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EXPERIMENTAL ARTICLES

Application of an Automated Colony Counter for Evaluation of the Viability of a Yeast Culture

E. O. Puchkov[1]

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Pushchino, Russia

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Abstract—Application of an automated colony counter for evaluation of the viability of microbial cultures was investigated with yeast cultures as a model. Statistical comparison of the results of automated and visual ("manual") colony counting is presented, as well as the results of the application of the bundled software to digital images obtained by light microscopy for determination of the cell concentration in suspensions. Automated counting is concluded to significantly accelerate the evaluation of culture viability by colony-forming capacity, provided that a certain requirements of sample preparation and analysis are observed.

Key words: yeast, *Saccharomyces cerevisiae, Cryptococcus terreus, Xanthophyllomyces dendrorhous*, viability, computer-aided colony counter, freezing, lyophilization.

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Cultivation of microbial cells in cylindrical plates on the surface or in the depth of culture medium to obtain detectable colonies was proposed by Julius Richard Petri in 1877. The procedure is still among the most widely used for quantitative determination of viable cells in various samples. Without going into details of a complex issue of the determination of microbial viability [1–4], let us note that the ability to form colonies reflects the ability of cells to grow under given conditions (ability to be cultured).

For enumeration of viable microorganisms in situ, their content is expressed as the number of colony-forming units (CFU) per unit of sample mass or volume. If microbial viability is assessed in pure cultures or microbial preparations (ex situ), the ratio between the CFU concentration and the total concentration of cells is used as the quantitative characteristic. Total concentration of the cells is determined by light microscopy.

Enumeration of colonies on petri dishes and determination of total cell concentration are labor- and time-consuming procedures. The first apparatus for automated colony counting on petri dishes was described in 1957 [5]. It was based on scanning the petri dish with a CRT beam. In the 1970s, a new generation of automated colony counters arose. They were based on computer analysis of digital images of colonies on petri dishes. Besides automated colony counting as such, these devices may be used to count colonies manually (visually), in any convenient time, repeatedly, if needed, using digital images and virtual markers with simultaneous documenting of results, saving the digital data, and their transmission. Several devices of this type are presently commercially available, including Scan500 (Topac, United States), Clinx BioCounter (Clinx Science Instruments, China), Color QCount (Sysmatec, Germany), Schütt colonyQuant (Schütt Labortechnik, Germany), Sorcerer Colony Counter (Perspective Instruments, United Kingdom), ProtoCOL SR, HR (Synbiosis, United States), and KOMPANKOL-M1 (Nabitekh, Russia). However, we found no systematic studies on the application of these devices for evaluation of the viability of microbial cultures. In particular, no data is available on the statistical comparison of automated and "manual" colony counting, as well as on the possibility to use the equipment and/or the bundled software to control cell concentration in suspensions using digital images obtained by light microscopy.

The aim of the present work was to investigate the feasibility of automated counters to develop advanced procedures for determination of microbial viability with yeast cultures as models. A KOMPANKOL-M1 apparatus (Nabitekh, Russia) was used in the work (http://ibpm.ru/content/view/36/18). Compared to other devices, it has a Russian software interface, enables data storage and processing with standard Microsoft Office tools, and is relatively cheap.

MATERIALS AND METHODS

Most of the experiments were carried out with *Saccharomyces cerevisiae* VKM Y-2549 (type strain); for some experiments, with the cultures of *Cryptococcus terreus* VKM Y-2253 and *Xanthophyllomyces dendrorhous* VKM Y-2786. The cultures were maintained on slants of agarized (1.5%) wort (7° B) medium. After

[1] Corresponding author; e-mail: nabiteh@rambler.ru

inoculation and incubation at 20–23°C, the samples were stored at 5–10°C for a time not exceeding 4 months.

Yeast lyophilization and freezing. After culturing on solid media, the cells were washed off with a sterile protector containing 10% sucrose, 1.5% gelatine, and 0.1% agar-agar in distilled water [6]. Lyophilization of 0.2-ml samples was carried out in glass ampoules on an Edwards (United Kingdom) apparatus in two stages: primary drying on an EF6 centrifuge-type apparatus (4 h, 0.1 Torr) and secondary drying on a 30S1 collector apparatus (18 h at 0.1 Torr) followed by ampoule sealing under vacuum. Lyophilized cells were rehydrated in sterile distilled water.

For freezing, the sample preparation was the same. Cell suspensions in polypropylene tubes (Nunc, Denmark), 0.2 ml per tube were placed into an MDF-Ultra Low fridge (Sanyo, Japan) (–68°C) for 24 h. Thawing was performed in a water bath at 36°C.

Determination of colony-forming capacity of the cells. Samples of intact cells were obtained by washing off the slants with sterile tap water; in case of lyophilized or frozen samples, washouts from the slants with the protector medium described above served as a control. The cell suspensions were thoroughly homogenized by pipetting, and the presence of cell aggregates was controlled by light microscopy. Only suspensions free from visible aggregates were used for plating. Series of tenfold dilutions in sterile tap water were prepared from initial samples. Resulting samples were plated onto Petri dishes with agarized (1.5%) culture medium containing wort (7° B). In order to ensure a uniform thickness of the medium all over the dish area, the medium was poured onto the dishes on a strictly horizontal surface. In case of inoculation onto the entire plate area by a conventional procedure, 0.2-ml samples were spread with a glass spreading rod. For implementation of the Miles and Misra procedure [7], drops of 0.02–0.05 ml were applied to any one of eight sectors drawn on a petri dish. The colonies were counted after incubation at 20–23°C for 3–4 days.

Colonies on the dishes were counted both manually and automatically using digital images obtained in transmitted light with an automated analyzer of microbial colonies KOMPANKOL-M1 (Nabitekh, Russia; http://ibpm.ru/content/view/36/18). For the publication, images are presented in black-and-white.

The results of colony counts were expressed as the number of colony-forming units per 1 ml of the initial sample (CFU/ml) with the standard deviation (SD) and relative standard deviation (%) calculated for three dishes of a given dilution.

Total concentration of cells was determined using a Goryaev chamber for counting of blood cells. A light microscope (Carl Zeiss, Germany) equipped with a 20/0.40 lens and a DSC-V3 digital camera (Sony, Japan) was used to obtain digital images of the cells in the chamber. Then, the cells were counted on these digital images with the KOMPANKOL-M software package of a KOMPANKOL-M1 microbial colony counter.

Determination of concentration of methylene blue stainable cells. Methylene blue solution (Sigma, United States) was prepared as follows [8]: 0.03 g methylene blue, 0.9 g NaCl, 0.042 g KCl, 0.048 $CaCl_2 \cdot 6H_2O$, 0.02 g $NaHCO_3$, and 1 g glucose were dissolved in distilled water to obtain 100 ml of solution. For cell staining, 0.09 ml of the methylene blue solution was mixed with 0.01 ml of a cell suspension. After a 30-min incubation at room temperature, the samples were analyzed in a Goryaev chamber as in the case of the determination of the total cell concentration using the images obtained by microscope equipped with a 40/0.65 lens.

Quantitative data were processed with MS Excel 2003, and the average values and standard deviation values were calculated using the software formula, and the rest of values, as is specified in the notes of the tables.

RESULTS AND DISCUSSION

Comparison of different methods of colony counting. To compare the results of manual and automatic colony counting with KOMPANKOL in the case of conventional spread-inoculation onto the entire area of a petri dish, the samples were divided into two groups (Figs. 1 and 2). Samples of each type were obtained from two sequential tenfold dilutions of the same initial suspension. Samples of the first group (Fig. 1a) contained 15–150 colonies (1–5 mm in diameter) per dish. Colonies were counted using the image of the whole plate. Samples of the second group (Fig. 2a) contained 150–1500 colonies (0.3–1.5 mm in diameter) per dish. In this case, the colonies were enumerated on five fragments of the fivefold magnified image (Fig. 2b) taking into account the ratio of the fragment area to the entire surface area of the dish (the software package includes such an option).

First of all, the data obtained (Table 1) evidence that automatic colony counting systematically resulted in lower values (Table 1, column "Error"). The value of this error did not exceed the values of standard deviation in either counting regimes. A trend was observed towards a decrease of the relative error with decreasing number of colonies per dish. The systematic tendency to underestimate the number of colonies in the automatic regime was due to the failure to distinguish between all merged colonies by adjusting the sensitivity of the KOMPANKOL using standard samples of a series (for example, Figs. 1a and 1b, colony groups 1–5) without losing small colonies (for example, Figs. 1a and 1b, colony group 6). The compromised sensitivity setting always results in some conglomerates counted as a single colony, while some of the small colonies are not registered. Besides, preliminary tests revealed that the results of automatic counting depended strongly on

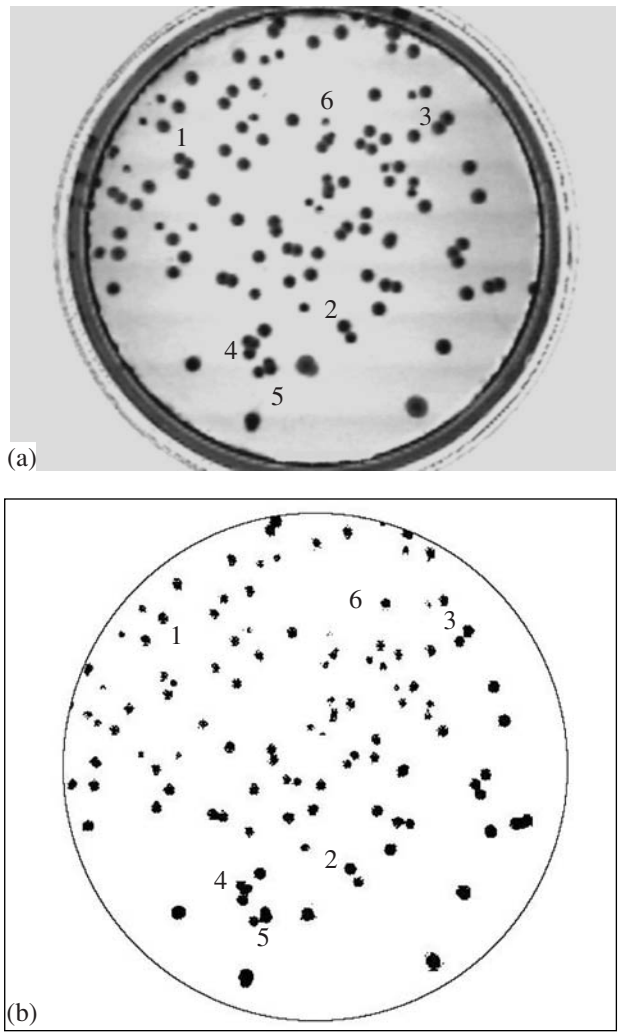

Fig. 1. An example of the type I sample. (a) digital image of *S. cerevisiae* colonies after inoculation over the entire area of a petri dish and (b) colonies treated as single units by the KOMPANKOL-M1 software after computer transformation of the image and sensitivity adjustment.

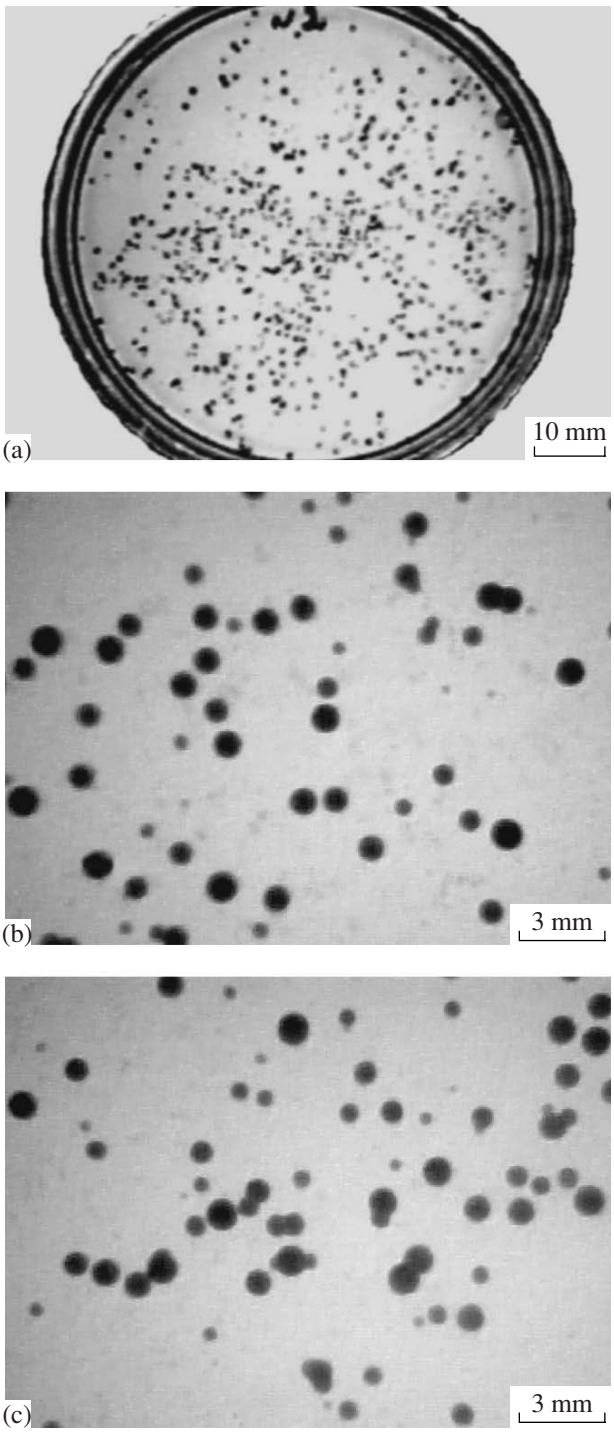

Fig. 2. An example of the type II sample. Digital images of (a) *S. cerevisiae* colonies after inoculation over the entire area of a petri dish and (b, c) two out of five randomly chosen fragments at five-fold magnification for the counting.

the evenness of suspension spreading over the dish surface.

Data of Table 1 also evidenced some divergence between results of manual counting in samples of types 1 and 2 of the same series (Table 1, column "Divergence"). The divergence was of a random nature because, calculated as is specified in the Notes to Table 1, it took both positive and negative values. However, here the tendency of decreased divergence with a decreasing colony number per plate held in this case too.

The Miles and Misra procedure [7] of inoculation of small volumes of samples of several dilutions on a single petri dish for determination of the colony-forming capacity is significantly less labor consuming. However, visual identification of small colonies is difficult; this procedure is therefore used mainly to estimate the colony-forming ability to the order of magnitude (or the degree of dilution) based on the presence/absence of colony growth in the section of the corresponding dilution (Fig. 3a and b).

Table 1. Determination of colony-forming unit concentration in *S. cerevisiae* yeast suspensions using a KOMPANKOL counter after conventional inoculation onto the entire surface of a petri dish: comparison between "manual" and automated modes

Series number	Sample type*	CFU × 10^8/ml ± SD (%)**		Error (%)***	Divergence (%)****
		Automatic counting	"Manual" counting		
1	I	6.8 ± 1.22 (18)	8.57 ± 1.46 (17)	26	
	II	5.2 ± 1.14 (22)	6.66 ± 1.66 (25)	28	22
2	I	2.34 ± 0.37 (16)	2.71 ± 0.41 (15)	16	
	II	2.61 ± 0.73 (28)	3.05 ± 0.98 (32)	17	–11
3	I	0.82 ± 0.09 (12)	0.90 ± 0.11 (12)	10	
	II	0.75 ± 0.10 (14)	0.87 ± 0.14 (16)	16	3
4	I	1.04 ± 0.15 (14)	1.16 ± 0.17 (15)	12	
	II	1.15 ± 0.11 (10)	1.25 ± 0.14 (11)	9	–7
5	I	0.71 ± 0.13 (18)	0.81 ± 0.16 (20)	14	
	II	0.69 ± 0.06 (9)	0.79 ± 0.13 (16)	15	2

Yeast cells were washed off with sterile water and plated on petri dishes in tenfold dilutions. Three samples of each type were used for colony counting.

Notes: * I, samples with colony density of 15–150 colonies per dish, colony diameter 1–5mm; II, samples with colony density of 150–1500 colonies per dish, colony diameter 0.3–1.5mm (see Figs. 1 and 2).

** Average concentration of colony-forming units in initial samples with standard deviation values (SD) and relative standard deviation in parentheses (in %).

*** Relative error of automated counting defined as ratio of difference between mean values in manual and automated counting to the mean value of manual counting (in %).

**** Relative divergence of counting results for samples of types I and II of the same series determined as ratio of difference between the mean values obtained by manual counting of samples of types I and II to the maximum mean value of the samples (in %).

Table 2. Colony-forming unit concentration in *S. cerevisiae* yeast suspensions determined using KOMPANKOL: comparison of conventional spreading over the entire dish surface and the Miles and Misra procedure [7]

Series number	Sample type*	CFU × 10^8/ml ± SD (%)**		Error (%)***	Divergence (%)****
		Automatic counting	"Manual" counting		
1	I	–	1.26 ± 0.26 (21)	–	
	M-M	1.28 ± 0.23 (18)	1.56 ± 0.26 (17)	22	19
2	I	–	1.71 ± 0.21 (12)	–	
	M-M	1.34 ± 0.21 (16)	1.55 ± 0.22 (14)	16	–10
3	I	–	1.13 ± 0.12 (11)	–	
	M-M	0.91 ± 0.20 (22)	1.0 ± 0.12 (12)	10	–12
4	I	–	1.2 ± 0.13 (11)	–	
	M-M	1.14 ± 0.16 (14)	1.28 ± 0.19 (15)	12	6

Yeast cells were washed off with sterile water and inoculated onto petri dishes in tenfold dilutions. Three samples of each type were used for colony counting.

Notes: * I, samples with colony density of 15–150 colonies per dish, colony diameter 1–5mm after conventional inoculation onto entire dish surface (see Fig. 1); M-M, samples of plates prepared according to Miles and Misra technique [7] (see Fig. 3).

** Mean value of colony-forming units concentration in initial samples with values of standard deviation (SD) and relative standard deviation in parentheses (in %).

*** Relative error of automated counting defined as the ratio of difference between mean values in manual and automated counting to the mean value of manual counting (in %).

**** Relative divergence between counts of I and M-M samples of a single series defined as the ratio of the differences between mean values of manual counting (in %).

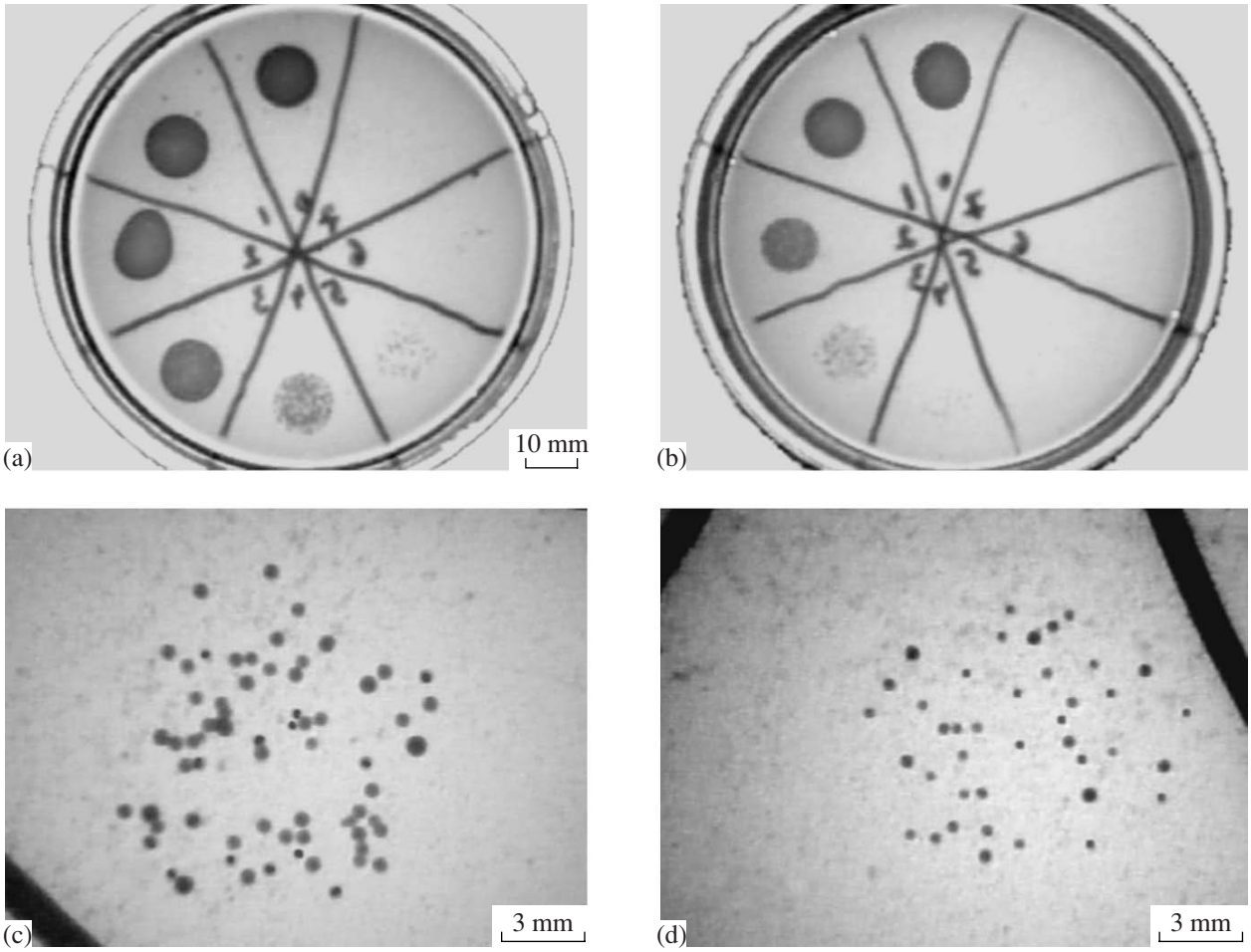

Fig. 3. An example of samples of *S. cerevisiae* colonies after inoculation according to the Miles and Misra procedure [7]. (a) and (b) represent digital images of the whole plate obtained before and after lyophilization correspondingly. The surface is divided into eight sectors each containing cell suspension sample from the zero to the seventh tenfold dilution; (c) and (d) are digital images of the sectors containing the fourth and fifth dilutions at five-fold optical zoom of the plates before and after lyophilization, respectively (images a and b).

When cells were inoculated according to [7], colonies of at least 0.3 mm in diameter were found to be distinguished reliably on digital images (Fig. 3c and d). Similar to the conventional inoculation procedure (Table 1, column "Error"), colony counting with KOMPANKOL revealed a systematic divergence between the data of automated and manual counting (Table 2, column "Error"). A satisfactory agreement between colony counts obtained by the conventional inoculation procedure and the one described in [7] (see Table 2, column "Divergence") was observed.

Determination of the total cell concentration and the portion of methylene blue-stained cells. The feasibility of the counting of yeast cells in a Goryaev chamber using digital images and the KOMPANKOL software was investigated (Fig. 4). The results (Table 3) indicate the possibility of determining yeast concentration using this procedure in both manual and automatic modes. The divergence in results between the modes is due to the same reasons as in the case of the colony count (see above).

Digital images of yeast cells in a Goryaev chamber may be used to count the cells stained with methylene blue (Fig. 5) in the manual operation mode of KOMPANKOL. However, we were not able to adjust the sensitivity of the apparatus in order to distinguish between stained and unstained cells automatically.

Determination of the viability of yeast cells after lyophilization and freezing. The feasibility of computerized colony counting combined with methylene blue staining for evaluation of culture viability after exposure to extreme factors related to conservation by low-temperature freezing or lyophilization was of particular interest. When the content of merged colonies was high, only manual colony counting was to be used (Table 4, series 2 and 3 and Table 5, series L). In one of the samples after lyophilization (Table 4, series 4), viability evaluation was hampered by significant aggregation of cells which couldn't be eliminated by pipetting.

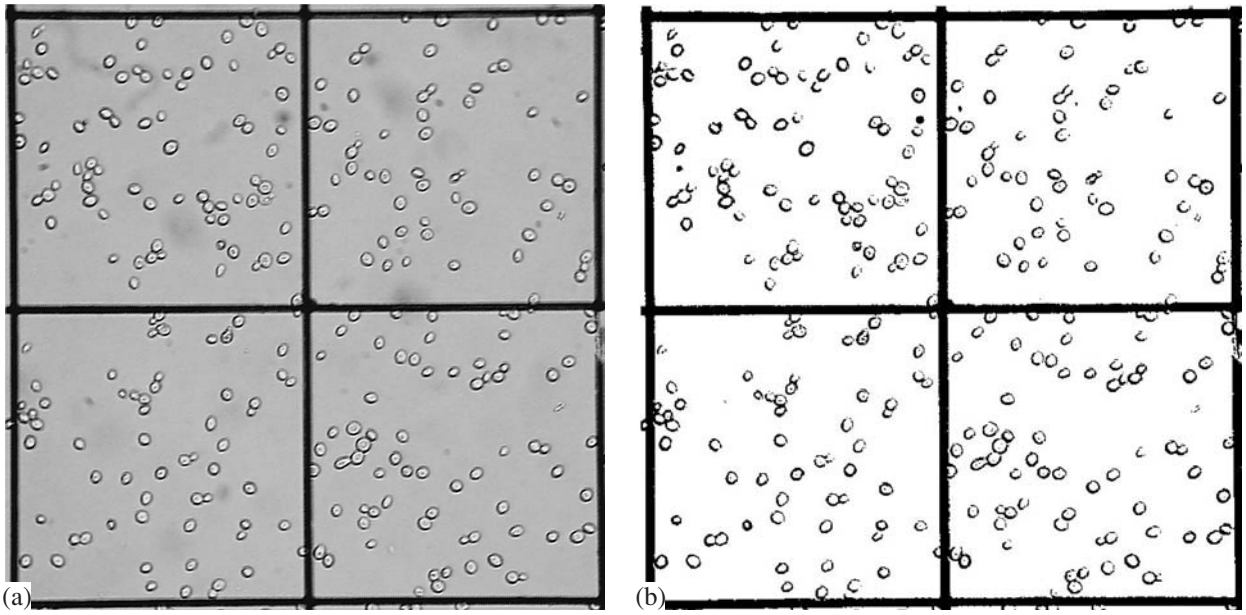

Fig. 4. Determination of total cell concentration of *S. cerevisiae* cells in a Goryaev chamber using digital images. (a) initial digital image of the cells in four large squares and (b) cells treated as single units by the KOMPANKOL-M1 software after computer transformation of the image and sensitivity adjustment. Square side length is 200 μm. Sample preparation conditions are indicated in Materials and Methods.

Apparently, cells collapsed in the process of lyophilization [9]. In general, the results are evidence of a satisfactory agreement between the results of manual and automated counting in both the control and experimental samples (Tables 4 and 5). For a number of *S. cerevisiae* samples, significant divergence was revealed between the data on viability evaluation obtained by the colony-forming ability and by the assay of methylene blue staining after freezing and lyophilization (Table 4). Previously, such a possibility was indicated by other researchers [2].

To conclude, we should particularly note that the major principles of application of computer colony counters for evaluation of colony-forming ability and total concentration of microorganisms studied in the work on yeast cultures using KOMPANKOL may be applied to other microbial objects, as well as apparatus of other manufacturers (see Introduction). First of all, computer-based counting allows one to accelerate the evaluation of viability of microbial cultures by their colony-forming ability. The rate of colony counting on a single dish (or in a single field of view) in the automated mode after the sensitivity setup did not exceed 1 s, this was independent of the number of colonies. From our experience, manual counting using digital images may be performed at a rate of two colonies per second. Taking into account automated documentation and the possibility of performing counting at any convenient time, even the manual mode improves significantly the procedure of colony counting on petri dishes. Finally, an additional advantage of a computer counter demonstrated in the present work is the possibility of quantifying the total cell concentration using digital images obtained by light microscopy.

However, a number of requirements are to be complied with in order to utilize the advantages of computer counting and to avoid crucial mistakes. First of all,

Table 3. *S. cerevisiae* yeast cell concentration determined with Goryaev chamber using digital images: comparison of the manual and automatic modes of KOMPANKOL operation

Series number*	Number of cells × 10^8/ml ± SD (%)**		Error (%)***
	Automatic counting	"Manual" counting	
1	1.1 ± 0.11 (10)	1.3 ± 0.22 (17)	18
2	0.86 ± 0.14 (16)	0.98 ± 0.14 (14)	14
3	1.47 ± 0.25 (17)	1.6 ± 0.19 (12)	10

Notes: * In each experimental series cell suspensions from different slants were used.

** Mean value of cell concentration in the samples with standard deviation (SD) and relative standard deviation in parentheses (in %). In each series data of three independent measurements of at least 600 cells in a Goryaev chamber in both modes in the same fields of view were used for calculation. Undivided mother and daughter cells were considered as a single cell.

*** Relative error of automatic count defined as the ratio of difference between mean values of manual and automated counting to the mean of manual counting (in %).

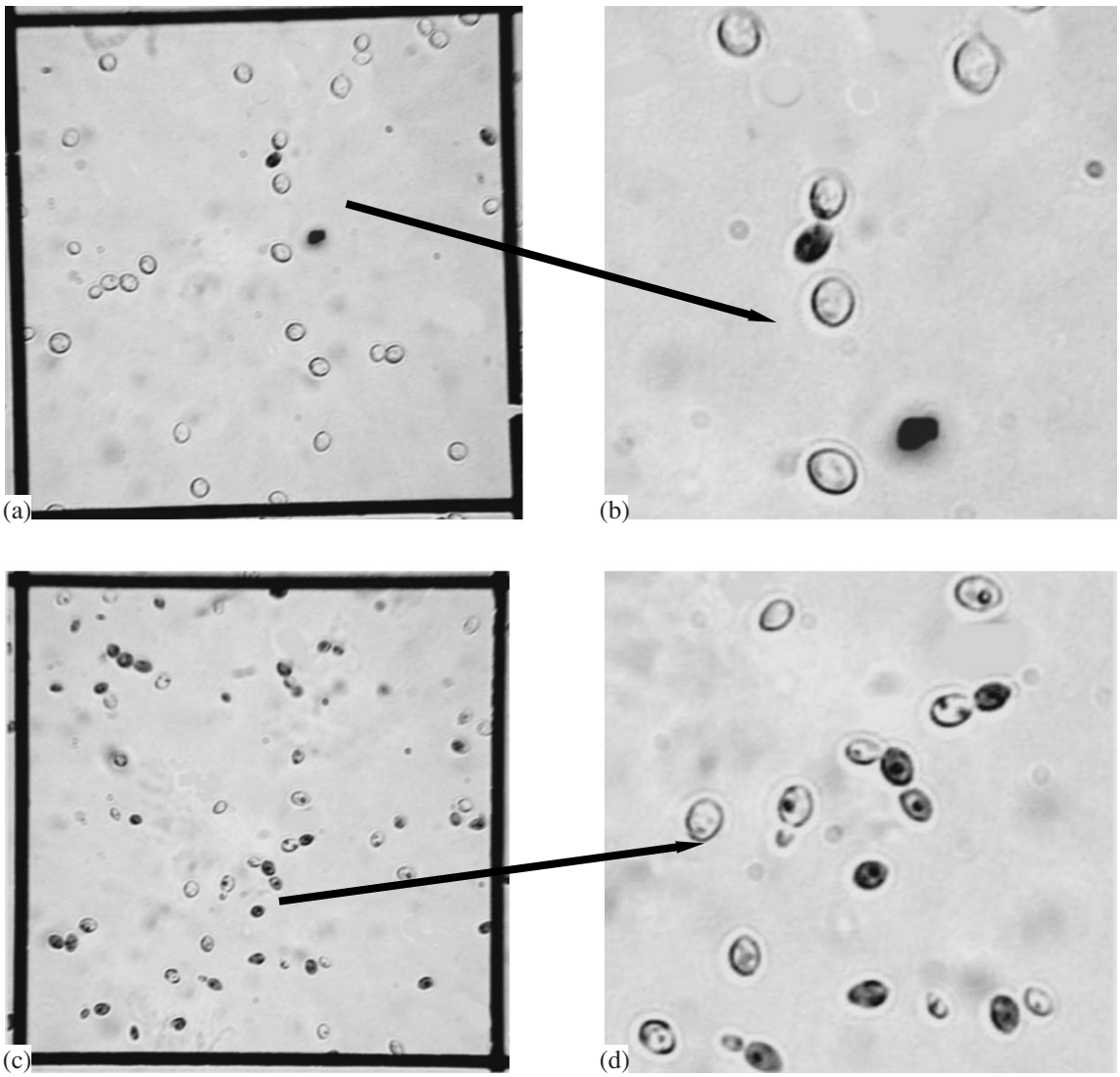

Fig. 5. Determination of portion of *S. cerevisiae* cells stained with methylene blue in a Goryaev chamber using digital images: (a) and (c) are intact and lyophilized cells, respectively, in the large square of a Goryaev chamber; (b) and (d) are magnified fragments (shown with arrows) of images (a) and (c), respectively. Cells stained with methylene blue appear dark. Square side length is 200 μm. Sample preparation conditions are indicated in Materials and Methods.

prior to plating, the absence of cell aggregates should be verified by microscopy. Without this control, viability evaluation based on the colony-forming ability of microorganisms under study is somewhat doubtful: does a colony derive from one cell or from an aggregate? Moreover, paradoxical and false conclusions are possible. For example, in paper [10] only data on the colony-forming ability of *Saccharomyces carlsbergensis* Sa.23 before and after freezing at –20°C and –30°C (see Table 1 in [11]) were provided. The data is evidence of a 1.6-fold increase in the viability of the culture after freezing. These results, however, may be due to aggregates disintegrating after freezing.

Particular attention should be paid to the choice between the modes of manual and automated colony counting. An automated count is performed with a certain error, the value of which depends on the number of merged colonies, on the ratio between numbers of small and large colonies, irregularities of the medium thickness over the dish surface, etc. The choice between modes of automated and manual counting is to be made depending on the aim of the experiment and of the sample characteristics. Generally, the manual mode is more suitable for a small number of samples. The automatic mode is advantageous in speed when analyzing a great number of serial samples. It is reasonable under conditions of reliable standardization of the procedures of sample preparation, including optical characteristics of the medium and colonies, even spreading of the samples over the surface of the medium, incubation duration (size of colonies), etc. Accuracy and reproducibility of the analysis results in the automatic mode depend

Table 4. Evaluation of *S. cerevisiae* yeast viability with KOMPANKOL after lyophilization and freezing

Series number	Sample type*	Viability ± SD (%)**		MB staining (%)***
		Automatic count	"Manual" count	
1	C	105 ± 7	95 ± 10	92 ± 4
	L	15 ± 10	18 ± 5	5 ± 5
	F	76 ± 12	80 ± 19	45 ± 20
2	C	–	98 ± 15	95 ± 5
	L	5 ± 20	15 ± 18	0
	F	55 ± 10	48 ± 14	30 ± 15
3	C	95 ± 11	100 ± 8	5 ± 5
	L	–	17 ± 20	0
4	C	101 ± 12	98 ± 7	95 ± 1
	L	–	–	–
	F	65 ± 16	75 ± 12	35 ± 12

Notes: * C, L, and F stand for control, lyophilized, and frozen samples, respectively.

** Viability was determined as the ratio between colony-forming units concentration and total cell concentration (in %). Colony-forming units concentration was assessed after cell inoculation according to the Miles and Misra procedure [7]. Calculations were performed using data of three samples of each type.

*** The MB staining column represents the portion of cells not stained with methylene blue (in %).

Table 5. Evaluation of *Cr. terreus* and *X. dendrorhous* viability with KOMPANKOL after lyophilization and freezing

Yeast culture	Sample type	Viability ± SD (%)	
		Automatic count	"Manual" count
Cr. terreus	C	83 ± 7	90 ± 10
	L	–	30 ± 20
	F	66 ± 20	81 ± 14
X. dendrorhous	C	92 ± 6	98 ± 12
	L	25 ± 11	35 ± 8
	F	75 ± 10	78 ± 15

Note: Designations and methodological conditions are as indicated in Table 4.

directly upon the quality of sample preparation. Anyway, a computer colony counter as any advance apparatus, does not substitute for a qualified researcher, but assists him!

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