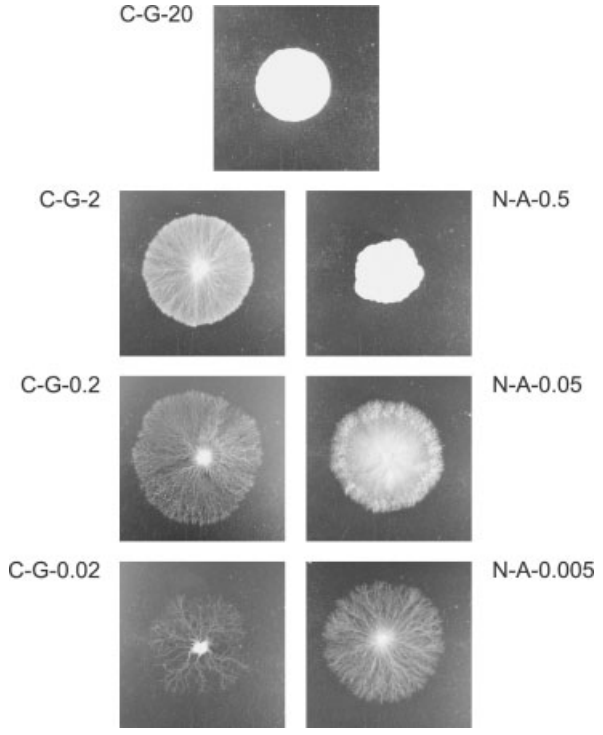


Figure 2. Morphology of *Y. lipolytica* colonies growing under various degrees of nutrient limitation. The growth media were derived from a standard minimal medium described in [12] by the selective dilution of either the nitrogen or carbon source. Left column: Carbon-limited colonies growing on glucose as the only carbon source. Right column: Nitrogen-limited colonies growing on ammonium sulfate as the only nitrogen source. Short notations referring to medium compositions are provided next to the images (see Tab. 1 for details). Cultivation time was 21 days. The width of the images represents 2.8 cm.

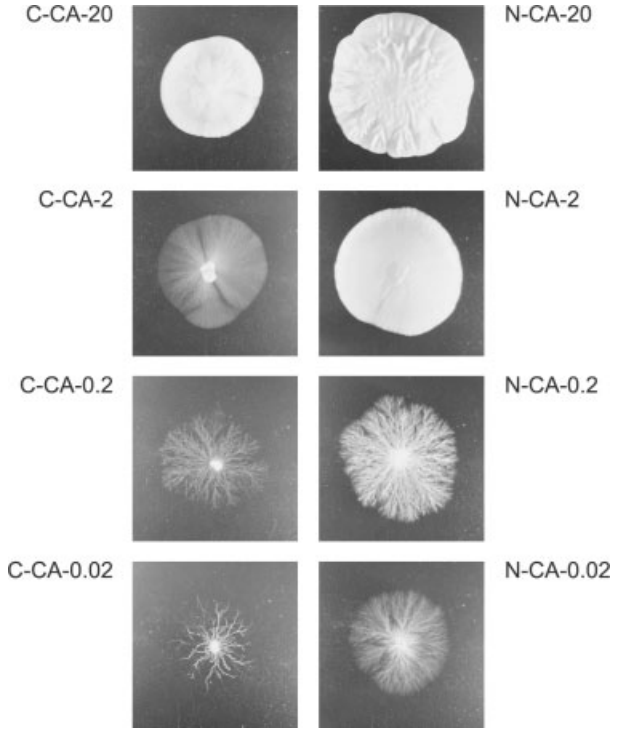


Figure 3. Morphology of *Y. lipolytica* colonies growing under various degrees of nutrient limitation. The growth media were derived from a standard minimal medium described in [12] by the selective dilution of either the nitrogen or the carbon source. Left column: Carbon-limited colonies growing on casamino acids as the only carbon source. Right column: Nitrogen-limited colonies growing on casamino acids as the only nitrogen source. Short notations referring to medium compositions are provided next to the images (see Tab. 1 for details). Cultivation time was 21 days. The width of the images represents 2.8 cm.

(see Figs. 4 and 5). Only carbon-limited *Y. lipolytica* colonies exhibited sparsely branched filamentous structures at very low nutrient concentrations (see Figs. 2 and 3). Furthermore, under most conditions, no significant drop in the size of the colonies could be observed although the concentration of limiting nutrient decreased in an order of three magnitudes. In the case of *Y. lipolytica* grown under glucose or ammonium sulfate limitation, the transition to mycelial morphologies even coincided with a pronounced enlargement of the colony diameter (see Fig. 2).

The transition to mycelial colony patterns was caused by the differentiation from almost round (yeast-like) cells to elongated (filamentous) cells. In *Y. lipolytica* and *C. boidinii* colonies the differentiation of cells was only prevented at high nutrient concentrations (see solid colonies on the medium C-G-20 in Figs. 2 and 4). The change in morphology facilitated the directed growth of individual cells away from the inoculation site and was the prerequisite for the formation of mycelial colony patterns. This behavior corresponded to a change from an exploitative to an explorative growth strategy, since a larger area can be covered at less expense of biomass in search of new nutrient resources.

3.2 Adaptation to Decreasing Nutrient Concentrations

To study the adaptation of mycelial yeast colonies to nutrient availability *quantitatively*, the model organisms were cultivated on various carbon (glucose, casamino acids) and nitrogen (ammonium sulfate, casamino acids) sources at two different degrees of nutrient limitation. Since the results of the experimental characterization of colony development were meant to be compared to one-dimensional models [11, 14], colonies were cultivated on rectangular growth fields and inoculated as a line. The cell-density profile of the colonies was estimated after 35 days of incubation by taking microscopic images along the longitudinal axis of the colony, calculating the OD profile from the intensity of the transmitted light, and calibrating OD for local cell number. The extension of the colonies was followed using a ruler. Although colonies grew in a rectangular shape, the extension of the mycelia along the longitudinal axis of the growth field was referred to as colony diameter (d_{col}).

The time course of the colony diameter and colony extension rates provided in Fig. 6 and Tab. 2 represented the average of at least four replicate runs. Under glucose-limiting

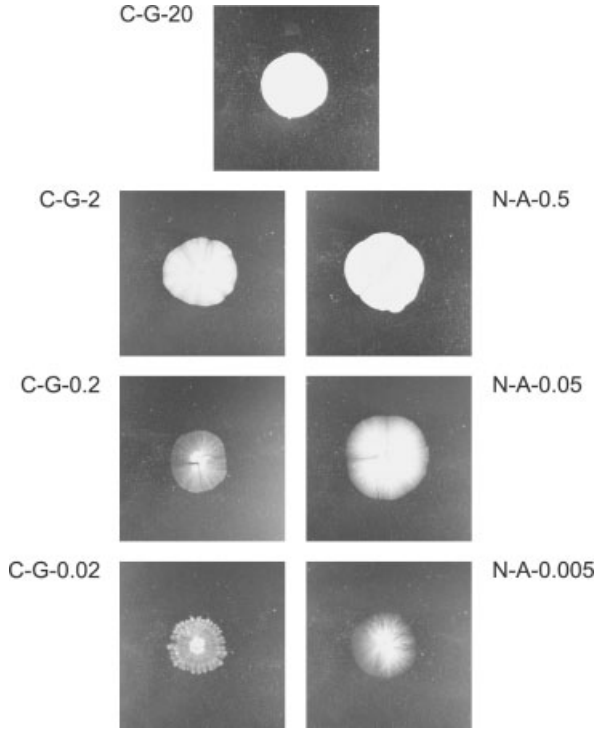


Figure 4. Morphology of *C. boidinii* colonies growing under various degrees of nutrient limitation. The growth media were derived from a standard minimal medium described in [12] by the selective dilution of either the nitrogen or the carbon source. Left column: Carbon-limited colonies growing on casamino acids as the only carbon source. Right column: Nitrogen-limited colonies growing on casamino acids as the only nitrogen source. Short notations referring to medium compositions are provided next to the images (see Tab. 1 for details). Cultivation time was 21 days. The width of the images represents 2.8 cm.

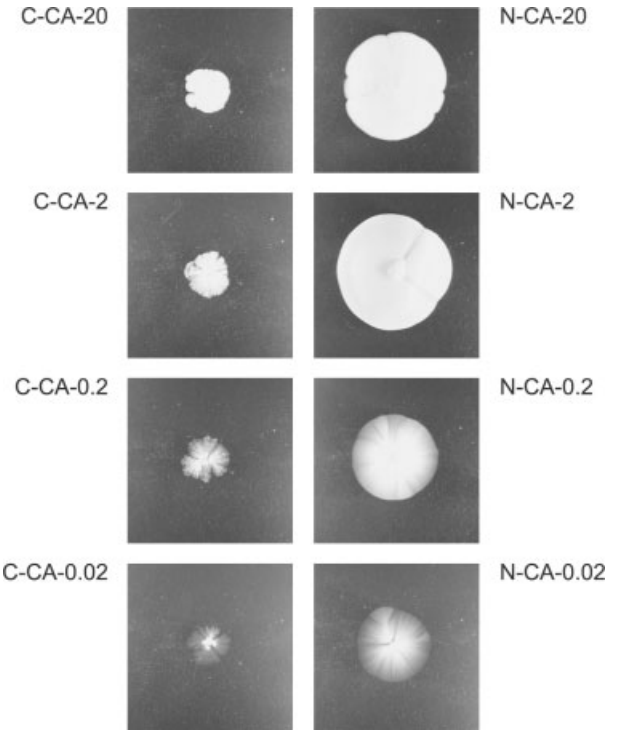


Figure 5. Morphology of *C. boidinii* colonies growing under various degrees of nutrient limitation. The growth media were derived from a standard minimal medium described in [12] by the selective dilution of either the nitrogen or the carbon source. Left column: Carbon-limited colonies growing on casamino acids as the only carbon source. Right column: Nitrogen-limited colonies growing on casamino acids as the only nitrogen source. Short notations referring to medium compositions are provided next to the images (see Tab. 1 for details). Cultivation time was 21 days. The width of the images represents 2.8 cm.

conditions, the final colony diameters of *C. boidinii* colonies varied strongly between the different batches of the experiments (maximum 14 mm, minimum 8 mm). The origin of these deviations was not ultimately identified. However, to increase the reliability of the data, colony extension rates and final diameter of glucose-limited *C. boidinii* colonies were estimated from the average of 5 independent batches of the experiments (each batch comprising at least 4 replicates).

Under most imposed conditions, colony diameters increase linearly until the edge of the growth field is reached (see Fig. 6, Tab. 2). As the only exceptions, carbon-limited *C. boidinii* colonies, as well as colonies of the same yeast cultivated on 20 g/L casamino acids as the only nitrogen source, stopped growing at a final colony diameter which is significantly smaller than the extensions of the growth field (see Fig. 6, Tab. 2).

Interestingly, the extension rate of *mycelial* yeast colonies was found to be independent of the initial nutrient concentration (see Tab. 2), a result which was consistent with findings for the growth of higher fungi (see [15] and references therein). Carbon-limited *Y. lipolytica* colonies extended with a rate of approximately 1 mm/d. Under nitrogen limitating conditions, the growth of the yeast was faster on casamino acids (1 mm/d) when compared to cultivations on ammonium sulfate (0.7 mm/d). Under all cultivation conditions, the growth of *C. boidinii* colonies was found to be somewhat slower with extension rates of 0.5–0.7 mm/d. According to the following equation

$$\Delta t_p = \frac{2 \cdot l_C}{v} \tag{14}$$

the replication interval (Δt_p), necessary to form a new cell, was calculated from the average length of pseudohyphal cells (l_C) and the growth rate of the colony diameter (v). l_C was estimated at different cultivation times and various locations within the colony.

Cell-density profiles varied significantly with nutrient availability (see Figs. 7 and 8). (Since colonies were symmetrical in shape, the figures only show the cell-density profiles in one half of the colony. The coordinate origin represents the inoculation site.)

The morphology of the cell-density profiles was dependent on both, the applied yeast species as well as on the particular cultivation conditions. Carbon-limited *C. boidinii* colonies were characterized by a rapidly declining cell density and a small final colony diameter when compared to the size of the growth field. The colony-density profiles of *C. boidinii*

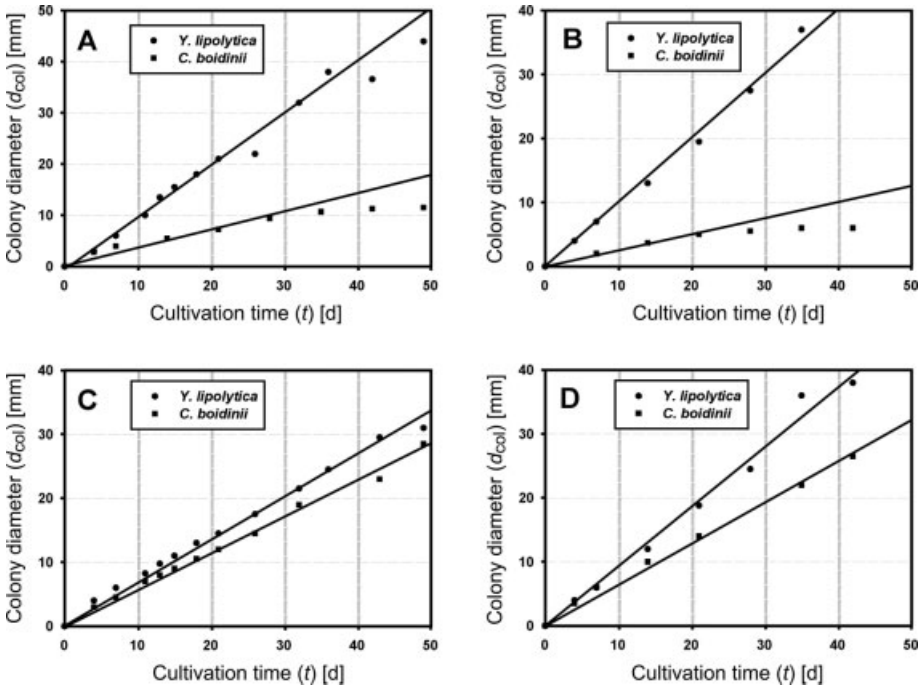


Figure 6. Colony diameters of *Y. lipolytica* and *C. boidinii* colonies *vs.* cultivation time.
(A) Carbon-limited colonies growing on glucose (C-G-2).
(B) Carbon-limited colonies growing on casamino acids (C-CA-10).
(C) Nitrogen-limited colonies growing on ammonium sulfate (N-A-0.05).
(D) Nitrogen-limited colonies growing on casamino acids (N-CA-2).

in Fig. 7 (B, D) show the final stage in colony development. For all other cultivation conditions, the presented cell-density profiles only represent intermediate states, since colony growth continued for up to several weeks. *Y. lipolytica* colonies limited for carbon sources exhibited large areas of slowly declining or constant cell density, respectively (see Figs. 7A and C). Close to the inoculation site, however, regions of rather high cell density could be observed. In particular, when glucose served as the carbon source a sharp transition between these two sections was evident (see Fig. 7A). Although not explicitly investigated, the peak in cell number at the inoculation site appeared to correspond to the presence of undifferentiated yeast-like cells.

Under severe nitrogen limitation, both yeasts formed colonies that finally covered the whole growth field (see Tab. 2). In *Y. lipolytica* colonies, the development of regions with slowly descending cell-density profiles had already become evident after 35 days of cultivation (see Figs. 8A and C). Since growth of *C. boidinii* was somewhat slower, colonies had not yet exhibited areas of constant cell density (see Figs. 8B and C). However, as colony development continued for several weeks the formation of cell-density plateaus could be expected for this yeast as well.

With increasing nutrient limitation, the total cell number within the mycelium declined. In the case of carbon limitation, the drop in total cell number was proportional to the

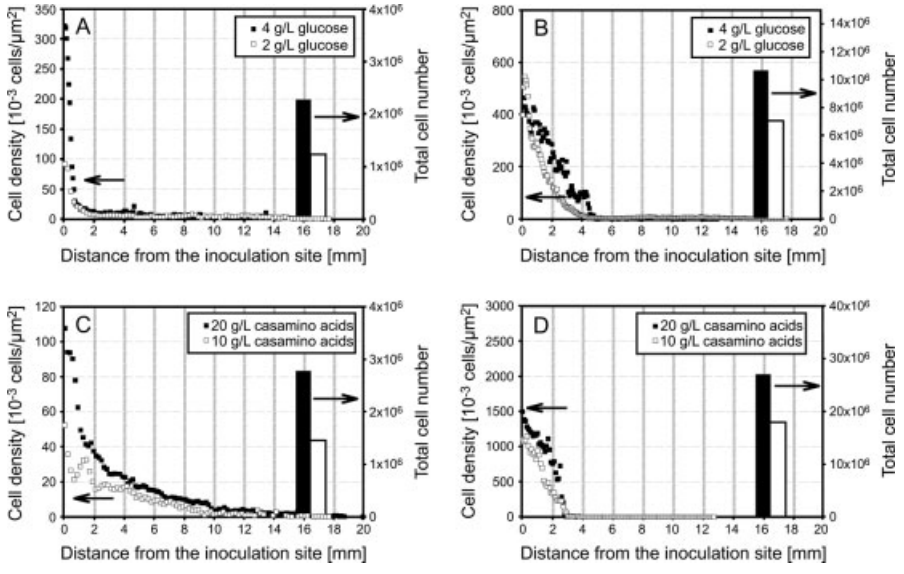


Figure 7. Cell-density profiles and total cell numbers of *Y. lipolytica* and *C. boidinii* colonies growing under carbon limitation. The profiles were estimated after 35 days of cultivation.
(A) *Y. lipolytica* growing on glucose as the limiting carbon source (media C–G–4 and C-G-2).
(B) *C. boidinii* growing on glucose as the limiting carbon source (media C-G-4 and C-G-2).
(C) *Y. lipolytica* growing on casamino acids as the limiting carbon source (media C-CA-20 and C-CA-10).
(D) *C. boidinii* growing on casamino acids as the limiting carbon source (media C-CA-20 and C-CA-10).

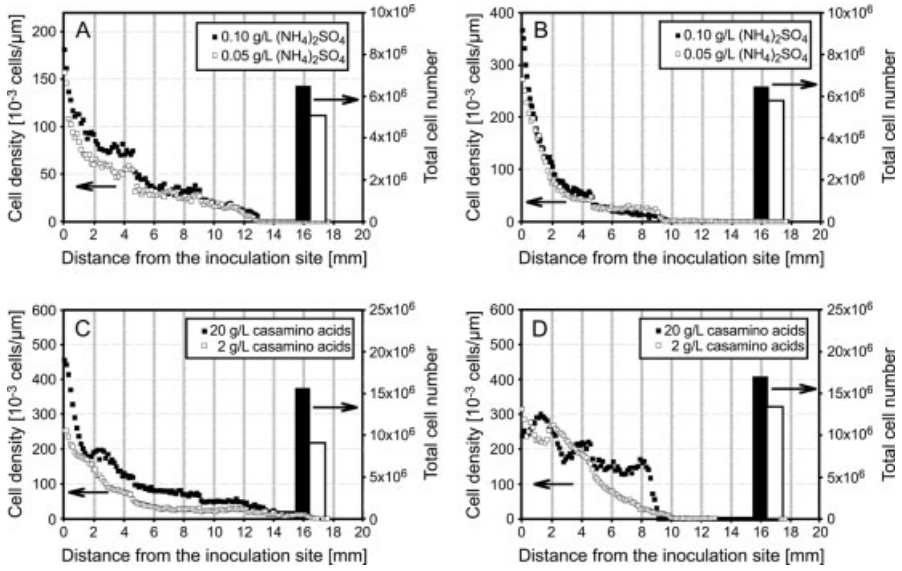


Figure 8. Cell-density profiles and total cell numbers of *Y. lipolytica* and *C. boidinii* colonies growing under nitrogen limitation.
The profiles were estimated after 35 days of cultivation.
(A) *Y. lipolytica* growing on $(NH_4)_2SO_4$ as the limiting nitrogen source (media N-A-0.1 and N-A-0.05).
(B) *C. boidinii* growing on $(NH_4)_2SO_4$ as the limiting nitrogen source (media N-A-0.1 and N-A-0.05).
(C) *Y. lipolytica* growing on casamino acids as the limiting nitrogen source (media N-CA-20 and N-CA-2).
(D) *C. boidinii* growing on casamino acids as the limiting nitrogen source (media N-CA-20 and N-CA-2).

decrease in nutrient availability (see Fig. 7). Under nitrogen-limiting conditions, however, a deprivation of the limiting nutrient source did not directly correspond to the observed decrease in total cell number (see Fig. 8). The reason for this behavior became obvious when the microscopic images of cells in carbon and nitrogen-limited colonies were compared (see Fig. 9). In nitrogen-limited populations, the cells vacuolized right behind the growing colony margin and strongly incorporated storage carbohydrates. This finding was consistent with reports on the intracellular accumulation of storage carbohydrates in *Saccharomyces cerevisiae* [16] and *Candida oleophila* [17] under severe nitrogen limitation. As a consequence of this behavior, OD measurements on nitrogen-limited colonies could not be related to cell number but rather corresponded to the accumulation of storage carbohydrates. Thus, the balancing of the limiting nutrient nitrogen on the basis of OD estimations was impossible under these conditions.

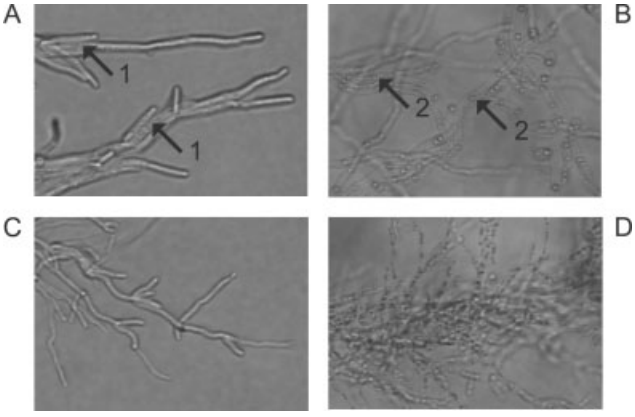


Figure 9. Comparison of the microscopic morphology of *C. boidinii* cells growing in (A, B) nitrogen-limited (medium N-A-0.05) or (C, D) glucose–limited (medium C-G-2) colonies.
(A, C) Cells at the colony boundary.
(B, D) Cells in the colony interior.
Cultivation time was 28 days. The arrows indicate 1) vacuolated cells and

In summary, a quantitative method for the estimation of cell distribution within carbon-limited yeast mycelia was developed. Since the presented method is non-invasive, this openes up the possibility to monitor the development of cell-density profiles at different stages of the cultivation. Indeed, these investigations are under way and will provide a deeper understanding of regulatory principles in the growth of mycelial yeast colonies. The investigations presented here showed that yeast populations of *Y. lipolytica* and *C. boidinii* adapted to decreasing nutrient concentrations by the transition from solid to mycelial colony patterns. Under the imposed experimental conditions, colonies that exhibited a mycelial morphology responded to the increasing nutrient limitation by a drop in the cell density within the population.

In contrast, the extension rate of the colony diameter remained constant even under severe nutrient shortage. This behavior facilitated the exploration of a comparatively large surrounding area at an economical expense of biomass and energy, and might be regarded as a general adaptation mechanism. Besides the similarities that were observed for both yeasts under all cultivation conditions, strong differences in the colony development of *Y. lipolytica* and *C. boidinii* existed. They had already become evident when the colony morphologies were compared visually (see Figs. 2–5). A *quantitative* description of these differences was provided by the estimation of extension rates, and cell-density profiles of the populations, respectively (see Figs. 6–8). The most striking example for discrepancies in the growth behavior of the model yeast was found under carbon-limiting conditions (see Figs. 6 and 7): Here, *Y. lipolytica* colonies exhibited large areas of constant cell densities, and proceeded to extend un-

til the growth field was finally covered. In contrast, colonies of *C. boidinii* stopped their extension at a comparatively small diameter, and developed a cell-density profile which declined from the inoculation site outwards. The observed variations of morphological measures indicated that colony development of the model yeasts was likely to be regulated by different mechanisms [11].

Furthermore, *C. boidinii* formed small colonies when limited by carbon sources while it developed mycelia that covered the whole growth field under nitrogen limitation (see Fig. 6, Tab. 2). Therefore, it was possible to conclude that the regulatory mechanisms acting during colony development were not only dependent on the yeast species but also on the particular cultivation conditions. In search of the underlying construction principles in yeast colony growth, mathematical models could serve as valuable tools to elaborate morphological parameters that are characteristic for one or the other mechanism [11]. As will be shown in Part 2 of this study [14], the measurements presented in this paper may serve to

i. parameterize the models correctly, so that
ii. simulations can be used to validate different hypotheses based on a quantitative comparison of model predictions and experimental results.

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Symbols used

A	[μm^2]	area covered by one sampling point in the OD measurements
c_N	[g/L]	nutrient concentration
d_C	[μm]	cell diameter
d_{col}	[mm]	colony diameter
g	[–]	correction summand for the intensity of transmitted light
I_B	[–]	intensity of transmitted light in the blank
$I_{B,max}$	[–]	maximum intensity of transmitted light in the blank
I_S	[–]	intensity of transmitted light in the sample
$I_{S,n}$	[–]	normalized intensity of transmitted light in the sample
K	[μm^{-2}]	calibration factor for the calculation of cell density from OD
l	[μm]	length of the growth field
l_C	[μm]	cell length
N	[–]	number of sampling points
n_C	[–]	number of unit (pseudohyphal) cells on a standard growth field
n_S	[–]	counted cell number on a growth field
$n_{S,n}$	[–]	normalized cell number on a standard growth field
OD	[–]	optical density
OD_{sample}	[–]	optical density of the sample
t	[h]	cultivation time
Δt_p	[h]	replication interval (generation time)
V_C	[μm^3]	volume of a cell
v	[μm/h]	extension rate of the colony diameter
w	[μm]	standard width of the growth field
x	[mm]	spatial coordinate
Y	[g/g]	biomass yield on the nutrient
ρ_C	[g/cm^3]	density of wet biomass

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