XVI. STUDIES ON THE GROWTH OF YEAST.

III. A FURTHER STUDY ON THE INFLUENCE OF VOLUME OF MEDIUM EMPLOYED.

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(Report to the Medical Research Council.)

(Received December 18th, 1926.)

IN a previous communication [1925, 1] I have recorded the growth of single-cell cultures of yeast in various volumes of medium. At the time of publication of those experiments I calculated that in the series of volumes employed I had included the different volumes of medium per yeast cell inoculated which were used by Liebig and Pasteur. I subsequently found that owing to an error in all my calculations the volumes which I had actually studied were not in cubic millimetres as recorded, but in thousandths of a cubic millimetre. I had therefore only studied concentrations of yeast equal to and greater than those used by Pasteur, and even in the largest volumes I had not employed such a low concentration of yeast as did Liebig. On this account the experimental evidence of the communication did not justify any conclusions as to the causes of the differences in growth observed by Pasteur and Liebig. The more general conclusions concerning the relationship between volume and growth are obviously not affected by any alterations in the units in which the volumes were recorded. In the experiments detailed below I have extended the range of volumes to include that which corresponds with the concentration of yeast employed by Liebig.

EXPERIMENTAL.

The experimental details will be found in the previous paper [Peskett, 1925, 1]. The yeast used was the same strain of *Saccharomyces cerevisiae*, and the cells whose growth was studied in these experiments were washed before inoculation by the technique described elsewhere [Peskett, 1925, 2]. The stock and parent cultures were grown on medium 5. All cultures were grown at 25°. Medium 1 consisted of the basal salt mixture plus 50 g. per litre commercial cane sugar; in medium 2 the latter was replaced by recrystallised cane sugar, and medium 5 was composed of medium 2 plus 10.0 cc. per litre yeast extract.

Volumes. In Exp. 1 the volumes were approximately 5.0 and 0.5 mm.³. If a yeast cell can be considered as a sphere of 10 μ diameter, *i.e.* about

5×10^{-7} mm.³ volume, then single cells in these volumes would correspond to concentrations of 0.01 mm.³ and 0.1 mm.³ of yeast per 100 cc. of medium respectively. These concentrations are approximately those used by Liebig and Pasteur, as will be seen from the discussion in my previous communication [1925, 1, p. 471].

In Exp. 2 the volumes were 39.7 mm.³ and 0.04 to 0.01 mm.³, *i.e.* roughly 10 times larger than Liebig's, and 10 times smaller than Pasteur's per yeast cell; in addition three cultures were studied in which the volumes were intermediate.

Preparation of the cultures. For use in Exp. 1 a silica capillary pipette was calibrated to deliver 5.0 mm.³ of mercury determined by weighing. A suspension in medium 1 of washed yeast cells was prepared of such a dilution that 5.0 mm.³ of it could be distributed in 10 drops of approximately equal diameter, a few of which would contain a single cell. Thus hanging-drops were prepared of about 0.5 mm.³ volume, some of which contained a single cell.

The 5.0 mm.³ drops of medium 1 were measured from the capillary pipette on to slides, each of which had a deep circular depression about 13/16 inch in diameter. The drops were each inoculated with a single washed cell transferred by means of a capillary pipette from hanging-droplet preparations in medium 2 which had been prepared previously. Particular care was taken to search these droplets thoroughly, and in every case only those were used which contained one cell beyond all doubt. Immediately after inoculation the cultures were covered by a cover-slip sealed in position with paraffin wax. The under surface of the cover-slips had been treated before sterilisation with a commercial preparation to prevent fogging owing to condensation of water. This did not come in contact with the cultures and therefore did not vitiate the results.

The error in measuring the drops in Exp. 1 was fairly considerable. The pipette delivered 4.9 mm.³ of mercury ± 0.07 mm.³. In measuring medium this error must at least have doubled owing to wetting of the surface by the fluid. Thus the probable error was at least 3 % for the larger drops, and it may have increased up to 10 % for the smaller though every effort was made to keep the diameters of the latter constant within 0.1 mm.

In Exp. 2 the largest volumes were measured (1 drop) from a Dreyer pipette which delivered 252 drops of medium 1 per 10.0 cc. The volumes of the other drops were calculated from measurements of their diameter and depth. The largest and intermediate drops were set up and inoculated as in Exp. 1, the smallest were prepared in the usual manner from a suspension of washed cells in medium 1.

Every precaution was taken to prevent contamination of the cultures.

Observation and bacterial control. The cultures were examined at intervals of about 4 hours, and the growth recorded. In the case of the larger drops it was often necessary to warm the cover-slip to dispel the thin film of water which had condensed thereon, in order to get clear definition of the cells under

Fig. 1. Growth of washed single cells in medium composed of recrystallised salts and commercial cane sugar.

Volume (mm.³)
5.0
0.5

Fig. 2. Growth of washed single cells in medium composed of recrystallised salts and commercial cane sugar.

Volume (mm.³)
40
1.4
1.3
1.0
0.04
0.02
0.008

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the microscope. If this precaution was observed the cells were counted quite easily, using a low-power objective and high-power ocular.

In Exp. 2 great difficulty was experienced in locating the single cells in the largest drops: in three cases out of the five they were not found until the third observation (10 hours).

At the end of the growth period all the cultures were examined for contamination, either by fixing and staining *in situ*, or by transferring a loopful to nutrient agar, which was then incubated at 25° for 2 days followed by a further 2 days at 37°. In the latter case smears were prepared from the agar slopes and stained and examined in the usual manner. Any infected cultures were excluded from the results.

RESULTS.

In each experiment I have recorded on a graph the logarithm of the number of cells present at each observation. Buds were considered as fractions, computed on the basis of the relationship between their diameters and those of the cells from which they were growing. The graphs are shown in Fig. 1 (Exp. 1) and Fig. 2 (Exp. 2).

In Table I, I have compared in each experiment the total number of bacteria-free cultures which were prepared with the number of these which actually showed growth.

Table I.

Volume	Exp. 1		Exp. 2	
	Total	Growing	Total	Growing
Largest	6	4 (66 %)	5	4 (80 %)
Intermediate	—	—	4	3 (75 %)
Smallest	12	8 (66 %)	6	4 (66 %)

DISCUSSION.

It is clear from Table I that the incidence of mortality among the cultures is fairly constant in spite of the differences in volumes. In Exp. 2 the greatest mortality occurred in the smallest volumes, possibly on account of relatively greater evaporation from the smallest drops. It seems unlikely therefore that Liebig's failure to obtain growth was due to death of the cells inoculated.

In Exp. 1 the behaviour of all the cultures up to the 20th hour was very uniform, and closely comparable to that observed in Exp. 2. After the 20th hour there was a marked retardation of growth in two of the cultures in the larger volumes (Nos. 10 and 13). At the same time one of the cultures in the smaller volumes was retarded to an even greater degree (No. 3), and in another case (No. 1) there was some retardation at first. In the latter case the drop touched the surface of the slide at the 24th hour, after which growth could no longer be observed. At the conclusion of the experiment I could only count a total of 13 cells on the slide and cover-slip, but I have omitted this observation from the graph on account of its uncertainty. With the exception

of these four cultures, uniformity of growth was observed from the 20th hour to the end of the experiment.

In Exp. 2 the largest variations in growth were observed in the three cultures in intermediate volumes (Nos, 5, 6, 7). I am inclined to attribute these variations to the relatively large degree of evaporation which must have occurred, since the drops were placed in large depression slides similar to those used for the largest drops. In this connection it is perhaps significant that the greatest variation occurred in the smallest drop (No. 6). Apart from these variations the growth of all the cultures in this experiment was strikingly uniform.

In Exp. 2, although the conditions were similar to those of Exp. 1, the final growth obtained was far less. I cannot at the moment account for this difference, but it does not appear to affect the point at issue.

Thus, not only in volumes approximately 10 times larger or smaller than those used by Liebig and Pasteur, but also in volumes comparable with those which these observers employed per yeast cell inoculated, the growth of single cells of yeast appears to be uniform. Liebig's failure to obtain growth does not therefore appear to have been due to any effect of volume *per se* on the vitality or growth of the yeast.

It would seem more probable that the absence of "bios" from Liebig's cultures affords the true explanation. For, as the following experiment shows, it is possible to obtain growth of a single washed yeast cell in a volume as large as 4.0 cc.

Growth of single yeast cells in large volumes.

In the following experiment I have studied the growth of single cells in volumes of 0.08, 1.0 and 4.0 cc. Hanging-droplet preparations of single washed cells in medium 2 were prepared, and the cells transferred by means of a capillary pipette as in the experiments reported above. The 0.08 cc. cultures were grown in depression-slides, the 1.0 and 4.0 cc. in test tubes of suitable size. The medium used was medium 5. Growth was determined after 48 hours at 25° by a cell-count in a Bürker counting-chamber. In the case of the 0.08 cc. cultures the cells were washed off the slide with 1.0 cc. of medium 2 into a small test tube, the medium being delivered drop by drop from a calibrated Dreyer pipette. Examination of the slide after this process showed that very little yeast remained on it.

Table II.

Exp. 3. Inoculum one cell. Thousands of cells present in each culture after 48 hours at 25° are recorded in the third column.

Volume cc.	Culture No.	Growth $\times 10^3$	Error $\times 10^3$
0.8	1	914	25
0.8	2	892	25
1.0	3	551	25
4.0	4	712	60
4.0	5	1215	80
4.0	6	922	130

The results are recorded in Table II. The distribution of cells was rather uneven, and the figures for the counts were low (about 1 for each large square of $1/25$ mm.²). There is thus considerable error in these figures. In the last column of the table I have recorded the probable error of the counts, calculated by taking extreme values of the figures obtained for each row of 12 squares.

All the cultures in this experiment were free from contamination. With the exception of No. 3, the growth of all the cultures was approximately uniform, and there was no evidence of any inhibitory effect in the 4.0 cc. cultures. The figures are too few to justify any comparison of the effects of the different volumes, especially as the experiment has not been repeated. It is clear, however, that provided that the composition of the medium is suitable a single yeast cell can increase a millionfold in 48 hours when inoculated in a volume of medium 8,000,000,000 times its own. The growth of single cells in a medium composed of recrystallised cane sugar and salts (medium 1) has never in my experience exceeded 20-fold, and has generally been about 10-fold in 48 hours. These observations are in accord with those of Hansen [1879] and Kossowicz [1903]. The former inoculated a series of flasks with very small quantities of yeast, and, to obtain pure cultures, only employed those flasks which showed single colonies after incubation. But, as Lafar [1910] points out, cultures arising from a single cell can only be obtained from gelatin plates actually examined under the microscope, and this was the method subsequently adopted by Hansen [1883]. It cannot therefore be stated with absolute certainty that Hansen grew single cells in comparatively large volumes of fluid medium, but he certainly obtained growth from very small inocula. Kossowicz failed to observe multiplication, as judged by the naked eye, of single cells in media composed of salts and pure sugar, while large inocula ("viele Zellen") showed rich development under similar conditions. He was inclined to attribute the growth of the latter to "carry over." We must conclude therefore that failure of growth of small inoculations of yeast is due to deficiencies in the composition, and not to the volume, of the medium used. The explanation of the Liebig-Pasteur controversy as a result of differences in amount of "bios" present appears to be justified.

SUMMARY.

1. The growth and vitality of single cells of yeast does not appear to be affected by the volume of medium employed, provided that the composition of the medium is suitable.

2. The differences of growth observed by Pasteur and Liebig cannot be ascribed to differences of the volume of medium used per cell inoculated, but were probably due to the presence of different amounts of "bios." This conclusion, although stated in a previous communication, was not justified by the results reported at that time.

In conclusion I desire to thank Prof. Peters for his interest and advice. I am indebted to the Medical Research Council for a part time grant, and to the Trustees of the Christopher Welch Fund for the purchase of apparatus.

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