LXVII. STUDIES ON THE GROWTH OF YEAST.

IV. A NEPHELOMETRIC METHOD OF COUNTING YEAST SUSPENSIONS.

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Report to the Medical Research Council.

(*Received March 17th, 1927.*)

Below I have recorded details of a convenient method for determining the concentration of cells in suspensions of yeast. It is based on nephelometric comparison of the yeast with standard suspensions of barium sulphate. McFarland [1907] has described a rough method for the estimation of the number of cells present in bacterial suspensions, which is essentially similar. In his earliest experiments he compared the suspensions against a dark background, but subsequently he designed an apparatus in which they were observed by direct illumination. Richards and Wells [1905], in improving the earlier method of Stas [1894], found that greater accuracy could be obtained with indirect illumination and varied the amount of light which was allowed to fall on to the tubes containing the suspensions. They determined the relative lengths of tube which were exposed to illumination when the turbidities appeared equal. In the method which I am about to describe, the tubes receive equal illumination and are compared against a dark background. A series of standard suspensions is used.

The method possesses the following advantages.

(1) It is rapid and reasonably accurate.

(2) It can be applied to the direct estimation of the number of cells present in growing cultures of yeast, and observations can be made on the cultures while growth is in progress without risk of exposure to infection.

(3) No expensive or elaborate apparatus is required.

(4) Although the preparation of a series of standard suspensions takes a considerable time, these standards appear to keep indefinitely. I have in use at the moment a series which was prepared eighteen months ago.

Standard suspensions of barium sulphate.

General considerations. The conditions employed in the precipitation of barium sulphate have been shown by Folin [1905] to influence to an important extent the character and composition of the precipitate obtained. In the

preparation of the standard suspensions every effort was made to keep the conditions absolutely uniform, in order to secure uniformity in the precipitates. Moreover, the special conditions recommended by Folin for the precipitation of potassium sulphate solutions have been adhered to as closely as possible.

To avoid the errors which would occur in the measurement and dilution of suspended barium sulphate, each standard suspension was prepared separately.

The test-tubes selected to contain the standards, and those used for the growth of yeast cultures, were of uniform bore.

While the excess of barium in the suspensions was probably adequate to prevent the growth of any organism, mercuric chloride was added as an additional precaution. Denis [1921] has shown that this does not interfere with the precipitation of barium sulphate. The mercuric chloride which I employed gave no precipitate on addition of acidified barium chloride solution.

For the sake of convenience I have considered as a dissolved substance the barium sulphate present in a suspension, and expressed its "concentration" in terms of molarity. If m be used to express molar concentration $\times 10^{-5}$, or $M/100{,}000$, then the concentrations of barium sulphate in the series of standards which I have used are as follows:

1000 m, 900 m, 800 m, 700 m, 600 m, 550 m, 500 m, 450 m, 400 m, 350 m, 300 m, 250 m, 200 m, 160 m, 100 m, 80 m, 60 m, 40 m, 20 m, 10 m.

The cloudiness of any suspension containing less than 10 m $BaSO_4$ is too slight for accurate comparison with yeast suspensions. Concentrations greater than 1000 m have not been employed, as the precipitate settles too rapidly. This difficulty might have been overcome by the addition of ammonium nitrate, as suggested by Denis.

Method of preparation. The solutions employed were:

barium chloride, approximately $M/4$,
mercuric chloride, saturated solution,
potassium sulphate $M/40$ and $M/200$ (Kahlbaum).

From the two solutions of potassium sulphate further dilutions were made, namely, $M/2000$ from $M/200$, and $M/400$ and $M/4000$ from $M/40$. Each suspension was prepared as follows. To 5 cc. mercuric chloride solution in a 250 cc. flask I added successively 2 cc. pure concentrated hydrochloric acid, the requisite volume of potassium sulphate solution, and sufficient barium chloride solution to give an amount of barium in solution at least equal to that present in the precipitated form. For example, in preparing 200 m $BaSO_4$ I used 100 cc. $M/200$ potassium sulphate and added 5 cc. barium chloride. The barium chloride was added drop by drop at the rate of 2·5 cc. per minute, without shaking or moving the mixture in the flask, which was allowed to stand undisturbed for a further five minutes. The flask was then filled to the mark with distilled water, shaken very thoroughly, and a sample was transferred as rapidly as possible to a clean test-tube. The tube was immediately sealed in a flame.

Apparatus for comparing turbidities of suspensions.

For comparing turbidities I have used the apparatus shown in Fig. 1. When in use, the hinged lid (*F*) is closed and observation is made through the apertures in it (*A*). As a rack to hold the tubes I have used a modified Cole and Onslow's comparator (*B*) [Cole, 1926] in which the four compartments employed are as close together as possible, and are drilled out through the bottom to allow light to pass up from below. All the inner surfaces of the comparator as well as the apertures at the back of the posterior compartments are covered with matt-surfaced black paper. As a source of light (*C*) I have used a 40-watt gas-filled lamp with opal globe, but owing to the heat it produces I have found it necessary to cool the whole apparatus by means of an air current from an electric fan. To secure approximately equal illumination of the tubes I have inserted between the comparator and the lamp four pieces

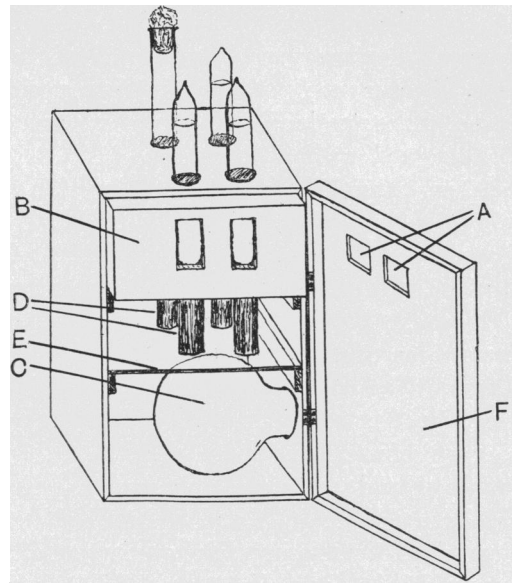

Fig. 1.

of glass tubing (*D*) of uniform length and diameter. These are lined with black paper and rest below on a glass plate (*E*). Above, they are fixed into the compartments of the comparator by means of rubber tubing. The lamp is arranged so that two samples of a suspension of yeast (or barium sulphate) display the same turbidity when compared in the apparatus; this being considered as a sufficiently accurate criterion of equal illumination of the tubes. The apparatus should be used in a dark room, but I have obtained quite good results in daylight by fixing a long tube of cardboard with one end around the windows (*A*), and applying the eye to the other end.

Estimation of yeast suspensions in coloured and colourless fluids. In comparing the turbidity of suspensions in colourless fluids I have placed two tubes

of distilled water in the anterior pair of compartments in the comparator, and the tubes containing the suspensions in the posterior pair. This is the arrangement shown in Fig. 1, in which a tube containing a yeast culture is pictured in the left-hand compartment, and a standard in the right. A small degree of colour in the yeast suspension does not interfere with the comparison.

I have had little occasion to estimate suspensions which were coloured to such a degree as might interfere with the estimation. But in the estimation of the most highly coloured suspensions a control tube of the coloured fluid (containing no suspended matter) must be substituted for the tube of water in front of the standard suspension. At the same time a screen must be placed between the source of light and the standard tube to compensate for the cutting off of light from the suspended particles, owing to the colouring matter. This screen should be chosen of such a colour that, on looking down through a tube of distilled water placed over it, and through a control tube of the coloured fluid, the same tint is observed, the tubes being filled to the level of the upper margins of the windows in the front of the comparator. Obviously this procedure does not perfectly control the colour factor, but preliminary experiments have shown that it yields very good results in the estimation of suspensions of yeast in highly coloured fluids.

In all comparisons of turbidity the suspensions were shaken as thoroughly as possible and compared in the apparatus immediately. Growing cultures of the yeast "381" readily form uniform suspensions if the tube is held upright between the thumb and fingers and shaken with a slight rotatory movement. This is especially the case up to the third day of growth. Subsequently, the yeast tends to adhere to the bottom of the tube and some pigment formation commences which renders more difficult the comparison with the barium sulphate standards, particularly in the case of rich development of the yeast. I have frequently observed that up to the third day of growth the cultures contain a very high proportion of single cells, and only a few cell-aggregates containing more than two cells. As growth continues larger aggregates generally predominate, and these tend to adhere to the walls of the tube. By admitting a small glass bead I have been able to obtain suspension of the cultures more easily, and experiments are in progress to determine whether this procedure is injurious to the yeast.

Thus the method is most suitable for the estimation of young growing cultures of yeast and becomes less accurate and convenient as the age of the cultures increases. This does not appear to me to be a great disadvantage as the growth of most cultures is very slow after the 70th hour.

Results.

The method has been applied to the estimation of suspensions of two species of yeast. Results are tabulated below (Table I) and recorded graphically in Fig. 2. They may be considered as calibrations of the barium sulphate suspensions in terms of yeast.

For each suspension of yeast a figure is given in the third column of the table denoting the concentration of cells it contained, and in the fifth column showing the concentration of barium sulphate which was required to display the same turbidity. In cases where the turbidity was not exactly matched by a standard I have judged the concentration of the standard which would have matched it by reference to the two standards nearest to it. In these cases I have shown the figures for turbidity in parentheses.

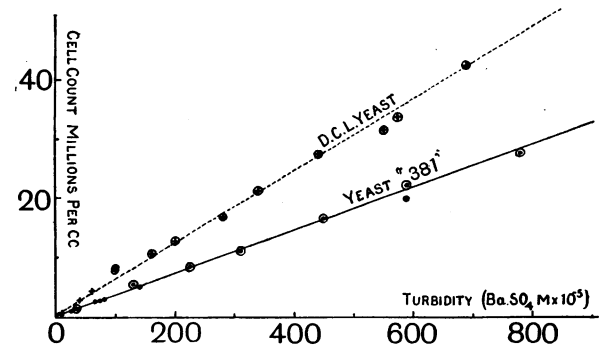

Fig. 2.

The remarks in the fourth column of the table need some explanation. In determining the number of cells present by a count a Bürker chamber was used, four samples were usually counted, and the mean taken. The maximum deviation of the figure for any individual sample from the mean was 21 %, the minimum 1 %, while in most cases the deviation was about 8 %. In the earlier determinations only the large squares of the chamber were counted. Such procedure necessarily limits the concentration of cells which can be determined by a direct count—without dilution—to some value between 0·25 and 5·0 millions per cc. But any suspension containing more than 50,000 cells per cc. possesses turbidity to a degree which can be matched by one of the standard suspensions. Thus in many cases where the turbidity of a yeast suspension was determined its cell content was not actually estimated by a count, but was calculated from the dilution used in preparing it. The remarks in the fourth column of the table refer to the method employed in obtaining the figures recorded in the preceding column. "Observed" values were determined by cell-count, whereas "calculated" values were calculated from the dilution of the suspension which was used.

The counts of suspensions II and III show that the discrepancy involved in diluting suspension II ten times was 14 %. But the counts of the four samples of suspension III showed a deviation of 21 % from the mean, presumably because the concentration of cells was low. In more recent determinations, of which suspensions XXIX and XXX are typical examples, larger concentrations have been counted directly using the small squares in the Bürker chamber. The error in diluting suspension XXIX ten times to

prepare XXX was 4·5 %. Thus when counts have been made of diluted suspensions the results have agreed with the values calculated from the dilution within the limits of experimental error. I therefore feel justified in including in my results those observations in which direct counts were not actually made, but in the graph such observations have been charted with distinguishing marks, *i.e.* enclosed in small circles (⊙ and ⊕).

Table I.

Fresh "D.C.L." Yeast.

Suspension number	Method of preparation	Millions of cells per cc. = C	Observed or calculated	Turbidity in terms of $BaSO_4$ $M10^{-5}$ = T	T/C
I	Suspended in water containing phenol	31·5	Calculated (from mean of II and III)	550	17·46
II	25 cc. I in 250 cc.	2·93	Observed	40	13·66
III	50 cc. II in 500 cc.	0·34	,,	(4)	11·78
IV	25 cc. I in 100 cc.	7·9	Calculated	100	12·66
V	100 cc. II in 500 cc.	0·59	,,	(9)	15·26
VI	Another sample suspended as I	42·2	Calculated (from VII)	(690)	16·37
VII	50 cc. VI in 500 cc.	4·22	Observed	60	14·24
VIII	25 cc. VI in 100 cc.	10·55	Calculated	160	15·18
IX	50 cc. VI in 100 cc.	21·1	,,	(340)	16·12
X	30 cc. VI in 100 cc.	12·66	,,	200	15·80
XI	40 cc. VI in 100 cc.	16·88	,,	(280)	16·60
XII	65 cc. VI in 100 cc.	27·42	,,	(440)	16·08
XIII	80 cc. VI in 100 cc.	33·76	,,	(575)	17·05
XIV	25 cc. XIII in 100 cc.	8·44	,,	100	11·86
				Mean =	15·01

Yeast "381." This is a pure strain of *S. cerevisiae* which I have used throughout a series of studies on yeast growth. "381" is the catalogue number in the National Collection of Type Cultures. Suspension XV was prepared from a 48 hours' growth at 25°, on medium containing cane sugar, salts and yeast extract. The cells were thoroughly washed on the centrifuge before use.

XV	Suspended in water containing phenol	27·7	Calculated (from XVI)	(780)	28·16
XVI	5 cc. XV in 50 cc.	2·77	Observed	(75)	27·07
XVII	40 cc. XV in 50 cc.	22·16	Calculated	(590)	26·65
XVIII	30 cc. XV in 50 cc.	16·62	,,	450	27·09
XIX	10 cc. XV in 50 cc.	5·54	,,	(130)	23·46
XX	25 cc. XVI in 50 cc.	1·39	,,	(35)	25·20
XXI	5 cc. XX in 50 cc.	0·14	,,	(3)	21·43
XXII	25 cc. XVII in 50 cc.	11·08	,,	(310)	27·99
XXIII	25 cc. XVIII in 50 cc.	8·31	,,	(225)	27·07
XXIV	Isolated observations on various cultures	0·24	Observed	(8)	33·33
XXV		5·0	,,	(140)	28·00
XXVI		2·91	,,	80	27·50
XXVII		2·68	,,	(65)	24·27
XXVIII		0·96	,,	(27)	28·14
XXIX	Another sample suspended as XV	19·75	,,	(590)	29·96
XXX	5 cc. XXIX in 50 cc.	1·87	,,	60	32·07
				Mean =	27·34

Discussion.

If the figures in the third and fifth columns of the table are plotted on a graph, as is shown in Fig. 2, the points fall on a straight line, the slope of which depends on the species of yeast considered. There is a direct relationship, therefore, between the number of cells present per cc. and the "concentration"

of barium sulphate which is required to give the same turbidity. By determining the latter in the case of a given suspension of yeast its cell-content can be estimated by reference to the graph, or by calculation from the constant T/C. If the lines plotted, or the mean value of the constant T/C, can be considered as representing the true relationship between turbidity and number of cells, and the fact that they are derived from a number of observations seems to justify this assumption, then it will be seen that, for the yeast "381," the maximum error of any single observation is 17 %, while over the greater part of the graph the error is not greater than 3 %. For "D.C.L." yeast the corresponding figures are 21 % and less than 7 %. These figures compare favourably with the average error involved in a direct cell-count. I have not considered the error of the figures for suspensions showing a turbidity less than 10 *m* $BaSO_4$ for reasons already stated.

The graph shows that the method can be used conveniently for the estimation of the concentration of cells in yeast suspensions between 0·5 and 35 millions per cc., in the case of yeast "381." The estimation is less accurate for smaller concentrations, and is not possible if there are less than 40,000 cells per cc. In the case of concentrations greater than 35 millions per cc. the suspension of cells must be diluted to come within the range of the standards.

It would perhaps be simpler to use some suspension other than barium sulphate as a standard, *e.g.* gum mastic. One standard suspension of such a substance could be employed, and the turbidity of the cell suspension determined in terms of the dilution of this standard which would be required to display the same cloudiness. I have not attempted any modifications of this nature, as the method in its present form has quite fulfilled all my requirements. Indeed, I hope that it may be modified for use in the estimation of cells other than yeast, *e.g.* bacteria, blood corpuscles, etc.

SUMMARY.

A method is described for the rapid estimation of the number of cells in suspensions of yeast, based on a comparison of the turbidity of the suspension with that of standard suspensions of barium sulphate.

In conclusion I desire to thank Prof. Peters for his advice and criticism, and the Medical Research Council for a part-time grant.

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