POTENTIALLY UNLIMITED MULTIPLICATION OF YEAST WITH CONSTANT ENVIRONMENT, AND THE LIMITING OF GROWTH BY CHANGING ENVIRONMENT.

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I.

A small amount of yeast seeded into a given volume of a suitable culture medium grows at a constant rate for a short time, after which the growth rate gradually decreases until the yeast just maintains itself at an equilibrium crop. The experiments reported in this paper show that the constant rate of growth can be maintained for as long as the environment can be maintained effectively constant. The part of the usual sigmoid growth curve of the yeast population after the inflexion point is merely a measure of the retarding effect of the accumulation of waste products. If the retarding influence is avoided the growth curve for a population of yeast is an exponential curve. Consequently, an attempt to analyze directly the nature of the S-shaped curve to determine the basic nature of the growth process can not be successful. This is why the method for such an analysis proposed by Robertson (1923) has not been profitable, even with the most general conditions.[1] The growth in diameter of mold colonies gives a sigmoid curve (*cf.* data in Fawcett, 1921), but as Crozier (1926) has indicated this curve is complicated by changes in the substratum not unlike those to be discussed in the case of yeast.

The experiments were made partly in the Department of Zoology of the University of Oregon, and partly at the Laboratory of General Physiology of Harvard University, and will be referred to as the Oregon and the Cambridge experiments respectively. The origin of the strain of yeast used in the Oregon experiments,

[1] Richards. O. W., The growth of the yeast *S. cerevisiæ*. I. The growth curve, its mathematical analysis and the effect of temperature of the yeast growth, *Ann. Bot.*, 1928, clxv, 271.

the culture technique, and the method used for determining the growth have been described.[1]

The Cambridge experiments were made with another strain, No. 2335: *S. cerevisiæ*, Hansen, furnished through the kindness of Professor F. W. Tanner of the University of Illinois. Its growth is the same as that of the strain used at Oregon, except that the equilibrium crop is numerically slightly different.[2] Except as specifically stated, the technique used in the Cambridge and in the Oregon experiments is the same.

II.

The effect of crowding was mentioned, but not tested, by Carlson (1913). Clark (1922) found that crowding did not lessen the growth when air and fresh medium were forced through the culture for 5 hours. I have found that rocking the cultures of yeast slightly increased the yield but did not change the nature of

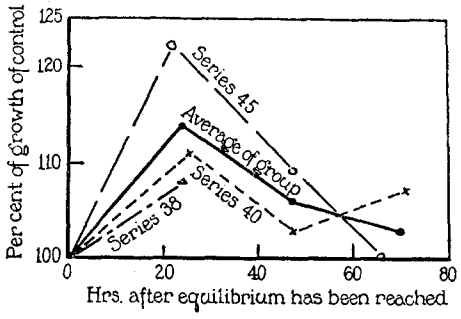

FIG. 1. The effect of reseeding a culture that has reached an equilibrium of growth.

the growth curve. The increased yield is not sufficient to justify the use of a rocking device.

Other experiments[1] have shown that a decrease of food in the medium is not a causal factor in the decrease of the growth rate. When the growth rate is diminished by a lack of food it is usually due to the exhaustion of an essential substance rather than to a general decrease of all of the nutrient substances (Slator, 1921). This is supported by the fact that diffusion seems to be adequate to furnish nutrient material to the individual cells (Slator and Sand, 1910).

Two series of experiments were made by growing the same seeding of yeast in the same volume of culture medium in both petri dishes and in test-tubes. The layer of medium in the petri dishes was about 1 mm. thick, so that the surface was

[2] The significance of these differences is discussed in the previous paper,[1] together with the method used in averaging the results of the different experiments.

proportionally large as compared with the small surface and the greater depth of the test-tube cultures. The amount of growth in each case was so nearly the same that the probable errors of the determinations overlapped throughout the time of the entire cycle. This shows that oxygen is not a complicating factor in the present study.

If the figures for any given series are studied the equilibrium crop is seen to be reached actually by oscillations. The maximum variations amount to less than 10 per cent for the test-tube cultures used in the present study. These oscillations vary from series to series and average out when several series are combined which gives the smooth line on Fig. 4*A*. This indicates that the oscillations are caused by changes in the environment rather than due to primary causes, as Ludwig (1926) seems to infer from the greater magnitude of the oscillations (about 30 per cent) found in his four 1-liter mass cultures.

TABLE I.

Size of Cells and Freezing Point Determinations at Equilibrium Conditions.

	Average cell size*	Δ	Per cent size	Per cent Δ
Control	91.5	0.434	100	100
Replaced	130.0	0.514	142	118
Doubled	127.8	0.577	139	132

* The difference of average size (arbitrary units) in these experiments and the previous ones is merely due to different enlargements of the photographs, as they are from the same strain of yeast and under the same condition of culture.

If a culture that has reached equilibrium growth at about 100 hours after seeding be reseeded with the usual seeding of cells, 40 to 48 hours old, we find a slight but definite increase in the number of cells present. The new growth soon ceases and the result is a new equilibrium at a higher level (Fig. 1), which seems to show that there are present in the medium products either toxic to the new buds produced or actually preventing budding.

Withdrawing half of the culture medium seems to have little effect on the cells. The average size of the cells is 65.1, as compared to 63.7 area units[3] of the controls, a difference just a little less than the error of measurement and hence insignificant. If the medium is pipetted off from the cells, and an equal volume of fresh, sterile medium added, we find an increase in the total volume of the yeast present of

[3] These data were obtained by measuring the tracings of projected photographs of the yeast cells with a planimeter, and they are expressed in arbitrary *area* units. A more complete discussion of the size changes of the cells and of the method will be published in a separate paper.

about 100 per cent, and about 40 per cent increase in the number of cells present. The average cell size is 74.1 units or 116 per cent of that of the controls, at 70 hours after making the change.

Adding an equal volume of fresh, sterile medium to the amount already present increases the volume of yeast by about 80 per cent and the number of cells by about 30 per cent in the same interval of time. The average cell size in this group is 71.0 units, or 111 per cent of the average size of the control yeast.

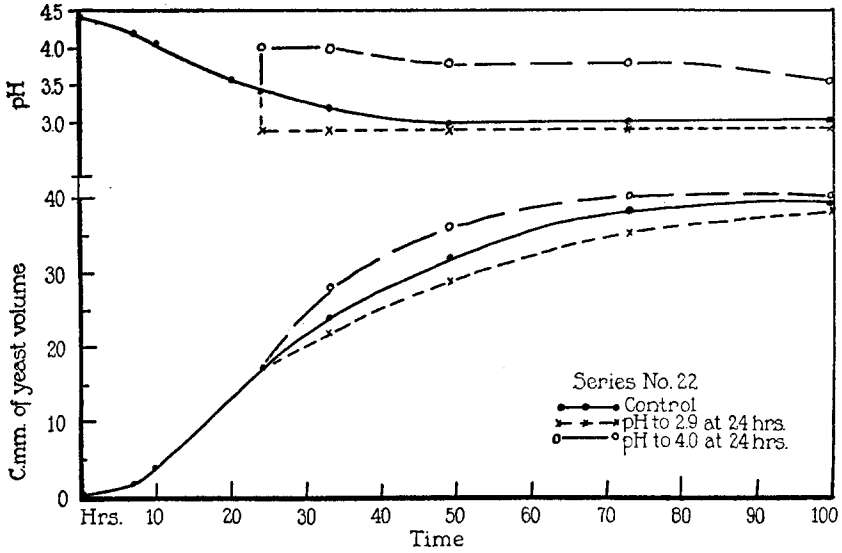

FIG. 2. The effect of changing the acidity of the culture medium on the growth of yeast.

These results support the view that the inhibition of the growth of the population is due to toxic waste products present in the medium. Doubling the amount of the solution merely halves the amount of toxic substances present, while replacing the medium greatly reduces their concentration. The increased yield was considerably greater with the latter condition than with the former. The changes of osmotic pressure in such experiments, proportional to the depressions of the freezing point, are opposite to the changes in the size of the yeast cells, as shown in Table I. The size changes are therefore not simply the result of osmotic pressure changes.

III.

The curve showing the change of acidity of the culture medium as the population grows is similar to the growth curve inverted (Fig. 2).

The acidities were determined colorimetrically by means of a drop method. The values were found to be slightly more accurate than 0.1 pH when checked with the aid of a hydrogen electrode.[4]

The effect of the acidity of the medium may be tested by dividing a number of tubes into groups, at about 24 hours after seeding, and changing the acidity in some while retaining the rest for controls. Some of the tubes were brought, with a few drops of N/10 HCl, to a pH below the value that they were expected to reach. The other group of tubes was partially neutralized with $Ca(OH)_2$ to about the acidity that they had at the time of greatest velocity of growth. Fig. 2 shows that acidity reduced the yield of yeast and that partially neutralizing the medium increases the crop.

Continued partial neutralization increases the crop and the growth curve is observed to be more symmetrical. However, the equilibrium conditions are merely delayed and not prevented, so the acidic changes are not solely responsible for bringing the growth to an equilibrium. The anion of the acid added may be deleterious to the yeast as well as the increase in the hydrogen ion concentration. The Ca is probably not toxic as the amount added does not bring the total concentration of the Ca above the optimum concentration previously found (Richards, 1925). The optimum Ca concentration would cause less increase than that noted in these experiments.

The effects of changing the acidity of the medium are summarized in Table II. These effects are not due to the dilution of the medium, because a similar simple dilution has no effect on the growth. When NaOH is used for partial neutralization it is strongly toxic. A greater yield of yeast may be obtained by using K_2HPO_4 instead of KH_2PO_4 in the medium, but the shape of the growth curve is hardly altered. Further buffering makes the medium toxic.

These effects seem to be general, as Yeast 2342 of the American Type Culture Collection responds in the same way and the results were duplicated at Cambridge with a third strain. All of these strains are "bottom" yeasts and give similarly shaped growth curves (with numerically different levels of equilibrium).

[4] I am indebted to Professor J. B. Conant for having these determinations with the hydrogen electrode made in his laboratory.

The maximum velocity of the change in acidity of the medium occurs *after* the maximum velocity of the increase in the number of cells. The Cambridge experiments gave maxima of 30 and 50 hours for the growth rate and the rate of change of acidity respectively. This demonstrates that the increase in acidity is an accompanying rather than the primary cause of the decline of the rate of growth of the yeast population. This is strengthened by the fact that the difference between the growth at different acidities is not numerically proportional to either the pH or the C_H. The acidity is due to non-volatile organic acids, because boiling a tube that has reached a pH of 3.0 only changes it to pH 3.2. Saturating the medium with CO_2

TABLE II.

The Effect of Changing the Medium on the Growth of Yeast (Oregon Experiments).

Hrs. of growth	Percentages of the central group		
	Kept at pH 4.0	To pH 4.0 at 24 hrs.	To pH 3.0 at 24 hrs.
35	108	111	91
48	116	110	86
72	107	104	90
100	105	102	90

at 24°C. brings the acidity only to 3.3. The nature of the acid present will be considered again in Section IV.

When yeast is grown in sealed tubes CO_2 has been shown to be a limiting factor for growth (Slator, 1921). Many investigators have observed that CO_2 lessens the growth of yeast, but few quantitative investigations have been made. My observations do not support the suggestion made by Pearsall (1925) that optimum cell growth occurs at or near the "isoelectric point" for the cells, or of the tissues of higher plants. Pearsall quotes the isoelectric point for yeast suspensions, as determined by cataphoresis, as pH 3.1 to 3.3. Certainly the growth of yeast is slight at such acidities, and they are reached long after most rapid growth has ceased. However, the isoelectric point for cell suspensions is probably very different from that of the cell contents at the place where the bud is being formed. Until we can measure the "isoelectric point" of the cell constituents that are being modified into a bud, such a theory must be speculative.

IV.

Most investigations on the effect of alcohol on yeast have concerned the fermentation industry and yield little of value to the present study. The earlier data are summarized by Euler and Linder (1915).[5]

Clark (1922) found that the growth of yeast was logarithmic for about 15 hours, when it began to decrease. Experiments showed that alcohol concentrations in excess of 1.75 per cent slowed growth. Clark does not give his method for determining the amount of alcohol further than "by distillation." He concludes that the retarding effect of alcohol is due to a lowered rate of reproduction of each cell.

A few experiments with the strain of yeast used in Oregon confirmed the observations of Clark. The chief difficulty of such experiments is the accurate determination of the amount of alcohol present in the 10 cc. of medium used in each tube. The most satisfactory method was a modification of the Nicloux method used by Miles (1922). It gave accurate checks when as little as 1 mg. of alcohol was present per cc. Alcohol production detectable by this method does not take place until after 24 hours after seeding. The alcohol then increases, reaching a maximum at 40 hours, and then decreases until an equilibrium concentration is reached (Fig. 4*D*).

Comparison of the individual curves indicates that the rate of growth of the yeast population immediately declines after the alcohol concentration reaches 1 mg. per cc. of medium. This is equally true when the acidity is maintained constant, which suggests that it may be a real threshold concentration. That the acidity effect in slowing the growth of yeast is secondary to the alcohol effect is attested by the maximum acidity increase occurring 10 hours later than the maximum alcohol production. Pyruvic acid, which is an accompaniment of the alcoholic fermentation, first appears at about 44 hours, or about 5 hours before the acid production becomes maximal.

These relations were tested as follows: the tubes were divided into three lots and one lot was kept for a control, the other two receiving sufficient alcohol to make the concentration 12 mg. per cc. of medium, which is more than the amount usually produced. One lot was maintained at a pH greater than 3.9, by frequent neutralization, and the

[5] See Euler and Linder (1915), p. 283.

other lot was unchanged (Fig. 3). In both cases the addition of alcohol reduced the growth of the yeast. Holding the acidity relatively constant seems to stabilize the growth curve. A decreased amount of alcohol is associated with this partial neutralization and there is also

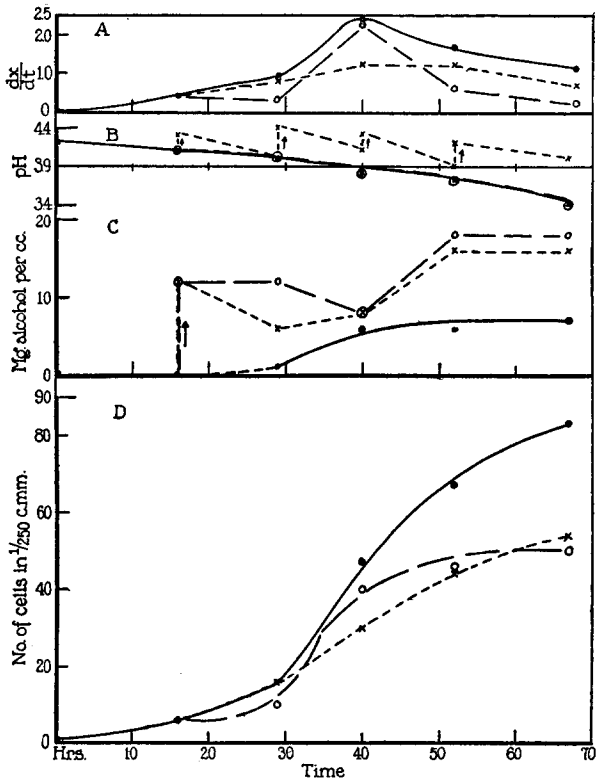

FIG. 3. The effect of alcohol on the growth of yeast. *A*. Velocity. *B*. Acidity *C*. Alcohol production. *D*. Growth curves. ● = Control group; ○, group + alcohol; x, group + alcohol kept at pH > 3.9.

a delay of the alcohol effect on cell proliferation. Adding alcohol only at first depressed, then accelerated, and finally depressed the growth more rapidly than in the control. The growth yield with the alcohol added was 59 per cent of that of the control, and with the alcohol added and the acidity controlled the yield was 65 per cent of

that of the control. The alcohol production was twice that of the control in the group maintained at approximately constant acidity, and slightly greater in the group not kept at constant acidity. The addition of the alcohol did not change the rate of acid production. This again suggests that the alcohol effect is the primary cause of the decrease in the rate of growth of the yeast population.

This strain of *S. cerevisiæ* is more sensitive to alcohol than that used by Clark (1922). The effect of alcohol may be determined by finding the percentage of cells that are actually budding at the time of greatest growth rate and at the time the population is just maintaining itself at a certain equilibrium. At 30 hours, time of maximum velocity of growth, and at 116 hours, culture at equilibrium, these percentages are 22 and 19, respectively, for the average of two series of experiments. As will be shown in a later paper, the distribution of the sizes of cells indicates that it is the larger buds that are selectively affected by the increased toxic products in the environment, rather than the division rate as suggested by Clark.

My experiments suggest that it is the increase of alcohol rather than the exhaustion of sugar that causes the decrease of the rate of the growth of yeast in the aerated, ammonia-molasses medium used by Balls and Brown (1925). They report that the sugar is used up by 8 hours of growth. Calculation shows that the velocity of the yeast growth begins to decrease steadily at about 5 hours. At 5 hours the alcohol content of the medium is from 1.6 to 5 mg. per cc., which is near the threshold found in the present experiments. Further, Balls and Brown failed to obtain any distinct increase in the growth of their yeast by renewing the sugar after it had been exhausted. Both of these facts suggest an alcohol effect on the growth rate instead of the exhaustion of the sugar. The growth curves of their experiments and of mine are very similar, which indicates that aeration increases the yield of yeast but does not change the sequence of events in the growth of the yeast population.

V.

One cannot run fresh medium over the cells without losses, if caking on a filter is to be avoided. The best method for getting rid of the alcohol seemed to be to very carefully pipette the medium off down to

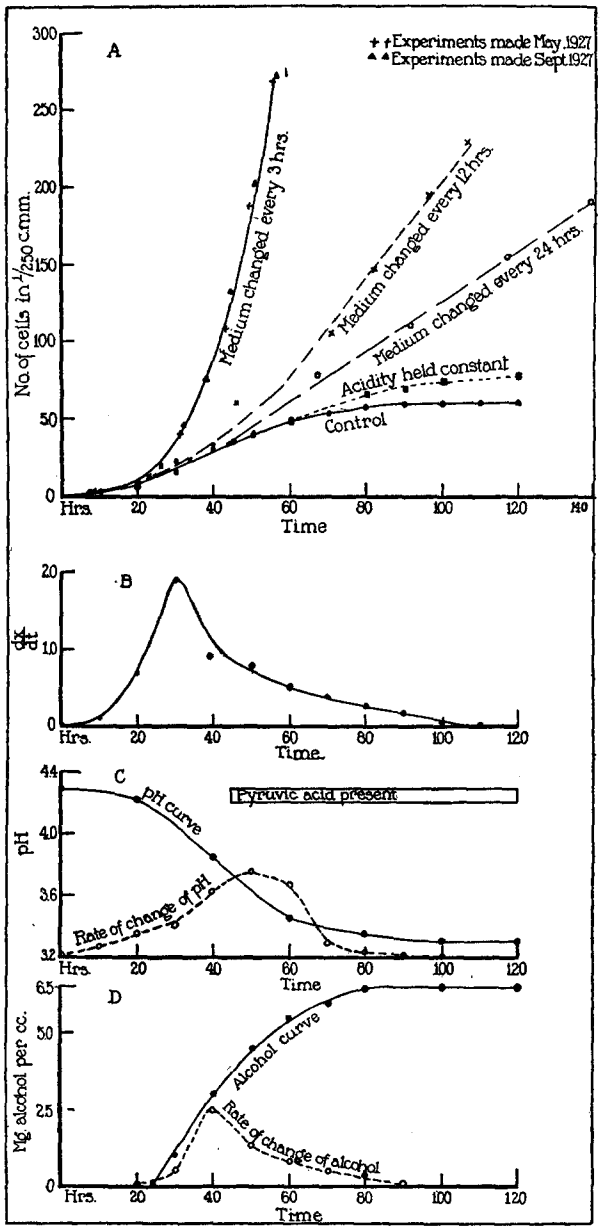

FIG. 4. *A*. Growth curves. *B*. Velocity curve of the control. *C*. The changes in acidity of the medium of the control. *D*. The alcohol production of the control.

as close to the cells as possible without actually removing the cells. This left about 0.3 to 0.5 cc. of medium, but the concentration of waste products contained in it would be greatly reduced when the original volume (10 cc.) was restored with fresh, sterile medium. The amount of alcohol left could be kept to an amount below the threshold value by frequently changing the medium.

Changing the medium at intervals of 24 hours, Figs. 4*A* and 5, shows a yield about three times as great as that obtained by the control group. The growth after 60 hours is practically linear to the time

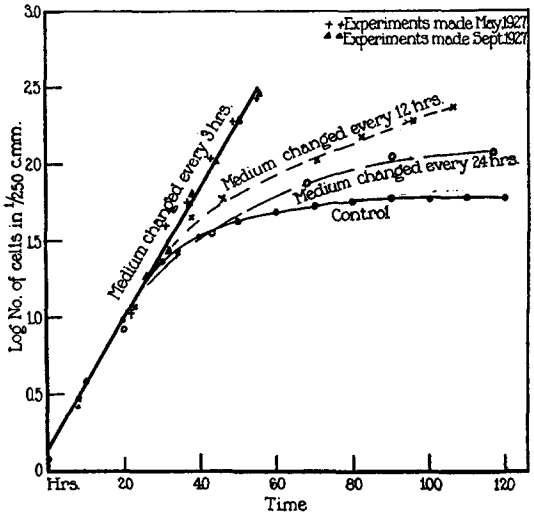

FIG. 5. Growth curves, logarithmic plot.

that the experiment was discontinued. When the medium is changed every 12 hours the growth more nearly approaches a simple logarithmic curve such as that given by the compound interest law (Fig. 4*A*), and the rate of increase shown by a logarithmic plot (Fig. 5) remains constant for a longer time.

Inspection of the times of departure from the control growth curve, or of the slopes of the growth curves after the departure from the linear segment, indicates that changing the medium every 4 hours should entirely prevent the inhibiting effect of the toxic products.

The medium was consequently changed every 3 hours. This gave

a relatively enormous growth that had to be discontinued at about 56 hours, as the number of cells became so great as to prevent changing the medium without losing some of them. A further increase in numbers would require dilution before they could be counted. Inspection of the logarithmic plot in Fig. 5 shows that the curve is linear within the experimental error. The smooth line was drawn through the points representing experiments made in May. The experiment was repeated in September and the points of the latter test are plotted on the previous graph. The agreement of the determinations indicates the orderliness and regularity of the growth of the yeast under these conditions.

Multiplication under the condition of an effectively constant environment as obtained by changing the culture medium every 3 hours is *potentially unlimited.* The increase in the number of cells per unit of time is in proportion to the number of cells present, and follows the compound interest law. This form of growth leaves no room for the assumption of any autocatalyst for multiplication, because the rate of increase is constant.

Slator (1913) has obtained logarithmic growth up to a concentration of about 10 million cells per cc. My yield is about 70 million cells per cc. This may be relatively a greater crop, as I have used a synthetic medium while Slator used a wort medium. Clark (1922) maintained logarithmic growth for almost 24 hours, or for a little more than a third of the time in the present experiment. By using larger dishes and improved technique for changing the medium this constant rate of growth could be greatly prolonged.

Consequently, when the food is adequate, the sigmoid part of the growth curve after the point of inflexion is merely a measure of the inhibiting effect of the toxic waste products of the cells, excreted into the environment. This suggests that the results of experiments regarding the relations of yeasts to vitamines, for example, can only be compared when the growth is maintained in a constant environment, and probably accounts for some of the current controversy in this field (*cf.* Tanner, 1925). It lends further weight to the opinion expressed by some investigators that probably the conditions of multiplication are more significant in influencing the rate and nature of growth than any "bios" (Tanner *et al.*, 1926).

It is quite possible that the growth of populations of other unicellular organisms is similar to that found for yeast. The fact that yeast grows by budding does not influence the rate of growth under constant conditions. The relations between the individual cell and those of the population will be considered in more detail, together with the changes in the sizes of the cells during growth, in a forthcoming paper. The effect of variable and constant environment on the growth of populations of yeast suggests that analogies between yeast and human populations, such as have been made by Pearl (1925), should only be made with the greatest of caution.

SUMMARY.

1. The decrease in the rate of growth of a population of yeast cells, which results in the maintenance of an equilibrium crop level, is shown to be due to substances excreted into the culture medium by the growing cells. These toxic substances tend to destroy the young buds, because the percentage of budding cells is about the same at the time of most rapid growth and at the time of the growth equilibrium.

2. Alcohol is the product which primarily causes the decline of the growth rate. For the strain of yeast used, under the particular conditions of these experiments, a concentration of alcohol of about 1 mg. per cc. is associated with the beginning of the decrease of the growth rate.

3. The increasing acidity of the medium, due to CO_2, pyruvic acid, and other organic acids, is also a retarding influence. It is a secondary factor, however, as the greatest increase of the acidity of the medium occurs after pyruvic acid, probably a by-product of alcoholic fermentation, appears.

4. When the medium is maintained effectively constant, by preventing the accumulation of these toxic products, the yeast grows at a *constant* rate and the yeast growth is *potentially unlimited*. The limit of growth found in actual experiments is due only to the size of the test-tubes and to the relative efficiency of the method used in keeping the medium effectively constant. The necessity of maintaining a constant rate of growth in studies on the relations of yeasts to vitamines and other products is stressed.

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