RECENT STUDIES IN YEAST
AND THEIR
SIGNIFICANCE IN INDUSTRY

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FIRST SESSION

Chairman: MR. H. J. BUNKER

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PHYSICAL PROPERTIES OF BREWING YEAST

by

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As is well known, the more familiar range of brewing yeasts belonging to the species *S. cerevisiae* and *S. carlsbergensis* includes strains differing widely in brewing properties, particularly as regards the detailed physical behaviour of the yeast cells when suspended in nutrient media such as wort. The properties in question involve (*a*) the ability of the cells to aggregate, or as it is often termed, to flocculate; (*b*) the ease with which they are entrained by bubbles of carbon dioxide and carried to the surface of the fermenting wort, behaviour on which is based the distinction between 'top' and 'bottom' yeasts; and (*c*) their ability to precipitate isinglass, a reaction used particularly in British breweries to remove unwanted yeast and proteinaceous matter from turbid beer. It will readily be appreciated that the importance of these properties arises from the fact that they control the extent to which the yeast is maintained in contact with the wort at various stages during the fermentation and thus, indirectly, the eventual composition of the fermented wort or beer. It must be admitted, however, that we are relatively ignorant of the precise factors determining properties (*a*) and (*b*) which evidently depend on subtle aspects of the structure of the surface of the yeast cell.

A whole body of new evidence[1] has recently come to light in these laboratories on the basis of which it must be supposed that the flocculation of yeasts is a reversible process involving interaction between specific structures located in the cell surface, for the cells of flocculent yeasts are not simply 'sticky' in the sense that they adhere indiscriminately to all solid surfaces. They show, rather, a marked preference for attaching themselves to other yeast cells and here again further restrictions operate as only certain kinds of yeast are found to interact. The simplest case is where the cells of one yeast flocculate each other but fail to react with foreign cells. Various self-flocculent yeasts of this kind may be distinguished by the changes in flocculation which occur when the acidity is changed, when specific sugars are added and, finally, by the effect of ethyl alcohol on the intensity of flocculation. A somewhat broader specificity is shown in the phenomenon of mutual flocculation in which the cells of one yeast besides attracting each other also attract those

of certain other yeasts, which themselves may or may not be self-flocculent. Indeed, cases are known where neither member of a pair of mutually flocculating yeasts is self-flocculent to an appreciable degree. Such considerations have proved to be important in understanding the complex behaviour of the mixtures of yeasts which are often used in British breweries. For instance, mutually flocculating yeasts may tend under the conditions of commercial practice to be selected as a group from among a given heterogeneous mixture of strains in the course of successive fermentations.

Turning now to property (*b*) of yeast, namely that of collecting at the surface of the fermentation vessel, this has been shown to depend essentially on the ability of the cells to collect at the interface between the liquid phase and the escaping bubbles of carbon dioxide. The latter, incidentally, may conveniently be replaced by air in laboratory experiments. The tendency of top yeasts as opposed to bottom yeasts to collect in the interface may in fact be demonstrated by quite simple means, for instance by inspecting the surface of a drop of an appropriately buffered suspension of the cells.[2] It seems that even non-flocculent yeasts may form the yeast 'head' typical for top yeasts although, as might be expected, attractive forces between the cells may help to stabilize a film of yeast at the surface and, incidentally, draw into the surface other strains of indifferent head-forming ability.

In seeking a deeper explanation of these phenomena one is naturally led to explore the finer details of the structure of the cell surface, for it is clear in a general way that the specific forces which seem to govern the mechanical behaviour of suspensions of yeast cells originate in a specific structure of an unknown nature. One method of approach to this problem has consisted in studying the migration of various strains of yeast on being placed in an electrical field. An interesting preliminary conclusion drawn from these studies,[2] and from further studies using chemical techniques, is that phosphate groups play an important part in the structure of the outermost surface of the cells of many brewing yeasts. Furthermore, in many but not all cases they are found to be responsible for the major part of the negative charge located at the surface. No evidence has been found, however, that the phosphate groups as such play a part in flocculation or in the development of a yeast 'head', but their presence at the surface seems to be essential if rapid fining is to occur [the property mentioned above under (*c*)]. As first demonstrated by Wiles[3] and now confirmed in detail by work in these laboratories,[4] fining results from interaction between positively-charged particles of the collagen isinglass and negatively-charged

yeast cells. It is worthy of note that an analogous process of precipitation of positively-charged by negatively-charged yeast cells can be demonstrated but that the conditions under which the reaction takes place are not those which favour flocculation as it commonly occurs.[2] Indeed the latter, as already mentioned, would seem to be the expression of more specific forces of attraction, but until more is known about the nature of these forces it seems unlikely that an explanation will be forthcoming of the variations in flocculation which are commonly encountered in the brewery.

References

[1] Eddy, A. A., *J. Inst. Brew.*, 1955, **61**, 307, and unpublished observations
[2] Eddy, A. A., & Rudin, A. D., in preparation
[3] Wiles, A. E., *Proc. European Brew. Convention* (Brighton) 1951, p. 84
[4] Rudin, A. D., *J. Inst. Brew.*, 1956, **62**, 414

Discussion

Mr. J. H. St. Johnston: Can mutual flocculation of two types of yeast occur between a flocculent and a non-flocculent yeast and would that perhaps account for the regularity of proportion of flocculent and non-flocculent yeasts separated by the Burton Union system of fermentation?

Dr. Eddy: Yes it can and, as you suggest, may account for the behaviour you mention.

Mr. R. B. Gilliland: What effect would a small quantity of beer have on the differential character of the lysine medium?* Would it be possible to plate out a small quantity of sediment from a bottle of beer?

Have you done any quantitative recovery experiments to find if a small percentage of wild yeasts in a mixture could be accurately estimated?

Dr. Eddy: I think one would be advised to remove even traces of beer by washing in the centrifuge. Recovery experiments indicate that the wild yeasts are accurately estimated in the presence of excess brewing yeast.

Mr. A. E. Wiles: I note that the lysine medium, whilst effective in many cases, failed to detect some troublesome wild yeasts, e.g., strains of *S. carlsbergensis*. Is there any correlation between the mutually flocculating A and B types and their electrophoretic behaviour?

* Used for detection of contamination of brewing yeast strains with yeasts of foreign genera.

Dr. Eddy: Yes, the first comment is perfectly true. No correlation has so far been found between the A and B types and their respective electrophoretic behaviour.

Dr. J. I. Webb: What is the effect of lysine on the growth of Brettanomyces?

Dr. Eddy: All the strains tested have been found to grow.

Mr. M. H. Proctor: In the two groups of yeasts, (*a*) those showing a strong negative charge and (*b*) those with no charge or positively charged, is there any obvious difference in type or quantity of nucleic acid in the cell or specifically in the cell wall?

Dr. Eddy: Our studies of the composition of the cell wall have not proceeded sufficiently for me to be able to answer this question, but speaking from the experience of other workers I would say that there is little nucleic acid in the wall itself.

Dr. F. Winder: If the tendency of the top yeast to rise is to be attributed to a tendency to collect in the air/water interface, this presumably is due to a hydrophobic surface on the cell. Is this supported by a tendency to collect in an oil/water interface? The fact that the collection on the surface increases as fermentation progresses is in keeping with the fact that the lipid content of micro-organisms tends to increase as the culture ages.

Dr. Eddy: I have attempted experiments of this kind, using olive oil droplets in water suspension, but have so far failed to find evidence of accumulation at the interface. As regards your second point: I do not think that one can generalize on the matter of lipid content having regard to the variety of substances covered by the term 'lipid.'

RESPIRATION AND THE GROWTH OF YEAST

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Introduction

IN spite of numerous investigations concerning respiration and growth of *Saccharomyces cerevisiae*, certain fundamental relations between these two processes have been neglected almost completely in the past. The purpose of this paper is neither to give a general review of the question nor to discuss what is well known about respiration, growth and their mechanisms, but to bring out some of the salient discrepancies between the currently accepted picture and the experimental facts.

1. Measurement of respiration during growth

The manometric estimation of oxygen consumption using either Warburg or Barcroft respirometers is one of the techniques most widely used at the present time. In the early days of manometric techniques, it was found that the amount and the surface area of alkali employed for absorbing CO_2 in the Warburg procedure have to be quite large in order to permit a rapid absorption of the CO_2 evolved during vigorous fermentation,[1] and some thirty years ago Dixon[2] introduced the use of filter papers placed in the alkali centre well for this purpose.

Apparently it has not been realized, however, that this method presents a systematic error, irrespective of the concentration and surface of the alkali used. This error is due to the kinetic properties of the system and cannot be avoided. The extent of the error can be estimated precisely, however, and adequate corrections can, if necessary, be made.

In a Warburg vessel the rate of absorption of CO_2 by alkali follows a first-order equation

$$d[CO_2]/dt = K_1[CO_2]$$

The constant K_1 depends on the volume of the vessel, the alkali concentration and surface area, temperature, rate of shaking etc., but it should not change in the course of the experiment. Assume for a moment that the CO_2 output in a yeast fermentation is linear in time, i.e. $+d[CO_2]/dt = K_2$. An equilibrium will be established and the manometer will show no deflection if

$$+ d[CO_2]/dt = - d[CO_2]/dt.$$
$$K_1[CO_2] = K_2, \text{ or } [CO_2] = K_2/K_1.$$

This means that there will always remain a stationary level of CO_2 in the vessel even in the presence of alkali and that this stationary level may vary considerably during the experiment. When the rate of CO_2 evolution (K_2) changes, the stationary CO_2 level changes and the manometer shows a deflection which, in the case of oxygen-consumption measurements, will be attributed to changes in the respiratory rate. Variation in rate of CO_2 evolution unavoidably affects the determination of respiration.

For those not working with yeast, the error is an extremely small one, because the rates of CO_2 output and O_2 uptake are of the same order of magnitude, but it may be considerable for those interested in yeast fermentation problems.

Under usual conditions (vessel of 20 ml. total capacity, 2 ml. of buffer 4·5, 110 oscillations per minute, temperature 28°c, using 0·2 ml. of 20% KOH plus filter paper), the K_1 values are of the order of 0·3 – 0·4 min.$^{-1}$. If we place in the vessel 1 mg. dry weight of yeast having initially a Qco_2 of 360, then

$$[CO_2] = \frac{360}{60 \times 0{\cdot}3} = 20\mu l.$$

If, in the course of experiment the rate of fermentation increases by one half, an underestimate of 10 μl. of O_2 uptake will be made. If a brewer's yeast with a Qo_2 of, say, 20, is used in the experiment, the error will be 50%. It is easy to calculate that the error is important only if the rate of CO_2 evolution is several times higher than the rate of O_2 uptake and if there is a change in the CO_2 rate. And this is precisely what happens when yeast is growing. There are several possible ways to obviate the difficulty, such as to estimate the error and correct for it, to try to increase the constant K_1 by putting e.g., alkali into side-arms, or to measure oxygen uptake by a quite different method, e.g., an amperometric one.[3]

2. Influence of respiration on growth

It is a truism that a respiring yeast grows better than a purely fermenting one, by which it is generally implied that both parameters of growth,[4] the rate (measured during the exponential phase) and the yield (measured by the ratio of cellular matter synthesized to organic matter used up), are higher in the presence of respiration than in its absence. The experiments can be performed in two ways: (*a*) by comparing aerobic and anaerobic growth of the same yeast (this is the usual way), (*b*) by comparing aerobic growth of a normal

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strain and a respiratory-deficient mutant derived from it. Ephruss and his collaborators[5-9] have studied these 'petite' mutants which lack cytochrome oxidase and are therefore unable to respire.

If such an experiment be performed on freshly isolated mutants the expected result is obtained. Fig. 1A shows aerobic multiplication of normal and mutant yeast in a fully aerated medium in which glucose is the only limiting factor of growth. Both the rate and the yield are much lower for the mutant than for the parent strain. After a certain number of transfers, however, the rate of growth of the mutant increases and eventually becomes identical with that of the normal strain.[6,8,10,11] Fig. 1B shows the same two strains as before but after about 450 transfers corresponding to several thousands of cell generations; they differ no longer in the rate of growth but only in the yield. Now, in spite of this acceleration, the mutant has preserved all its fundamental metabolic and enzymic deficiencies. It still lacks cytochrome oxidase and the cytochrome-*c* reductases of succinate, of α-glycerophosphate and of DPNH. It is clear therefore that a new, more efficient type of fermentative metabolism has been developed which, without consuming oxygen, suffices to assure the maximal growth rate. We know neither the

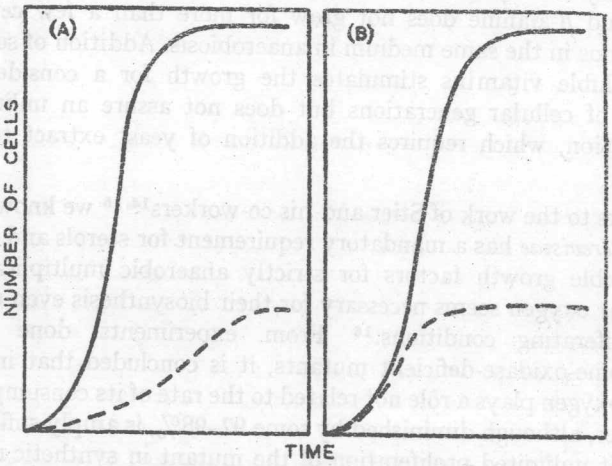

FIG. 1. *Rate of multiplication of normal and mutant yeasts.*
———Normal haploid strain 59R.
– – – – respiratory-deficient mutant derived from it 59RA.
Aerated medium containing glucose as the sole growth limiting factor.
A: freshly isolated mutant, B: the same, after approx. 450 transfers in pure culture. Schematic, after references 6 and 10.

biochemical basis of this phenomenon nor the mechanism of training which *a priori* may be due to selection of fitter respiratory-deficient mutants or to some physiological adaptation. It should be pointed out that, in natural conditions, this phenomenon could very rarely appear, if at all, because the 'petite' mutants would be wiped out by selection before the training could occur. It would appear that these fast-growing, permanently non-respiring 'petite' strains may be of great interest for some practical problems discussed at this Symposium.

Quite an analogous situation is found by studying anaerobic growth of normal yeast. Before discussing it, however, some remarks concerning anaerobic growth seem necessary. It should have been apparent from a careful examination of Pasteur's data[12] that the low yields of anaerobic growth could not be explained solely by the low energy-efficiency of sugar fermentation. Pasteur found in certain experiments that the yield of yeast in anaerobiosis was 50 to 100 times smaller than in the presence of oxygen. A purely energetic difference could have explained a difference of a factor of 15 at most, but not a factor of 100 (cf. references 6 and 13). This discrepancy is due to the fact that anaerobiosis increases the requirements of vitamins and growth factors. For instance,[6] a strain that grows aerobically *ad infinitum* in a synthetic medium containing only biotin and β-alanine does not grow for more than a few cellular generations in the same medium in anaerobiosis. Addition of several water-soluble vitamins stimulates the growth for a considerable number of cellular generations but does not assure an unlimited proliferation, which requires the addition of yeast extract to the medium.

Thanks to the work of Stier and his co-workers[14,15] we know now that *S. cerevisiae* has a mandatory requirement for sterols and other lipid-soluble growth factors for strictly anaerobic multiplication. Molecular oxygen seems necessary for their biosynthesis even under non-proliferating conditions.[16] From experiments done with cytochrome-oxidase-deficient mutants, it is concluded that in this respect oxygen plays a rôle not related to the rate of its consumption. The latter, although diminished by some 97–98%, is amply sufficient to permit unlimited proliferation of the mutant in synthetic media not supplemented with sterols. We have to distinguish therefore between two ways by which oxygen affects yeast multiplication. In the first, the major one, it acts as a terminal acceptor for electrons and completely changes the pattern of energy production in the cell. In the second, its action is nearer that of a trace element or a growth factor.

Harris[11] in this laboratory has confirmed Stier's results that normal yeast can grow *ad infinitum* under anaerobic conditions when supplemented with ergosterol and Tween 80. The experiments were done in an apparatus (cf. reference 15) which permitted all manipulations to be performed in an atmosphere of nitrogen containing less than 0·00005 parts of oxygen. In the course of successive transfers amounting to several hundreds of cell generations, a considerable increase of the growth rate was observed.[11,17] The final anaerobic value was quite similar to the maximal one in air. Such a rapidly growing anaerobic yeast is, however, perfectly capable of respiratory adaptation, i.e. it is able to synthesize all the lacking respiratory enzymes when exposed to oxygen. It contains only a very small proportion (approx. 1%) of 'petite' mutants.

The question arises whether training of the mutant and training of the normal yeast are similar. In the author's laboratories, normal baker's yeast has been trained to grow anaerobically almost as fast as it grows aerobically, and also respiratory-deficient mutants to grow aerobically as fast as does the normal strain. These two cases appear to be parallel, but we know now that they are different. Normal yeast (anaerobically fast-growing) gives rise occasionally to 'petite' mutants, which have the slow aerobic growth rate and not the fast one. Conversely, a 'petite' trained to grow fast aerobically will not grow as fast in the absence of air. In other words, at least two different mechanisms of supporting the maximal rate of cellular synthesis are compatible with the absence of respiration.

3. Influence of growth on respiration

If commercial baker's yeast is put in a buffer containing glucose, in the presence of air, it is found that the Pasteur effect operates and almost all the non-assimilated glucose is oxidized to CO_2 and water, ethanol is never formed in large quantities, the rate of respiration is very rapid and the rate of fermentation is slow. Since the appearance of Meyerhof's paper,[18] this classical picture is very well known to every student of biochemistry.

What happens if the same experiment is performed in a growth-sustaining medium? In other words, what is the influence of the general synthesis of cell materials on respiration and fermentation? Surprisingly enough, very few experiments have been done. It has been noticed by Meyerhof[18] and by Warburg[19] that yeast cultivated in the laboratory has in general a much lower respiration rate than commercial baker's yeast. Some ten years ago Swanson & Clifton[20] found that growing yeast first consumes oxygen slowly, then more rapidly. They concluded that young cells of baker's yeast, even cultivated aerobically, carry on their most active synthesis by

fermentative assimilation. This predominates until all the hexose has been used, oxidative assimilation then taking place from accumulated ethanol. Their data, although not always convincing in details (e.g. the manometric experiments were carried out under conditions where the systematic error of the method, discussed previously, must have been very large), are fundamentally correct. More recent carbon-balance studies by Lemoigne *et al.*[21] demonstrate that growing baker's yeast ferments glucose even if it is optimally aerated. During exponential growth, ethanol accumulates to the extent of more than 80% of hexose used and only in the last phase of growth which may show a diauxie phenomenon is ethanol respired. These findings seem paradoxical. Respiration of glucose is energetically more efficient than fermentation; oxidative assimilation as measured by intracellular carbohydrate synthesis is also much greater than the fermentative assimilation; the variety and versatility of carbon intermediates and building-blocks formed through the respiratory tricarboxylic acid and pentose cycles, is much greater than what can be produced by a relatively rigid Embden–Meyerhof–Parnas pathway. Last, but not least, the Pasteur effect which is so efficient in the absence of growth, should operate and prevent fermentation from gaining the upperhand. Yeast, however, grows wastefully most of the time, and at the end hastily dissimilates the alcoholic product of its own dissipation.

The reason for this lies in a regulatory mechanism of the yeast cell which has been called 'counter-Pasteur-effect'[13]. The Pasteur-effect describes a relationship between rate of fermentation and rate of respiration. It is a uni-directional relationship in which the first process depends on the second one and it operates at the level of enzyme *function*. The rate of fermentation does not influence, or influences relatively little, the rate of oxygen uptake (except for rendering the measurements more susceptible to the systematic error, *vide supra*). The counter-Pasteur-effect describes a relationship between the rate of synthesis of respiratory enzymes and the rate of fermentation, the former being in general inversely proportional to the latter. It operates at the level of enzyme *formation* and is therefore of great importance during growth and practically of no importance during an experiment of the type described at the beginning of the paragraph.

Respiratory enzymes in aerobic growth cycle

A study has been made of the fate of respiratory enzymes in the growth cycle.[22] Results of a typical experiment are given in Fig. 2. A baker's yeast with classical characteristics (spectrum showing strong bands of cytochrome *a*, *b* and *c*, high respiratory rate of

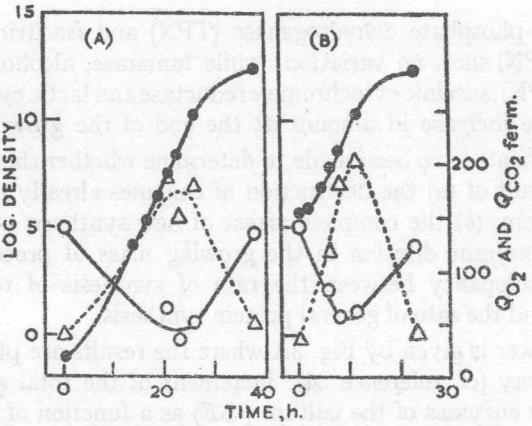

FIG. 2. *Evolution of metabolic parameters of normal yeast 59R growing in a synthetic, aerated medium containing 3% of glucose*

The parameters, $Q^{air}_{O_2}$ and $Q^{air}_{CO_2\,ferm.}$ are measured, under resting conditions, on washed samples and expressed per h. and per mg. dry weight of cells (from reference 22). Inoculum 100 times as great in B as in A.

● growth △ Q_{CO_2} ferm. ○ Q_{O_2}

Qo_2 140 and low aerobic fermentation of $Q^{air}_{CO_2\,ferm.}$ 38) is inoculated into a synthetic medium containing 3% of glucose. From time to time samples are taken out, cells are rapidly chilled and washed, their metabolic parameters are measured under standard resting conditions in the presence of glucose or ethanol, and finally the cytochrome spectrum is observed and certain enzymic activities assayed after breaking the yeast and extracting the enzymes. The two experiments shown in Fig. 2 differ only in that the size of inoculum in the first was 100 times smaller than in the other. In the first experiment the culture went through 14 cellular generations, while in the second one through only 7. The phenomenon was similar in both cases. From the commencement of growth the respiratory capacity fell, reached a minimum during the exponential phase and was finally restored to its original value during the phase of negative acceleration of growth. The $Q^{air}_{CO_2\,ferm.}$ varied in an almost exactly opposite manner. The Meyerhof quotient remains practically constant. The concentration of certain respiratory enzymes falls considerably during the exponential phase and explains the drop in Qo_2 values. Cytochrome *c* content is diminished by about 80%, as is that of cytochrome oxidase, but the concentrations of other constituents of the respiratory system do not vary in a parallel manner.

Glucose-6-phosphate dehydrogenase (TPN) and *iso*citric dehydrogenase (TPN) show no variation; while fumarase, alcohol dehydrogenase (DPN), succinic cytochrome-*c* reductase and lactic cytochrome-*c* reductase increase in amount at the end of the growth cycle.[23]

Experiments have been made to determine whether these changes are the result of (*a*) the destruction of enzymes already existing in the inoculum, (*b*) the complete arrest of new synthesis of enzymes with concomitant dilution in the growing mass of protoplasm or (*c*) the discrepancy between the rate of synthesis of respiratory enzymes and the rate of general protein synthesis.

The answer is given by Fig. 3A where the results are plotted in a different way (cf. reference 24): increment of the total amount of respiratory enzymes of the culture (ΔE) as a function of the increment of the total mass of the culture (ΔM). The curve is not horizontal, which means that the cells do synthesize respiratory enzymes during the exponential phase. Regression is linear within 2% limits of error, from which it is concluded that an equilibrium is established between the two rates of synthesis. The value of the slope gives the *minimal* value of Qo_2 that can be reached only when the enzymes already existing in the inoculum are sufficiently diluted by the growing mass of protoplasm. Further, this minimal value is maintained as long as the equilibrium is maintained. Approximately at

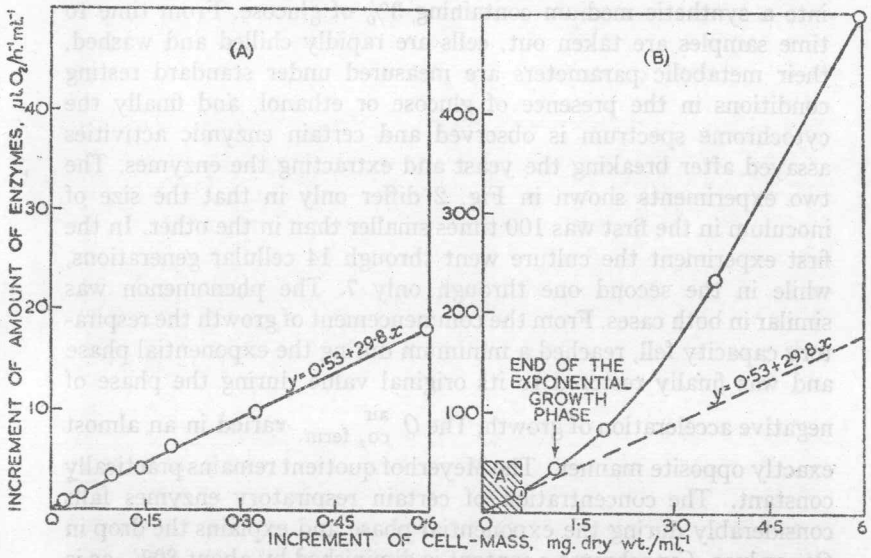

FIG. 3. *Evolution of the differential synthesis of respiratory enzymes during aerobic growth in a glucose-containing medium*

Co-ordinates are expressed per ml. of the culture. Modified, after reference 22

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