LXIX. STUDIES ON THE GROWTH OF YEAST.

I. THE INFLUENCE OF VOLUME OF CULTURE MEDIUM EMPLOYED.

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EVER since the controversy between Pasteur [1872] and Liebig [1870, 1871] as to the growth of yeast in a synthetic medium the problem has remained unsettled. It will be remembered that Pasteur claimed originally [1859] that he could obtain a good growth of yeast "dans un milieu formé uniquement de sucre, d'un sel d'ammoniaque et d'éléments minéraux." Although the work of Willaman and Olsen [1923] shows that it is highly probable that the cane sugar which Pasteur used was contaminated with growth-promoting substances, yet Fulmer, Nelson and their supporters at the present time claim that they can produce a normal growth of yeast on their Medium "F" which is composed of chemicals of the highest possible degree of purity. On the other hand, there are still a number of workers who claim that for the normal growth of yeast some substance of a "bios" nature is indispensable (Funk, Eddy, Williams *inter alios*). In fact, a crystalline substance with well-defined physical properties and marked "bios" activity has recently been described by Eddy, Kerr and Williams [1924]. [For further references and details of this controversy see *Med. Res. Council Report*, 1924, p. 54.]

Whatever may be the true solution to what we may call the "bios problem," the differences between the results obtained by Pasteur and Liebig have yet to be explained on an experimental basis. The following communication is an attempt to supply such an explanation in the hope that it may throw light on the "bios problem."

Liebig, in accord with his view that fermentation was a process which only occurs in non-living matter, emphatically opposed the idea of Pasteur that it was an expression of the living yeast cell. In support of his contention he stated that he had repeatedly attempted to obtain a growth of yeast under Pasteur's conditions without succeeding in doing so. In these attempts, as we shall see below, he did not inoculate as much yeast in his medium as Pasteur had done. The main difference therefore between the experimental conditions of these two observers lay in the different volumes of medium which

were employed for each yeast cell originally present. With this difference in mind, the experiments detailed in the following communication were undertaken. Their object was to determine the effects, if any, of alterations in the volume of culture medium used on the growth of single cells of yeast. The experiments fall into two classes, one in which the yeast was grown in a medium as free from "bios" as possible, the other in which various concentrations of "bios" extracts were added.

Owing to the difficulty of obtaining in any given experiment a large number of active single cell cultures, observations were also made on cultures containing two cells. The justification of the use of results obtained in two cell cultures as corroborative evidence of the behaviour of single cells will be given in the next communication (Part II).

EXPERIMENTAL.

General.

In view of the influences on growth which may occur owing to the presence of bacterial contamination (*vide infra*) the technique has been further modified to prevent contamination as far as possible and to determine with greater certainty those cases in which it may have occurred. With the exception of these modifications the general procedure was essentially the same as in the experiments previously described [Peskett, 1924]. The following media were used:

- Medium 1.* Basal medium + 50 g. per litre commercial cane sugar.
- Medium 2.* Basal medium + 50 g. per litre recrystallised cane sugar.
- Medium 3.* Medium 2 + 0.025 mg. per cc. of solid "bios complex."
- Medium 4.* Medium 2 + 1.0 cc. yeast extract per litre.
- Medium 5.* Medium 2 + 10.0 cc. yeast extract per litre.
- Medium 6.* Medium 2 + about 1.0 cc. "Kepler" malt extract per litre.

The basal medium, recrystallised cane sugar and "bios complex" were prepared as previously described. The yeast extract was prepared according to the method of Eddy, Kerr and Williams [1924] from fresh "D.C.L." yeast. After shaking with fuller's earth, the filtrate was made up to the volume of autolysate from which it had been prepared for use in these media.

It was also found to be more satisfactory to rinse the cover slips in glass-distilled water instead of acid alcohol before drying. The cultures were grown at 25°, and the yeast used was the same as in the previous experiments.

Preparation of drop-cultures.

Hanging-drop preparations were made by means of a silica pipette in the manner described previously. In view of the difficulty of removing vaseline from the cover slips before fixing and staining, this substance was only used to the extent of two small dabs placed at opposite corners of the depression

in the slide. This was quite sufficient to allow the cover slip to be picked up readily with the slide. When in position, the cover slip was sealed at its edges with warm paraffin wax.

Parent culture.

As in the previous experiments, great difficulty was experienced in obtaining a large number of drops containing single cells which would grow satisfactorily. The mortality among the drop-cultures seemed to vary to a certain extent according to the manner in which the parent culture from which they were derived was obtained. After many trials it was found most satisfactory to use a culture which had been grown at 25° in 2.0 cc. of Medium 3 for not less than 2 or more than 4 days; the best results being obtained when a small loopful of a culture at least 14 days old was used for inoculation. To avoid the risk of contamination by wild yeasts, the stock culture was occasionally started afresh by transferring a colony derived from a single cell in a drop preparation to a fresh tube of Medium 3.

Drop volumes.

By direct measurement with a high power objective and a calibrated fine adjustment of the microscope the depths of a large number of different-sized drops of Medium 2 were determined. At the same time their diameters were measured by means of an eyepiece micrometer. These drops had been dusted with lycopodium powder to enable their surfaces to be focussed. Since the shape of the drops was found to be approximately that of a segment of a sphere, the usual formula was applied, viz. $V = 1/3\pi (3r - h) h^2$. From this formula the volumes of these drops were calculated and plotted on a curve against their diameters.

In the following experiments only the drop diameters were measured, and from these the volumes were read off from the curve obtained above. It will be seen therefore that the values given for drop volumes, though recorded in cubic millimetres, are only empirical.

Observation and staining of the cultures.

In order to minimise errors of evaporation and condensation of the drops, which might occur owing to changes of temperature while the cultures were under observation, a warm stage (at 25°) was used.

Fixing and staining the cultures. The technique described previously involved a long period of drying before the cultures could be fixed. Since in many cases the cultures became contaminated during this process a considerable amount of time has been spent in trying to overcome this difficulty. The technique finally adopted was as follows:

At the conclusion of the period of observation the position and approximate size of the drop-cultures were marked on the upper surface of the cover slip by means of Indian ink. The cover slip was then removed from its slide and

the upper surface coated with a thin layer of paraffin wax. A small ring and an identification mark were then etched round each ink mark by means of hydrofluoric acid, the excess of acid being washed off carefully with a few drops of water. The cover slip was warmed to evaporate the greater part of the water from the drops, and whilst still warm any traces of paraffin wax or vaseline were removed with a rag moistened with xylol. The preparation was then immersed for a few seconds in a dilute solution of collodion¹, and transferred rapidly to 80 % alcohol. After a few minutes in that fluid it was transferred to 50 %, and thence to 10 % alcohol. The preparation was then stained and mounted as described in the previous experiments. The position and size of the cultures having been fairly accurately marked, it was only necessary to search these circumscribed areas for bacterial contamination.

It was found that, by using this method, only in a very few instances did any of the yeast cells of a given culture become detached from the preparation. Moreover, the final preparations were comparatively free from granular débris, even when viewed under the highest powers of the microscope, provided that the stains were filtered before use. For these reasons I have described the method at some length in the hope that it may be useful to other workers in this field.

RESULTS.

Method of consideration.

In each experiment I have plotted on a graph the number of cells present at each observation in every culture which showed growth. All the cultures considered were found to be free from bacterial contamination according to the method described. Fig. 1 illustrates the results obtained in a typical experiment.

Fig. 1. Growth of two-cell cultures on synthetic medium.

Culture No. 3 (▼) showed the slowest growth.

Cultures No. 5 (△) and No. 7 (□) showed the quickest growth.

¹ Prepared by the addition of 5.0 cc. of collodion solution (containing 3 g. of pyroxylin in 75 cc. ether and 25 cc. alcohol) to a mixture of 25 cc. alcohol and 25 cc. ether.

In order to save space, the curves have been omitted and the results given in tabular form.

Arrangement of tables.

In one experiment the points plotted on the graph fell very close together, there being little variation in the growth recorded in the individual cultures. In this experiment (4 A) the drop volumes of all the cultures are given in the first column of the table, under "Average growth."

In other experiments (1 B, 6 B, 8 A) one culture in each experiment showed considerably quicker growth on the graph, and its drop volume has been recorded in the second column of the table, under "Quickest growth." The remaining cultures in each of these experiments, showing little variation in growth, are considered in the first column of the table.

In most of the experiments the graph obtained has been similar to that shown in Fig. 1. Here it will be observed that there is considerable variation in the growth recorded in the individual cultures. In these experiments the drop volumes of the cultures showing the quickest growth (*e.g.* nos. 5 and 7 in Fig. 1) have been recorded in the second column; whilst the volumes of those showing the slowest growth (*e.g.* no. 3 in Fig. 1) have been given in the third column of the table, under "Slowest growth." The volumes of the remaining cultures in these experiments are given in the first column.

To determine the rate of growth I have in all cases studied the growth curve from the last point before the first division occurred. Any acceleration of growth due to shortening of lag period alone has not been considered.

Growth on synthetic media.

Table I. *Drop volumes in mm.³.*

Exp.	A. Single cells			B. Two cells		
	Average growth	Quickest growth	Slowest growth	Average growth	Quickest growth	Slowest growth
1	8, 58, 23, 5, 64, 90, 90, 43, 31, 14	58	10	13, 23, 64, 6, 43, 13, 14, 31	43	—
2	—	—	—	17, 10, 6, 13, 146	23	23
3	—	—	—	4, 13, 8, 5	13, 13	5
4	4, 13, 4, 13	—	—	6, 3, 2.5, 2.5	2, 4	4

In Table I the growth of 46 cultures has been considered, in volumes varying between 2 and 146 mm.³. All of these were grown on "synthetic medium" (Medium 2) and found to be free from bacterial contamination so far as the technique allows it to be judged. In the range of volumes studied, there was little evidence of any constant influence of differences in volume on the growth. Thus, the evidence of acceleration in the larger volumes and retardation in the smaller shown in Exp. 3 B is counterbalanced by the uniform growth observed in the volumes shown in Exp. 4 A. In all the remaining experiments it will be seen that fairly uniform growth has been observed over a wide range of volumes; in every case in volumes larger than that in which

the quickest growth has been recorded and smaller than that in which the slowest growth occurred, and *vice versâ*.

In synthetic media, therefore, within the range of volumes studied, I cannot attribute any of the variations in growth which occurred to differences in the volume of culture medium employed.

Growth on media containing "bios" extracts.

Table II. Drop volumes in mm.³.

Exp.	Medium	A. Single cells			B. Two cells		
		Average growth	Quickest growth	Slowest growth	Average growth	Quickest growth	Slowest growth
5	5	143, 238, 74, 90, 157, 362	90, 45	77	—	—	—
6	4	19, 26, 22, 23, 36, 48, 2, 10, 6, 13, 13, 6	115	48, 29	17, 27, 31, 17	107	—
7	6	7, 13, 9, 4	57	9, 43, 13	64, 14, 23, 48	10	1, 0.5
8	3	178, 161, 213, 260	106	—	235, 178, 178, 161, 107	270	23, 74

In Table II the growth of 57 cultures, on media containing various concentrations of growth-promoting substances, has been considered. These were all free from bacteria as far as could be judged. The volumes varied between 0.5 and 362 mm.³.

In the first place, it will be seen that in five experiments (5 A, 6 A, 7 A, 7 B, 8 B) there were cultures which showed extremes of growth in both directions. In these experiments there were 16 such cultures. As regards 15 out of these 16 it is true to say that the cultures which showed quicker growth had larger volumes than the corresponding cultures which showed slower growth and *vice versâ*. In media containing "bios" there is, therefore, evidence that the quickest growth occurs in volumes larger than those in which the slowest growth is observed.

In order to simplify the consideration of all the experiments in the table I have drawn up the following analysis. Out of 18 cultures which showed exceptionally quick or slow growth 8 showed quick growth and 10 showed slow growth.

Of the eight showing quick growth the volumes of		Exp. nos.		Of the ten showing slow growth the volumes of		Exp. nos.	
Four were largest		6 A, 7 A, 6 B, 8 B		Three were smallest		7 B, 8 B	
Two were smallest		5 A, 8 A		None were largest		—	
Two were intermediate		5 A, 7 B		Seven were intermediate		5 A, 6 A, 7 A, 8 B	

In the 9 cultures in intermediate volumes there was no constant relationship between volume and growth. Of the remaining 9 cultures, 7 showed definite evidence of acceleration of growth in the largest volumes or of retardation in the smallest, and only 2 showed the opposite effect.

Secondly then, in view of the fairly uniform growth obtained in Exp. 5 A over a very wide range of volumes, and in consideration of the aberrant growth noted in the 9 cultures in intermediate volumes (considered in the analysis) it is difficult to ascribe any of the variations in growth which occurred to

differences in volume. But in those cases where extreme variations in growth occurred in association with extreme differences in volume, there was distinct evidence of acceleration of growth in the larger volumes and retardation in the smaller in the majority of cases (77 %).

Thirdly, in the media containing "bios" extracts I have not observed slowing of the growth in the largest volumes, and in only two cases out of eight did acceleration occur in the smallest volumes. Robertson [1923] claims that the growth of infusoria is approximately inversely proportional to the volume of medium in which they are grown. This is not true of the yeast used in these experiments.

Growth in presence of bacterial contamination.

Table III. *Exp. 9. Medium 2. Single and two-cell cultures.*

Cultures considered	After 72 hours at 25°	
	Grown	Not grown
16 sterile (control)	9	7
16 infected	1	15

In Table III I have recorded the results obtained in an isolated experiment in which some of the cultures were intentionally contaminated with bacteria. The infected cultures had been lightly inoculated with a pure culture of a short rod-like bacillus obtained from the laboratory air. In its cultural characteristics it resembled *B. fluorescens liquefaciens*. Although the growth recorded in this experiment was not good even in the sterile control cultures, there was a definite inhibition of growth owing to the presence of the bacteria, and a marked retardation in the single infected culture which did grow. The experiment emphasises the need for prevention of contamination by bacteria, and for means for their detection.

Mortality of the cultures.

The only experiments that I have been able to find in which the growth of isolated yeast cells has been studied in small volumes of medium are those of R. J. Williams [1919]. In these the percentage of growing cells obtained seemed to be very low, especially in his control medium, which closely resembled the synthetic medium used in my experiments. I have therefore drawn up Table IV, in which are compared the percentages of growing cells obtained in Williams' experiments and my own.

Table IV.

Medium	A. Single cells			B. Two cells			Observer
	Cultures considered	Number growing	% growing	Cultures considered	Number growing	% growing	
Synthetic	95	25	27	85	32	38	Williams
Synthetic	21	16	76	43	30	70	Peskett
"Bios"-containing	210	113	54	164	105	64	Williams
	73	37	50	34	20	59	Peskett

It will be seen that the percentage of growing cells obtained in the synthetic media was considerably greater in my experiments than in those of Williams. The percentages for the "bios"-containing media in my experiments are unduly low as an earlier experiment has been included in which the mortality was high. If this experiment is omitted the percentages are, for the single cell cultures 66 %, and for the two-cell cultures 87 %, in the media containing "bios."

The technique employed in the later experiments, *i.e.* as described in this communication, is capable therefore of yielding a high percentage of growing cells.

DISCUSSION.

The results obtained raise some points of interest.

In the first place the use of growth curves brings out clearly the variations, in some cases considerable, which may occur in the growth of individual cultures in spite of all precautions that may be taken to standardise the conditions of growth. Robertson [1923, p. 87] quotes a series of isolated observations of his cultures after a definite period of time as evidence that the growth of infusoria is approximately inversely proportional to the volume of culture medium employed. Such isolated observations appear to be of little value. For if we were to consider the growth at two definite times in the experiment shown in Fig. 1, say at 20 hours and at 70 hours, we should in the first case group together cultures 3, 1 and 4, and in the second case 5 and 6. In this way any significance of the relatively slow growth curve of culture 3 or of the relatively rapid growth of 5 would be lost. For this reason, observations of the growth have been taken at frequent intervals, and only complete growth curves have been considered in studying the effect of volume.

In the second place, it is interesting to compare the concentrations of yeast employed in these experiments with those used by Pasteur and Liebig, together with the growth obtained in each case. Pasteur inoculated "une tête d'épingle" of fresh yeast, containing 80 % water, into 100 cc. of medium and obtained a growth in one experiment amounting to 0.043 g. of yeast weighed dry. Assuming a pin's head to be approximately a sphere of 1 mm. diameter it would have a volume of about 0.5 mm.³. Pasteur, then, inoculated about 0.1 mm.³ of actual yeast and this multiplied about 400 times.

Liebig using 100 cc. of medium inoculated with what he describes as "une trace de levûre." The only definition of this term that I can find occurs on p. 31 of his communication where he states "J'emploie de la levûre en pâte de la grandeur d'une tête d'épingle que je délaye dans 10 centimètres cubes d'eau; je prends de celle-ci un centimètre cube." Apart from any water that he may have used to render the yeast "en pâte" Liebig probably inoculated about one-tenth the amount of Pasteur, *i.e.* about 0.01 mm.³. He failed to note any multiplication of the yeast.

If we can consider the yeast cell as a sphere of diameter about 10 μ , and

therefore of about 5×10^{-7} mm.³ volume, then the range of volumes studied in my experiments was from 4×10^6 to 3×10^8 times the volume of a yeast cell in the synthetic media, and from 1×10^6 to 7×10^8 times in the media containing "bios" extracts. Single cells in these volumes would correspond to concentrations varying from 0.025 to 0.0003 mm.³ of yeast per 100 cc. of medium in the former and from 0.1 to 0.00014 mm.³ of yeast per 100 cc. in the latter experiments. On the synthetic media the growth obtained represented a multiplication of 5 to 28 times the amount inoculated, and on the "bios"-containing media from 120 to 18,000 cells were obtained from a single cell. On medium containing commercial cane sugar (Medium 1) multiplication of about 300 to 500 times was observed over a similar range of volumes.

It will be seen therefore that growth similar to that recorded by Pasteur was only obtained in the media containing "bios," although the range of volumes in both sets of experiments included values which, for the number of cells present, were comparable with those which Liebig and Pasteur employed. In none of my experiments was there any evidence that multiplication had been less in the larger volumes. On the contrary there was some evidence to show that the quickest growth occurred in the largest volumes, in the presence of "bios" extracts. The differences between the results obtained by Liebig and Pasteur cannot therefore be ascribed to differences in the volumes of medium used per yeast cell inoculated, unless the yeast employed by them was very different from that used in my experiments. They would appear to have been due to the presence of different amounts of "bios" in the cultures.

In this connection I have observed that the appearance of the cells has been similar to that described by Pasteur ("une belle levûre très ramifiée, extrêmement jeune d'aspect, c'est à dire que les globules sont gonflés, translucides, non-granuleux") only in the media containing "bios." In the synthetic media the growth was composed of very granular cells.

By further experiments in still larger volumes, I hope to elucidate the cause of the delay in multiplication observed in large volumes by Kuster [1907] and Wildiers [1901].

Thirdly, provided that the concentration of "bios" used has not been so great as to induce the "autostatic phase" of growth, the results obtained in the media containing "bios" are difficult to bring into line with the theory of an extracellular autocatalyst for growth suggested by Robertson [1923, pp. 102 *et seq.* and p. 147]. In all the experiments in Table II the growth was of a progressively increasing character, and therefore presumably in the "autokinetic phase."

In accord with Robertson's theory we should expect to find the reproductive rate enhanced in the smaller volumes and retarded in the larger, as the cells should at the time of division lose relatively less autocatalyst in the smaller volumes than in the larger. The results given above show that any enhancement of reproductive rate that may occur cannot definitely be attributed to a smaller or a larger volume, and that on the whole the evidence

points towards acceleration in the larger. In any case, Robertson's theory cannot be applied to the yeast used as no evidence of retardation of growth in larger volumes could be obtained.

Finally, the method employed includes a control of possible bacterial contamination, the importance of which has been demonstrated experimentally. Moreover, it yields a higher percentage of growing cells than that described by Williams. I hope that it may be usefully employed for the study of growth in other unicellular organisms.

SUMMARY.

1. A method is described for the study of the growth of single cells. Using this method, I have obtained a high percentage of living cells, and I have been able to detect readily those cases in which bacterial contamination has occurred.

2. The growth curves of one and two-cell cultures of yeast, grown in the absence of bacteria and in different volumes of medium, have been studied with the following results:

(a) In the absence of "bios." No constant effects on growth were observed as a result of differences in the volume of medium used.

(b) In the presence of "bios." The quickest growth occurred in larger volumes than the slowest, but there was not constant evidence to show that the growth was directly proportional to the volume. It was, however, definitely shown that the growth was not inversely proportional to the volume, as is the case in infusoria according to Robertson's observations.

3. The results summarised in 2 (a) and (b) are not in accord with Robertson's theory of an extracellular autocatalyst for growth.

4. The results obtained show that the differences between the observations of Pasteur and Liebig cannot be attributed to differences in the volumes of media employed by these workers per yeast cell inoculated, and that they were probably due to the presence of different amounts of "bios."

In conclusion I wish to express my indebtedness to Mr H. M. Carleton for his suggestion of the use of collodion in fixing the cultures and his advice throughout the elaboration of the bacteriological technique, and to Prof. Peters for his advice and interested criticism throughout the work. My thanks are also due to the Medical Research Council for a part time grant, and to the Government Grant Committee of the Royal Society for a grant to cover expenses.

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