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Use of Image Analysis to Study Growth and Division of Yeast Cells

K. Tomankova, P. Jerabkova, O. Zmeskal, M. Vesela and J. Haderka
Institute of Physical and Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkynova 118, 612 00 Brno, Czech Republic
E-mail: zmeskal@fch.vutbr.cz

Abstract. *Studies of biological objects like yeast cells are usually complicated due to seemingly unpredictable changes in various properties of the living organisms. This study represents an attempt to show how various time dependent properties of yeast can be captured and precisely quantified by modern image analysis methods. During the course of this study, various complications which had been anticipated were encountered and resolved. As each strain differs by various properties like average size, shape, and cellular membrane thickness, the necessary adjustments to data capture and processing for automated data analysis were the biggest complication encountered. The study successfully shows the applicability of the chosen method of image analysis to various shapes of yeast, with cell shapes varying from elliptical, cylindrical citrical, tube-shaped, to oblong. The yeast properties' quantification was based on calculated fractal dimension and fractal measure of the studied strains. The information about speed of growth of the colony as well as about the changes occurring within individual cells was correlated to the change of the fractal parameters in the captured images of the yeast. The studied parameters were: growth of the cells, changes in the thickness of the cellular wall, number of cells present in the sample, and changes in the shape of the cells. Samples were probed with a Nikon Eclipse E400 optical microscope and a Nikon Eclipse E200 optical microscope with phase contrast. HarFA 5.1 (Harmonic and Fractal Image Analyzer) (HarFA) software was used for processing the obtained images. Custom developed HarFA software is designed to facilitate fractal image analysis.*

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INTRODUCTION

A recent development in digital imaging technology makes it relatively easy to obtain and store image data from experiments for later analysis of studied microscopic objects.[1] Obtained and archived data are then not only used for primary analysis, but also to verify the findings by other methods as well as to facilitate further development of existing image analysis methods.

The highest quality of the obtained and stored image data is a necessary precondition for obtaining reasonable results during the image analysis phase of the experiment. Therefore, all the data have to be stored in the formats preserving quality of the data like tagged image file format (TIFF) or BMP (for smaller data volume) formats.

The quality of the data is not only influenced by the sheer amount of data, but also by the precision of captured images. In order to achieve the required precision, various optical systems were evaluated in course of the study.

Another element contributing to the quality of obtained results is the choice of the observation method itself. To study the growth and division of cells one of the methods of direct microscopic observation has to be used. The most useful method seems to be one of the variants of the counting chamber method (Thom, Neubauer, or Bürker).

The calibrated chamber allows for observation of cell growth and multiplication with precisely defined surface area and depth.

Due to the length of the experiment itself it is necessary to ensure the chamber is filled with nutrition rich media. It is also necessary to compensate for loss of water due to evaporation during the experiment. Bürker compartment design allows for compensation of water loss and to provide additional nutrition to facilitate growth, water based nutrition rich media was periodically added to the chamber during the experiment without interruption.

Due to reasons stated above the observation of the biological objects in cultivation media in the counting (Bürker) compartment seems to be the most suitable method for our experiments.

The pictures can be recorded using classical or digital cameras, though a high resolution camera (three million pixels or above) is required. In case a classical camera is used, pictures would have to be digitalized later on. Due to the size of the yeast it was necessary to use equipment that allows direct connectivity between the camera and the microscope itself. The amount of detail in the measured object is crucial to the quality of the analysis output. It is necessary to ensure the size of the analyzed cell does not drop below 256 pixels in the final image. Smaller resolution would decrease the ability of the method to capture changes in the cell surface caused by budding. The minimal raster size then sets the experiment observation equipment requirements. A microscope with a magnification of 800× and a camera with a minimum 3 megapixel resolution are necessary to obtain suitable images.

More than 1000-fold accretion was mostly used throughout the experiment, but the exact level of magnification varied based on the probed cell (compensating for different absolute size of strains).

Various data about the probed object are obtained dur-

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ing analysis, for example, the length and area covered by the analyzed object, etc.[2–4] It is possible to analyze the whole obtained image, or only certain regions of interest and therefore calculate the local maximum of the properties.

The main advantage of the chosen image analysis method is that it works with the whole picture as a block, rather than with the individual pixels only. The method is based on the counting of repeated motifs and their close imperfect copies, with disrespect to the absolute position (in terms of transition or rotation) of the copy to the original motifs. The analysis of the repeated motifs is commonly known as "*fractal analysis*." The results of the analysis are described by two parameters: fractal dimension, *D* and fractal measure, *K*. Calculated fractal properties can then be used to estimate the total amount of present objects (cells in this case) or to determine other properties of the examined cell, like their shape, growth factor, aging of cell and colony growth, etc.[5,6]

There are other commercially available image analysis software on the market, for example AccuPress (from Aware, Inc.)—using wavelet analysis or other software for analyzing medical and biological images—Neurolucida (MicroBright Field, Inc.), Alice (Perceptive Informatics), respectively, or BioSystematica—used for image analysis of electrophoresis gels. All the software products described above allow for analysis of images of microbiological structures in stationary mode based on proprietary algorithms. In contrast to that, the product used throughout the experiment—Harmonic and Fractal Imager Analyzer[7] (HarFA) allows not only static, but also dynamic analysis of video streams. The video stream based analysis takes into account dynamic properties of analyzed cells and better captures the complexity of evolving dynamic systems such as a living cell. Based on many parameters of captured images (or frames in the sequence) in many different modes (RGB, HSB, HLS), HarFA allows selection of the best parameters for main analysis. In our case main analysis consists of the description of dynamic properties—growth and cell multiplication. These are described by fractal dimension and fractal measure obtained from analyzed images by wavelet (Haar) transformation.

MATERIALS AND METHODS

Yeast

The culture of yeast used for the experimental work was taken from Slovakia Collection Culture Yeasts (CCY)—Bratislava, Slovakia Republic. *Saccharomyces fragilis CCY 51-1-1*, form of cell: ellipsoid right to cylindrical. *Candida vini CCY 29-39-3*, form of cell: ellipsoid right to tube-shaped. *Kloeckera apiculata CCY 25-6-22*, form of cell: citrical. *Geotrichum candidum CCY 16-1-16*, form of cell: cylindrical right to ellipsoid. *Dipodascus magnusii CCY 42-1-2*, form of cell: tube-shaped.

Cultivation Media

Medium 1—Glucose (4 g), peptone (0.5 g), yeast extract (0.5 g), distilled water (100 ml). Medium was sterilized in 110 °C for the period of 10 min.

Medium 2—Beer worth with vitamins: worth (75 ml, *p*H 6,5, glucose (3 g), nicotine acid (20 mg), aminobenzoic acid (20 mg), adermin (20 mg), thiamine (10 mg), distilled water (50 ml). Medium was sterilized in 110 °C for the period of 10 min.

Yeast Strain	Cultivation Media
Saccharomyces fragilis CCY 51-1-1	Medium 1, Medium 2
Candida vini CCY 29-39-3	Medium 1, Medium 2
Kloeckera apiculata CCY 25-6-22	Medium 1
Geotrichum candidum CCY 16-1-16	Medium 1
Dipodascus magnusii CCY 42-1-2	Medium 1

Methodology

The images of cells were obtained using binocular light microscope with phase contrast Nikon Eclipse E200 and light microscope Nikon Eclipse E400 connected with a digital camera Nikon Coolpix 990 with resolution of 2048 × 1536 and TV card. The charge coupled device camera PixeLink with resolution of 1280 × 1024 and the Lucia Net 1.16.5 software were used in the process for monitoring of movement, adjustment, and focusing of the yeast. Images were taken using a planar chromatic objective with 40× magnification. The focus of the camera lens was fixed on the ocular of the microscope with 20× magnification. The green filter with wavelength 540 nm on microscope E200 was used to increase the quality of acquired images. Initiation of image capturing process was done by remote trigger, in order to prevent possible accidental defocusing of the camera. Bipolar and multipolar observation modes of the microscope were used throughout the experiment to obtain images most suitable for analysis.

Cultures of yeasts 17–24 hours old (see Materials and Methods section, Yeast subsection) were used throughout the experiment. Cultivation was done on agar at laboratory temperature (24 °C). Yeast cells were seeded from the test tube to the cultivation media. Cells were cultivated for a minimum period of at least 1 hour. This was done to allow for the yeast cells to adapt to the new surroundings and supplied nutrition. The sample was then transferred into the Bürker compartment by a sterile pipette once the adaptation period was over. The compartment is usually used for obtaining a count of cells in a visible field. To do so, the sample is first covered by a determination glass. The glass ensures no bubbles of the air are introduced into the sample. The covered compartment is then put on a sliding table of the microscope where the number of cells in each field of the determination glass can be counted. An advantage of using the Bürker compartment throughout the experiment was its ability to minimize movement of yeast and its ability to promote growth of cells in the horizontal area under the determination glass. Yet another advantage is the length of time period during which the sample is in a state capable of measurement. This is achieved by the presence of channels, where the excess cultivation media is kept. It is very important to secure the durability of the sample in the compartment for an appropriate length of time (several hours) and to prevent it from drying out. Keeping the sample alive over a long time period can be arranged either by reducing the

temperature in laboratory or by placing a Petri dish with cold water on the condenser. Another method is with the assistance of the micropipette—adding the cultivation medium to the channel of the Bürker compartment. The latter option has one major disadvantage, however, thanks to the volume of liquid inserted, an extended motion may appear (caused by the incoming stream of the cultivation media) in the sample and can a cause disappearance of examined cells from the visual field.

The compartment channel width visible in the obtained images was used was to calibrate the size of a single pixel in the image. Channel width of the compartment used was exactly 0.1 mm. Sizes of examined cells have then been calculated as real size represented by a single pixel multiplied by the count of pixels covering the cell in the same image.

It was necessary to ensure illumination of the sample by homogenous light during the entire experiment otherwise the quality of the recorded images would be inconsistent (blurred images with different shades of color) and would introduce error in the analysis results. The effect of illumination inconsistencies was mitigated by the use of the white balancing features of the digital camera. Another option would be to isolate the experiment from daylight in the lab and illuminate it by a consistent artificial light source only.

The lens must be continuously refocused on a cell during the experiment, because nutrient media dries out and the thickness of the liquid slightly decreases and causes cells to move. A cell also tends to rotate around its z-axis (vertical), shifts along the compartment, and finally sometimes during the cell division rotates around the horizontal (x,y)-axes. The last mentioned situation results in termination of the experiment as it makes it impossible to determine further changes in cell properties.

The limited depth of sharpness of the planchromatic 40× objective makes it difficult, if not impossible, to focus a complete cell with its bud due to differences in thickness of the parent cell and its bud. It has been confirmed throughout the experiment that small cells (*Kloeckera apiculata*) tends to move more rapidly, while big cells (*Geotrichum candidum*) are more or less static and do not move at all. Intensity of the cell motion depends on viscosity of nutrient media used and also on living conditions of the yeast cells in the environment. The experiment was focused on monitoring of the cells which stayed in the visible area throughout the whole division cycle and did not move too rapidly.

While taking images of cells in the experiment, two different strategies were evaluated. In the first strategy, the lens was focused on the "inside" of the cell. In second strategy, the focus was kept on the cellular wall. The best images of cells were captured when the camera focus was kept on the outer wall of the cell rather than on the inner parts of the cell.[7,8]

When the camera focus was kept on the inside of the cell, big organelles present in the cell were visible in captured images and made such images less suitable for the analysis. The analysis focuses on repeating motifs in captured images. Organelles present with the shape or similar to the shape of observed cells represent such motifs as well and thus introduce the error in the analysis result when present. Although it may be possible to preprocess captured images and remove shapes representing organelles programmatically we have chosen to mitigate the problem presented by organelles visibility by keeping camera focus on the outer wall.

The main advantage of taking images with the focus on the outer wall is a clear separation line between the environment and the area occupied by the cell in the resulting image. A clear separation line makes it easier to remove/ignore all noise in the image by setting an appropriate threshold value for further processing of the image data. The clear separation line between the cell and its surroundings was at times broken by a growing bud. The effect of the growing bud on the resulting image was gradually changing. At the beginning, new buds were too small and were underfocused, resulting in grayish areas on a side of cell in the captured images. In later stage when buds grew in size they became clearly visible and made the captured images better suited for analysis.

The described experiment was focused on tracking the single cell's growth, however, it was possible to monitor multiple cells in a single experiment as long as the time interval between the capture of the following images was short enough to track the movement of the cells and the visible area was big enough to ensure the cells stay visible during the entire experiment.[9]

The images were captured during the experiment with a constant time interval. Since the rate of growth varied between different strains, different intervals were tested and a 2 min interval was chosen as most appropriate to capture meaningful changes in the cell lifecycle while trying not to generate redundant data. All experiments were then measured with a constant time interval of 2 min between captured images and then saved in TIFF (without compression). Obtained images were cropped to contain only area of the interest using harmonic fractal analyzer *HarFA 5.1* and then saved in BMP format for further processing. Prior to cropping, the area of interest in the image was centred on the examined cell in order to eliminate the effects of cell movement throughout the experiment and to position the cell in the center of the resulting image. Resulting images were then compiled into a video file in AVI format with 7 frames/s ratio and each frame was represented by single image. The resulting video file therefore represented a 1:840 time compression of the experiment (e.g., 14 hours experiment would be represented by 1 min video file).

Such preprocessed images were then further modified in HarFA to obtain a thresholded black-and-white view of the cell. HarFa was then used to obtain fractal dimension and fractal measure from such images.[10]

HarFA

HarFA is the image analysis software developed by the Institute of Physical and Applied Chemistry, Faculty of Chemistry, Brno University of Technology. The software is used to determine fractals parameters of the analyzed objects using the box counting, wavelet, or Fourier transformation based

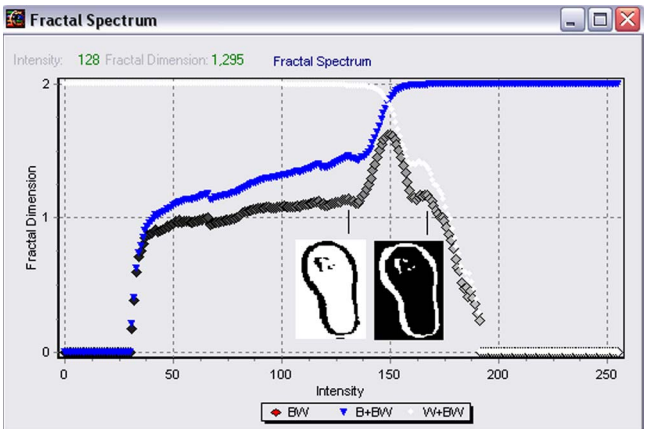

Figure 1. Fractal spectrum created by the programme HarFA, method range

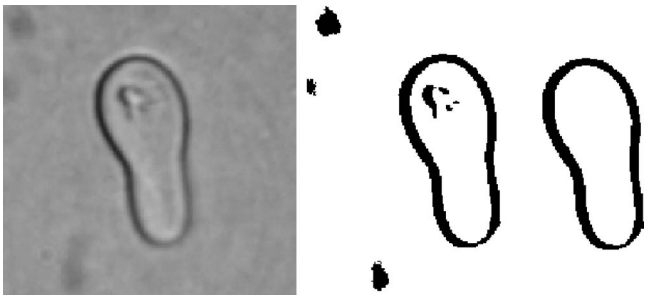

Figure 2. Original picture (left) is thresholded (by the thresholding method, center) and consecutively arranged by eliminating dirt (right).

methods. Very important functionality provided by the software is its ability to threshold images (transfer color image into black-and-white). The thresholding operation is critical for the successful image analysis and the setting of its parameters has a high impact on the obtained analysis results.

The optimal thresholding color value has to be determined first. This can be done for example using the range method (see Fig. 1). The optimal value is usually the value present on line intersections or in areas where significant changes to the shape of the object occur.[11]

The thresholding operation itself can be performed as soon as the thresholding color intensity is determined. In our experiment the thresholding color intensity was selected in the range of 0–135 points of the total 240 intensity scale (first rapid change). Before the thresholding operation, the image size was reduced by eliminating areas of the image outside of the area of the interest to the size of 530−530 pixels. Figure 2 shows all the individual steps taken during the thresholding: the filtration of colored components followed by the removal of the noise and the remnants of organelles, which would otherwise have a negative effect on the measured fractal dimension of yeast cells. As already mentioned above, visible organelles as well as big dark blobs in nutrient media would increase the error of the analysis and had to be removed prior the fractal properties calculation. The thresholded images were then subjected to the fractal image analysis using wavelet (Haar) transformation.[12]

Haar transformation or the wavelet transformation in general, provides a count of differently colored squares for different sizes of a laid mesh more effectively than the classical box counting method, provided that a square area of size 2^n is being analyzed. This transformation comes out from the system of orthogonal Haar functions which acquire values +1, 0, −1 multiplied by the power of number $2^i/2$, where $i=0,1,2,\ldots,n$. The first two Haar rectangular functions are identical with the Walsh functions. Higher Haar functions are obtained from the lower (i.e., previous) ones by changing the measure and shift. Based on the coefficients of the Haar transformation it is easy to determinate the count of black N_B, partially black N_{BW}, and white N_W squares for different sizes of mesh n ($1\times1, 2\times2, 3\times3, \ldots, n\times n$ pixels). The power of a square count is a function of the measure size. So it is again possible to determine the basic structure parameters, the fractal dimension D, and the fractal measure K of black areas (D_{BBW}, K_{BBW}), white areas (D_{WBW}, K_{WBW}), and partially black and white areas (D_{BW}, K_{BW}). These parameters can be used to evaluate ordering of the image, but also to specify the number of defined objects without having to count them explicitly.[13]

The five independent values were obtained from each of the analyzed images (image size was 530×530 pixels, but analysis matrix size only 512×512 pixels). The obtained values were then averaged to minimize the measurement error. Obtained properties were the average value of fractal dimension D and the average of fractal measure K. The segmentation of the observed object can be determined, from its fractal dimension. The segmentation is determined by the comparison of the fractal dimension of the object—area (D_{BBW}, D_{WBW}) or its border (D_{BW})) to the topological dimension of the area or curve. The flat (not segmented) area will have the fractal dimension (D_{BBW}, D_{WBW}) very close to the value of two. The flat (not segmented) curve will have the fractal dimension value (D_{BW}) equal to unity. During the segmentation of surface (e.g., during cell budding) the fractal dimension of the surface (D_{BBW}, D_{WBW}) is getting smaller, while the fractal dimension of the border region (D_{BW}) value is increasing.

The fractal measure dictates the number of pixels of the same color in the picture and gives information about how much of the area is filled by the observed object. The fractal measure is represented by a number of isochromatic pixels, absolutely or in percentage with respect to whole area of the image.[1,14]

A regression line establishing the relation between the square number of the size of mesh used can be calculated from the graph (Fig. 3). We can see three regression lines, one for each of the three fractal dimensions. For black ($D_{WBW}=1.4621$), white ($D_{WBW}=1.9903$), and partially black and white areas ($D_{BW}=1.1321$). The second column shows a fractal measure (log of the values divided by the $K_{\max}=262144$ (constant value for 512×512 matrix)). Again three different fractal measures can be obtained: one for black

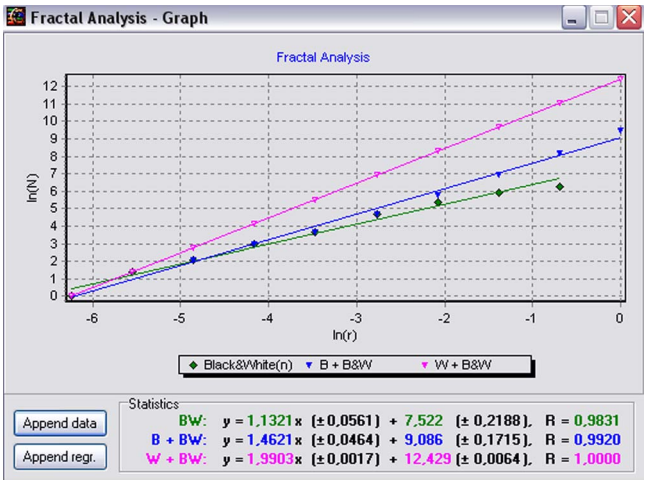

Figure 3. Fractal analysis of a representative picture.

($K_{BBW}/K_{\max}=3.4\%$), one for white ($K_{WBW}/K_{\max}=95.4\%$) and one for the black-white area ($K_{BW}/K_{\max}=0.7\%$) (see Fig. 3). The regression coefficient signifying reliability of the result is displayed in the fourth column. The fractal dimension of the individual image series is put into relation with real time.

To compare each individual experiment in the same or different species of yeast, the process differed only in the threshold value. The change was necessary due to differences in the thickness of the cellular wall between species. Some of the encountered differences between the results from different experiments were caused by changes in the focus (sharpness) and finally by differences in background light available in captured images of the sample. Effects of all those differences have been removed by setting appropriate thresholding values. Other parameters (temperature, etc.) of the experiment were standardized and kept at constant values throughout the experiment.

The fractal analysis was performed five times per each captured image, always in different parts of the picture: left upper part, right upper part, right lower part, left lower part, and central part of picture. The same means of the determining of the fractal and the fractal measure were used.[15]

The camera focus was kept on the outer wall of the cell during each experiment. The focus level was kept intact during the experiment as its changes would negatively impact obtained fractal parameters. Main constrains during thresholding were not to break continuity of cellular wall and to remove all existing visual noise. Effects of such noise, if not removed, would cause systematic error (constant value increase) in measured fractal properties, although progress (as far as time is concerned) would be the same. Trimming was done on 270×270 (analyze 256) pixels, 530×530 (analyze 512) pixels.[16]

RESULTS

The yeast *Kloeckera apiculata* was separated during observation.[17,18] The images were captured every 2 min; the cultivation media number 1 was used at the temperature

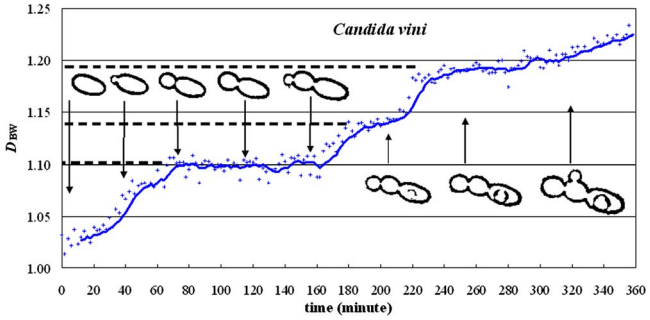

Figure 4. Dependence of yeast fractal measure on time.

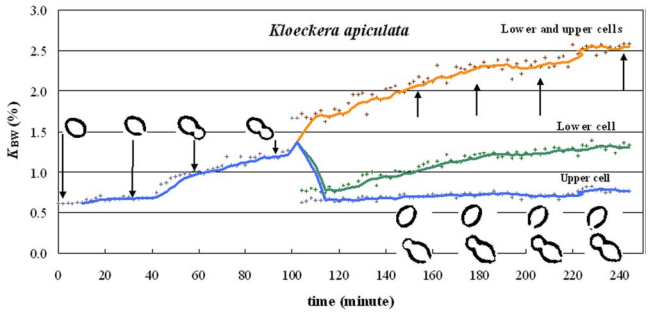

Figure 5. Dependency of yeast fractal dimension on time. Time constants are indicated by arrows.

24 °C. The captured images were then thresholded at the intensity range 0—130. The yeast began to bud after 9 hours and 30 min from seeding to the cultivation media. After another 90 min, the bud and its parent cells were completely split apart. During the separation the original cell was forced out by a new bud, which then began to bud at the same place. Fig. 4 represents the fractal dimension and the fractal measure of the original cell and also progress of life of the separated cells (daughter cells). The cells began division again shortly after finishing the first separation process. This can be seen in the graph as the initial increase of the fractal dimension and measure. The increase of the fractal measure is slower than that of the dimension. This species of yeast could be distinguished by the fast cell division process and by the fast separation of the individual cells from a chain. The disadvantage of observing this species is its tendency to form bigger colonies that tends to begin to turn upside-down and create a vertical spiral. Currently such turning makes it impossible to further focus the camera on cells in the colony. Due to this complication, the observation was done for only 4 hours after the cell division process was started. The size of the species maybe a reason for this observed tendency. This species was the smallest from all species used in the experiment. The cells of *Kloeckera apiculata* were very vital and changed their location vigorously.

Figure 5 shows a typical plot of the fractal dimension in a time for simple and multiple cell division of *Candida*. The three different phases can be recognized in the plot, each with a different characteristic growth followed by the saturation phase. The cell creates the store of the energy for

Table I. The time constants for saturation and the average increase in cellular mass as an index of the fractal dimension.

Yeast	K_{BW}/K_{max} (%)	τ_{BW1} (min)	τ_{BW2} (min)	τ_{BW3} (min)
Saccharomyces fragilis	0.0158	1.200	1.210	1.840
Candida vini	0.0105	1.105	1.145	1.190
Kloeckera apiculata	0.0154	1.270	1.400	—
Geotrichum candidum	0.0068	1.200	1.500	—
Dipodascus magnusii	0.0147	1.500	—	—

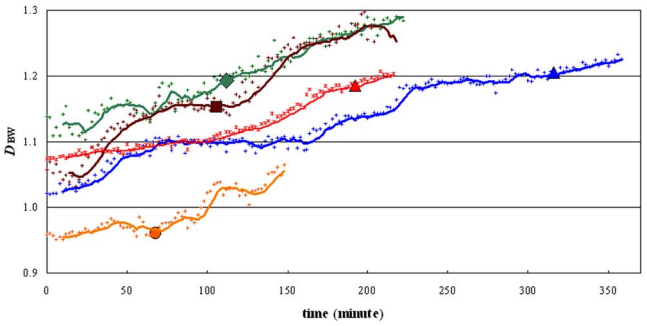

Figure 6. Comparison of the dependence of the fractal dimensions of different species of yeast on time: *Candida vini* (▲), *Kloeckera apiculata* (■), *Saccharomyces fragilis* (♦), *Geotrichum candidum* (●), *Dipodascus magnusii* (▲)

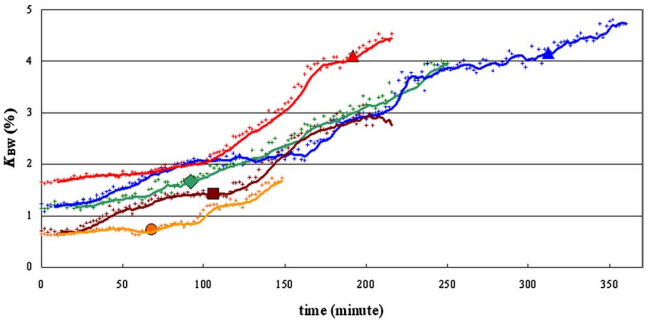

Figure 7. Comparison of the dependence of the fractal measure of different species of yeast on time: *Candida vini* (▲), *Kloeckera apiculata* (■), *Saccharomyces fragilis* (♦), *Geotrichum candidum* (●), *Dipodascus magnusii* (▲)

further growth and for the multiplication during each of the saturation phases. Table I shows the values of time constants of the saturation and that of the growth assuming exponential characteristic of the phases. The time constant τ_{BW1} represents the time when cell of the yeast bud for the very first time (the colony has only one daughter cell and one mother cell), τ_{BW2} represents the time when the cell of yeast buds for the second time (the colony has two daughter cells and one mother cell) etc. (see Table I). The first column shows the average percentage increase of the cell mass.

CONCLUSIONS

The article shows a possible use of the image analysis methods to study the dynamic properties of yeast cells. The fractal dimension and the fractal measure have been used to characterize the growth and the budding of the selected yeast strains. The change in those parameters in time shows and allows for a quantification of kinematics of the processes in the cells.

It is possible to use a culture younger than 48 hours (when cultivated on agar) for long term observation. The disadvantage is the loss of the ability to adapt the other cultivation surroundings and prolonged times between the individual periods of the cell division; 1 to 2 hours are usually sufficient for the adaptation to new surrounding after the transfer of the cells from agar to the liquid cultivation media.

The comparison of the progress of yeast cell size increase for all the five used yeast types can be seen on Figs. 6 and 7. The characteristic of the individual samples were different, but they all shared the basic trends: cells were growing and multiplying over a time.

Figure 6 shows that the original simple fractal structure of the cell with the fractal dimension of boundary $D_{BW} \approx 1$ exhibits growth over time owing to the increase in segmentation caused by budding. Such changes in fractal dimension would allow for experiments monitoring, for example, adaptation of the cells to a different living environment. Time intervals with constant fractal dimension were observed in the three of five tested strains (*Kloeckera apiculata, Candida vini, Geotrichum candidum*). Those time intervals show the saturation phase and nutrient extraction during the process of growing. The two remaining strains (*Saccharomyces fragilis a Dipodascus magnusii*) have shown continuous fractal dimension changes pointing to metabolic differences in comparison to other strains and to their ability to skip the extract nutrition/saturation phase.

Similar observations were made from plots of the changes in fractal measure in time (Fig. 7). The fractal measure shows how much the cellular wall grows in time. A more segmented wall results in the increase of the value of the fractal measure. The fractal measure K_{BW} (%) shows how much of the surface area of the whole image is covered by the cell. Figure 7 shows an increase from the initial value of $K_{BW} \approx 1\%$ to as much as 5% of the coverage. The wall size growth therefore is fivefold.

The highest expansion of fractal measure was observed for *Saccharomyces fragilis*. The reduction of the fractal dimension is product of appearance of the bud on a mother cell. The extraction of the nutrient by the cell is the cause for a part of the experiment captured as a line parallel to the time axis in the graph. The fractal dimension growth gets steeper with growth of more buds of the daughter cells and the fractal measure in that case is without any decrease. The fast reproduction rate makes *Kloeckera apiculata* an ideal observational object. The same is true for *Dipodascus magnusii* for its thick cell wall that simplifies the analysis of the captured images.

Furthermore correlations with the growth curves were observed. The growth of the cell can be characterized by the time constant from Table I. The time constants characterize the different phases of the growth of the yeast. The constant value shows *Candida vini* as the fastest growing (70 s), while *Dipodascus magnusii* is the slowest (90 s). The constants also shows that the daughter cell growth is getting slower in time. For example for *Saccharomyces fragilis*, where three generations have been captured in the experiment, the time required for next generation to come to life increases from 80 to 110 s.

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