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THE PHYSIOLOGICAL STATE OF MICROORGANISMS DURING CONTINUOUS CULTURE

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In the course of the last 10 years extraordinary attention has been paid to continuous culture. We can mark an increase in the number of papers which show that it is about to become a new method enabling us to deepen our knowledge about the multiplication of microorganisms and at the same time gives us a stable living material for experimental studies of variability and mutability and for biochemical analyses. It also points the way towards a substantial increase of productivity for a number of fermentations.

Up to 1945 there existed but a few papers describing the use of this method. Moreover these papers usually did not consider this method as a new and basic one and one which corresponds to the reproductive capacity of microorganisms better than the commonly used static methods. This is true for the work of Rogers and Whittier (1930) who tried to draw an analogy between a bacterial culture and a multicellular organism, and cultivated bacteria (*Streptococcus lactis*) at a rather slow rate of flow of the nutrient medium on the one hand, and using a microbial filter under which the nutrient medium was repeatedly renewed on the other hand. Their most striking results deal with the lactic acid production in continuous culture. Cleary, Beard and Clifton (1935) used the continuous method to study the basis of the stationary phase of common batch culture methods. The papers of Jordan and Jacobs (1944, 1947, 1948) also deal with an analysis of the stationary phase. Moyer (1929) used the continuous method only to increase the bacterial mass for chemical analysis. Among the theoretical papers from this period, only those of Utenkov (1929, 1942) and Málek (1943) considered continuous culture as a new experimental method from the very beginning. Utenkov, who was probably the first to take up the continuous flow method systematically, proceeded in 16 years of experimental work (from 1922) from the assumption that the batch culture method does not sufficiently reveal the true characteristics of microorganisms, and does not permit satisfactory regulation of the development of cultures. It is

only to be regretted that Utenkov's paper remained unknown to the world microbiological literature. It was from a similar of point view that Málek studied the continuous flow method (1943) and stressed its advantages in studying multiplication, variability and pathogenicity of microorganisms.

Practical exploitation of the method was only slightly developed. Lebedev (1936) developed the method of continuous alcoholic fermentation. In Germany, fermentations have been patented which are based on continuous flow fermentation (Lupinit, Norddeutsche Hefeindustrie, 1934); later on, a group of American research workers — Unger, Stark, Scalf, Kolachov (1942) and others — worked out procedures for continuous production of yeast and alcohol, and pointed out their advantages and greater productivity as compared with the static "batch" methods.

Later papers (after 1945), however, almost without exception, consider continuous flow culture as a new method. It has been studied technically both from the experimental-laboratory viewpoint (Castor (1947), Bactogen-Monod (1950), Chemostat-Novick and Szilard (1950), Anderson (1953), Málek (1943, 1952), Northrop (1954), Kubitschek (1954), Graziosi (1956, 1957), Davies (1956), Karush, Iacocca, Harris (1956), Formal, Baron, Spilman (1956), Perret (1956)) and from the laboratory-production viewpoint (Malmgren and Hédén (1952), Málek (1955), Elsworth et al. (1956), Pirt (1957)) as well as applied to microcultures (Pirfilev — personal communication, Rosenberg (1956)). A methematical treatment has been given for the theory of reactions in continuous flow systems in general (Denbigh (1944), Pasynskij (1957)) and in particular to the multiplication of microorganisms (Monod (1950), Northrop (1954), Herbert et al. (1956), Maxon (1955), Jerusalimskij (1958). The method has been considered from the point of view of experimental possibilities and perspectives (Monod (1950), Málek (1943, 1952), Maxon (1955), Novick (1955), Powell (1956)). The following theoretical problems have been studied by this method: induction of antibiotic properties (Sevage and Florey (1950)), the formation of mutants and other problems of genetics (Novick and Szilard (1950), Bryson (1953), Zelle (1955), Lee (1953), Moser (1954)) microbial adaptation (Verbina (1955), Graziosi (1956, 1957)), growth of fungi (Duché and Neu (1953), Hofsten et al. (1953)), bacterial multiplication (*Brucella*: Gerhardt (1946); *Aerobacter*: Herbert et al. (1956), Pirt (1957); *Escherichia coli*: Málek (1943, 1950); *Streptococcus haem. A.*: Karush et al. (1956); *Salmonella typhi*: Formal et al. (1956); *Mycobacterium tb.*: Švachulová and Kuška (1956)), and development of bacterial cultures (Málek et al. (1952, 1953, 1953b, 1955), Macura and Kotková (1953), Ševčík (1952), Jerusalimskij (1956, 1958)).

Other papers deal with the industrial application of the method (Harris et al. (1948), Victorero (1948), Adams and Hungate (1950), Šarkov and his school (1950), Elsworth et al. (1956), Málek et al. (1955, 1957)).

Experience has been acquired not only with the continuous culture of bacteria, yeasts and molds but also of some algae (Ketchum, Bostwick, and Redfield (1938), Myers et al. (1944) cf. Novick, Tamiya (1957)) and protozoa (Browning and Lockinger (1953), Vávra (1958)).

In this review we intend to consider only theoretical papers and results particularly with regard to the physiological state of microorganisms in continuous flow cultures. Therefore its mathematical aspect and the kinetics of growth of microorganisms will not be covered, since this question has been sufficiently studied in earlier papers (Monod, Northrop, Maxon, Herbert et al.).

Let us begin with a few remarks on terminology. When referring to "continuous cultures" we shall usually have in mind continuous flow cultures, as compared with continuous culture in the broader sense of the word, where culture with periodic renewal of nutrient medium is included (for this type of culture sometimes the word "semi-continuous" according to Maxon is used). Continuous flow culture is thus the extreme case of periodic continuous culture; the technical difference between these two is usually only in the length of the interval between additions of medium, because even in a continuous flow culture the nutrient medium is usually added intermittently. This difference is at times of great importance for the process. In contrast to continuous culture we shall consider the static single-run culture i. e. the common batch culture. Sometimes the term "dynamic culture" is used even for a batch culture when stirred. It is felt that this usage is incorrect because this type of culture remains basically static in character. It is typical of a true dynamic culture that a dynamic steady state is established.

What do we actually mean by the physiological state of microorganisms in continuous culture?

A lot of experience obtained in studying common static cultures have shown that the number of microorganisms follows the typical growth curve. However, only the exponential part of this curve is significant for multiplication of microorganisms in common continuous cultures, because only then is uniform multiplication taking place. Therefore, this part of the curve is usually treated as a whole quite uniformly, as no striking change of culture can be observed there. A number of papers (Malmgren and Hédén (1951), Hinshelwood (1947), Valyi-Nagy (1955) and many others) have shown that microorganisms are undergoing changes even in this exponential part of the curve. Thus, for instance, the ribonucleic acid content of cells does not remain constant during the exponential period, i. e. the period of uniform cell division, but usually drops very rapidly at the very beginning of the curve. On the other hand there are a number of reasons for the assumption that the beginning of multiplication coincides with the end of the lag-phase, i. e. with the late lag-phase, because then a great production of microbial material can be observed as a consequence of regular microbial proteosynthesis, as shown by an increase in enzymic activity, by a rise in the ribonucleic acid content, and by an increased sensitivity towards external conditions (Malmgren and Hédén (1951), Winslow, Walker et al. (1939) and other authors). All this indicates that the physiological state of microorganisms, as manifested by enzymic activity, sensitivity, proteo-

synthesis, RNA content etc., is undergoing changes in static cultures even during the phase of full multiplication. The conditions, however, which lead towards this change have not been sufficiently elucidated.

Now, to which part of the curve which has been thus divided can we compare the physiological state of microorganisms under conditions of continuous multiplication? A logical consideration leads to the conclusion that under conditions fully ensuring the multiplication of microorganisms, the physiological state will most likely correspond to the state of a culture in the late lag-phase or at the beginning of the log-phase. But what is the case when microorganisms are grown under conditions which are below this level, and when slower multiplication is taking place; does the physiological state of cultures then change in a similar sense as can be observed in static cultures?

This question appears to be rather important, since the physiological state can considerably influence multiplication itself and the kinetics thereof. Only when we have fully answered this question can we purposefully regulate continuous processes and exploit them for theoretical or practical ends. It is particularly important in cases when we intend to grow microorganisms over a long period with a maximum activity of multiplication, or when we wish to obtain products which in static cultures are associated with changes in the state of the culture, and appear therefore either at the end of the exponential, or during the stationary phase. The question arises, however, whether we can draw any parallel between continuous and static cultures in terms of the individual phases of development.

It is most probable that laws governing the multiplication of microorganisms in static cultures during the phase when the nutrient medium contains sufficient amounts of all necessary components, and when it is not affected by metabolites produced, are similar to those of continuous cultures. But even then there is an important difference between these two types of cultures: In static cultures the concentration of nutrients is usually excessive at the moment of beginning growth, when it is intended to last for several generations; in continuous cultures, on the contrary, the concentration of added nutrients is diluted into the whole volume of nutrient medium with the microbial culture, which can thus assimilate it immediately. When the basic nutrient factors or the source of energy are optimally balanced with respect to the requirements of a multiplying culture, we reach the stage where nutrients are assimilated on addition and cannot be determined in the culture fluid although constantly replenished. This is another aspect of the steady state of continuous cultures, and very important from the point of view of optimal exploitation of nutrients —a definite requirement in practical application. Is this difference in any way reflected in the physiological state of microorganisms in culture, as to the manner of absorbing nutrients and in the manner of their utilisation? Such a possibility cannot be excluded. What is the case when microorganisms are

cultivated in such a way that the rate of continuous feed is slower than would correspond to the growth rate as required by Monod for cultures in which self-regulation is operating? Is the answer given by merely slowing down multiplication, or is there a concurrent change of physiological state as is known in the latter stages of the exponential curve of a static culture? It cannot be excluded that some change could take place together with a different response of cultivated microorganisms to external conditions. Evidence on this would be of great experimental importance.

These are some of the questions that should help us to show the relation between the basic problem of the physiological state of cultures under conditions of continuous multiplication, and the application of this method in theory and practice. These questions have thus far been accorded little experimental attention, despite the importance for evaluation of obtained and obtainable results.

The attitude toward this question is connected with another problem of particular importance during the first period of study of continuous cultures, and of general biological importance: whether conditions of static or dynamic cultures better meet the physiological requirements of microorganisms. By the term "requirement" we have in mind the relation of microorganisms to their environment as developed in the course of their exposure to physiological conditions in nature.

Older papers — with the exception of those of Utenkov and Málek — assumed that the growth-curve characteristics derived from static cultures have an absolute validity, and that they reflect the natural development of the physiological state of microorganisms and their requirements. Cleary et al. (1935) therefore did not study continuous multiplication from the point of view of microbial multiplication, but rather in order to learn more about the stationary phase of static cultures. This attitude was apparently an expression of the fact that in that period a diagnostic raison d'être still prevailed in microbiology, for which static cultures are most convenient. Particular attention has been given to the study of the so called "maximum concentration" according to Bail (1929). There were however more profound biological reasons for this view: under natural conditions (e. g. in the soil etc.) microorganisms do not as a rule multiply in a homogeneous solution with a constant afflux of nutrients, but rather on structures where they behave similarly as in artificial cultures on solid media — they form colonies which are analogous to the well-known growth-curves of liquid static cultures (e. g. Vinogradskij (cf. Volodin 1952), Novogrudskij (1950)). From this the conclusion is usually drawn that microorganisms have developed a fixed form of multiplication which corresponds to the conditions of static cultures, and that even when they can multiply without limitation, cultures undergo developmental changes that correspond to the typical growth-curve of static cultures. The practical

consequence of such assumptions was that microorganisms were cultivated in continuous cultures with an afflux of nutrients at a rate considerably lower than would correspond to the growth rate. Another consequence of this thinking was that it was not generally believed possible to cultivate microorganisms in continuous cultures ad infinitum without some degeneration or change that would correspond to the stationary and decrease phases of static cultures. This opinion constituted an obstacle in accepting continuous culture methods and developing them in fermentation. This opinion has been shown to be wrong by work describing continuous cultures of microorganisms over long periods of time without signs of degeneration (Herbert et al. (1956), Málek (1955) and others).

A logical conclusion from the above findings would appear to be that static culture and the developmental changes observed with it are artefacts. The lag-phase appears as an artefact because, in the course of it, a culture changed by previous static cultivation must adapt itself to regular multiplication; the decrease of biosynthesis in the latter part of the exponential phase also appears as an artefact, as well as the whole of the stationary phase. The natural conditions which most fully correspond to the dynamics of microbial multiplication exist only in continuous culture. Therefore, only by using this method can we produce cultures which are really physiological, and correspond to the real physiological and biochemical characteristics of microorganisms. This view is supported by the results of continuous cultures which have shown that microorganisms can be grown under such conditions for indefinite periods in a vegetative form, with optimal results as to the production of living matter and optimal enzymic activity, etc. From the above the conclusion can be drawn that in static cultures only the initial, the late lag, and the exponential phases, can be considered as physiological, because only then can the synthesis of living matter, and all phenomena connected with it, proceed in an uninhibited manner. Everything else in the static growth curve appears to be an artefact caused by conditions inadequate for the multiplication of microorganisms.

This conclusion does not take into account the fact that some of the developmental changes in static cultures, at least in some microorganisms, have the character of fixed traits: above all the sporulation of bacilli (and, apparently, also of actinomycetes) and probably also the formation of resting forms of bacteria in the stationary phase. These traits are a biological fact which shows that in the course of development bacilli had to adapt to conditions somewhat analogous to those of static cultures. But the same microorganisms can be kept constantly in a vegetative state under suitable conditions. Furthermore a number of important products, which are certainly not a laboratory artefact, and which are of great practical importance, are formed only during the latter phase of static culture (antibiotics etc.). It cannot be affirmed

therefore that continuous flow cultures in their commonly used simple form correspond to all the physiological variables and requirements of microorganisms as which have developed. They meet the optimal requirements of fast vegetative multiplication of microorganisms; therefore, all the phenomena which are associated with the multiplication of microorganisms, be it the production of living matter, some basic enzymic processes, the influence of the environmental conditions on actively multiplying cells, or spontaneous mutability, can be conveniently studied only in continuous cultures. Only these cultures, when well set up technically and under constant conditions, can produce stable material for such investigation. On the other hand, in order to study phenomena caused by changes in environmental conditions due to the activity of microorganisms themselves, it is necessary to resort to a static culture, or to modify suitably a continuous culture method.

The majority of research workers using the continuous culture method base their views on the second assumption and do not take into account the possibility of changes of the physiological state under changed conditions. Theoretical papers devoted to the mathematical basis of multiplication (Monod, Herbert, Maxon and others) presume the existence of an ideal state in which the rate of growth and the activity of metabolic processes represent values dependent only very simply on the flow rate (dilution). This ideal state is hypothetically reached by assuming a system in equilibrium when the dilution rate (i. e. the ratio of emptied volume per unit of time to the volume of the culture) is equal to the multiplication rate (i. e. the number of divisions per unit of time) multiplied by 0,69 ($= \log_e 2$). Should some change in the physiological state of the culture occur, a new equilibrium would be formed, on the basis of which the experiment proceeds. Therefore no qualitative change in the physiological state of microorganisms is considered; it is taken to be constant in the course of a given experiment. The steady state which exists at different rates of flow is usually considered to differ only quantitatively. This abstraction is necessary as a basis for experimentation and it can be neglected within certain limits of multiplication rate. This assumption served as the basis for methods of the type of Monod's Bactogen or Novick's and Szilard's Chemostat, which choose an afflux of nutrients which remains below the level ensuring a maximum growth rate, and limit one important nutrition component. Other methods proceeding along the same line are those of the Turbidostat type, as used by Northrop, Bryson, Anderson and others, when a photo-cell helps to keep the density constant and the afflux changes in intensity. By means of both of the above-mentioned methods, the rate of growth can be automatically controlled and kept constant — in the first case by means of the self-regulating ability of the continuous flow system kept below the level of maximum growth rate, in the second case by means of an external

mechanism. Even workers using these methods to study important physiological processes (Monod, Duché and Neu, Karush et al.) or genetic processes (Novick and Szilard and others) work under conditions that disregard the physiological state and its changes. Thus they have the possibility of studying a number of important biochemical, physiological and genetic problems considerably more exactly, and with a more constant and more physiological microbial material than is possible in static cultures, but they do not fully exhaust the advantages of continuous methods for studying more thoroughly the biological factor itself and applying the results in practical fermentations. It follows from the conclusion of Monod's paper that he is aware of this simplification. Northrop stresses in the introduction of his paper that "any change in the concentration of any substance indicates a change in organism" and Maxon in his paper points out that a rigorous mathematical treatment of all factors is not even possible, and so far not useful, because a fermentation is a highly complex living system.

It seems particularly important to investigate to what extent we are justified in drawing a parallel between the slow growth achieved in the Chemostat by limiting one of the important nutrient factors (cf. e. g. Novick and Szilard 1950), and the faster growth in the physiological state. It remains a question what rôle a similar limitation might play in a continuous culture. This question is discussed by Powell (1956) from the mathematical point of view and in relation to the generation time of individual microorganisms. He proves that a continuous flow culture "discriminates heavily against organisms of unusually long generation time". Such long generation time certainly reflects a definite physiological state. Powell assumes in his conclusion that a continuous culture will stabilize itself within a certain range of physiological activity of microorganisms. But the question appears to be even more complicated: it is assumed, on the basis of a statistical treatment of the whole population, that the culture is homogeneous, but such is not the case even in a well stirred continuous culture. Let us therefore consider the effect of limiting one of the important nutrient factors: will the result of this procedure be that all the microorganisms present in the population consume an equal minimum amount of the limited factor and therefore multiply themselves more slowly (and in turn influence their physiological state), or that microorganisms metabolically more active consume the limited factor, depriving less active bacteria? In the latter case the culture can be divided into a portion with a normal generation time corresponding to temperature etc., and into a portion with an abnormally long generation time, or not dividing at all. The resulting generation time would reflect the statistical difference of these cases, but would not give a true picture of the physiological state of the microorganisms present. From this point of view it is interesting to note the remark of Karush et al. (1956) that

"the efficiency of utilisation of glucose increases with increasing growth rate". The above is intended to point out the importance of a more thorough investigation of growth limitation in continuous cultures achieved by limiting one important source. It is particularly important because under the conditions of limited growth, mutability has been studied. The relation of this phenomen to the physiological state deserves attention.

The assumption that only continuous culture is physiological is a starting point for practical applications in simple fermentative productions, as for instance, the production of yeast. Even with these simple processes, the question of physiological state can play an important role. For instance, in yeast cultivation the question arises of the behaviour of glycolytic activity when aeration is prolonged, or what will happen with maltase activity during prolonged culture on molasses. Similar questions arise as to physiological state when growth takes place on complex media, e. g. wood hydrolysates or sulphite liquors, and the capacity to utilise a mixture of carbohydrates, the role of diauxia, etc.

We have now compiled sufficient proof to show that the question of the physiological state of microorganisms is important for the knowledge of conditions and possibilities of continuous culture. But so far little attention has been given to this question. Utenkov proceeds from it rather systematically by his method, which he has called "microgeneration". He worked out in detail the possibilities of combination of the continuous flow method in various applications (e. g. aerobic culture, the possibility of combining media without interrupting the experiment, and the like) with static methods. His broad experimental work is available only in a brief summary of his doctorate thesis from 1942, where the principles of this method are presented together with a summary of results from 16 years of work (from 1922) concerning the development and variability of 40 different species of microorganisms under continuous flow conditions as compared with static conditions. As mentioned above, all his work is motivated by the endeavour to influence the physiological state of microorganisms through a suitable manipulation of cultivation. Utenkov is convinced that through a suitable choice of cultivation method it would be possible to keep microorganisms constantly at the different stages of their development. The continuous culture method represents for him the method for maintaining constant the stage of active vegetative multiplication and its practical exploitation. He studied with particular attention the first stage of continuous growth immediately after inoculation, as well as the occurrence and significance of atypical bacterial forms. He noticed that growth rate is higher in a continuous culture than in a static one, that the cultures reach a steady state, that under the conditions of continuous cultivation it is possible to preserve certain characteristics of microorganisms (e. g. virulence) which is of importance for the preparation of vaccines and for the possibility of

producing long-term (permanent) modifications under changed conditions. He furthermore treated theoretically, and partly experimentally, the question of mutants. In conclusion he stressed the advantage of the method for studying microbial associations, and for practical tasks, because it forms a basis for the application of automatisation of microbial growth etc... Even if these experiments are mostly descriptive and not sufficiently analytical, they represent the basis of continuous cultures, and what is even more important, they always concentrate upon the microorganism itself and on its physiological state and its relation to the environment.

Powell (1957) also touches on the physiological state of microorganisms, and considers the growth rate and generation time of bacteria in relation to the conditions of continuous culture.

The physiological state of yeasts under continuous culture conditions have been taken up by Plevako, who studied the dynamics of subcultures from individual yeast cells (the material of this symposium (1958)). Jerusalimskij and his coworkers are studying it in detail with cultivation of *Cl. acetobutylicum* (1958).

The question of the physiological state of microorganism under continuous flow conditions has formed also the basis of our studies (Málek et al. 1943 und other papers). We assumed that static batch cultures are in antagonism with the dynamics of a multiplying culture, and do not comply with that part of physiology of microorganisms which is manifested by multiplication. From this antagonism arise those complicated population and physiological changes of microorganisms as are known from common batch cultures.

What are the possibilities of studying the physiological state of microorganisms? For this purpose we must bear in mind the analogy with experiences obtained in work with static batch cultures, because only here have those parameters been studied which reflect the physiological state, although care must be taken in making such comparisons. The physiological state manifests itself above all in the qualitative aspect of multiplication, and in the proteosynthesis associated with it. If we permit an analogy with static cultures it is necessary to determine whether the culture and its characteristics correspond to the culture from the first part of the exponential curve, or from a later stage. It appears very convenient therefore to estimate the ribonucleic acid content, because this reflects the most marked changes in this period. The physiological state is also reflected in the qualitative changes in enzymic systems, cell resistance, etc.

As far as the first, purely quantitative, aspect is concerned, it is important to study multiplication and its utilization of living sources. By comparing the data obtained with mathematically predicted results, we can estimate

whether the system corresponds to the optimal capacity of vegetative reproduction. The mathematical basis has been studied in a number of papers, and can well be used for the treatment of simple continuous systems.

That is of course possible only in a perfectly homogeneous stirred culture. But the conditions of such a culture need not necessarily meet all the aspects of a continuous culture. Thus, for instance, a common problem that had to be solved by some authors is that even in fully stirred cultures, some bacteria settle preferently on the glass of the culture flask, and grow there. This phenomenon is particularly striking when cultures are grown in small flasks where the only movement is provided for by the nutrient medium dropping into the flask, and by its removal. Under such conditions the culture separates into two distinct parts even at a very fast rate of flow, as we have seen in a long-term culture of *Escherichia coli*; bacteria multiply in the nutrient liquid on the one hand — there they resemble the actively multiplying organisms as to shape and size — and grow in a thick film along the glass of the flask on the other hand — where they have the shape of small rods, multiplying apparently at a slower rate. The former are like those from the first part of the exponential curve, the latter like those from the stationary phase. But of course such a simple type of cultivation cannot provide us with any more exact data than those presented by a mere description.

Therefore we have developed an experimental method based on multistage cultivation, where several flasks are connected, one to the other, and the microorganisms pass with the flow of the nutrient medium from one into the other. In a system thus arranged it is possible to study the influence of different degrees of nutrient exhaustion in the medium, as well as the influence of particular metabolites on the change of the physiological state of cultures. The culture then contains several steady states at different levels. By changing the number and size of flasks, and the rate of flow, their ratio can be affected as desired, this makes it possible to study the conditions of origin of some stages of development of microorganism, e. g. sporulation, etc. It also gives us the possibility to study the influence of the physiological state of the culture on the production of various metabolites, such as antibiotics, etc. Finally, by using this method, we can study in detail the influence of various factors on the physiological state of microorganisms by adding them to the second, or some other flask where they are in contact with a fully multiplying culture. This opens new approaches to the knowledge of the adaptive ability to various factors. With actively multiplying cultures we may investigate their capacity to respond more actively to environmental influences, and mutability caused and influenced by external factors. Jerusalimskij presents a mathematical treatment of such a multi-stage system.

To study the physiological state of microorganisms during continuous culture in a multi-stage system we first chose *Azotobacter chrococcum* (Málek (1952),

Macura and Kotková (1953)). This microorganism undergoes — when cultivated statically — a complicated form of development from rods to chroococcus bundles, and from these to cystoid forms. In a number of experiments in a glass apparatus it could be observed that with a suitable and sufficient rate of flow of the medium, ensuring fast multiplication, it was possible to maintain it permanently in the form of motile rods with undifferentiated plasma, while at a slower rate of flow the rods were shortened, encysted, and chroococcus forms appeared. Particularly striking was the difference between microorganisms in individual stages. Even if the steady state at the various stages did not differ very much as to the number of microorganisms there were striking differences in shape: in the first stage there is a predominance of actively multiplying rods with undifferentiated plasma, in the second and third stage the chroococcus forms predominate. This difference could be shifted by changing the rate of flow.

Our second model was *Escherichia coli* and *Salmonella enteritidis* and we took the sensitivity towards temperature and towards formaldehyde (Málek et al. 1953) as the criteria of physiological state.

We proceeded from the assumption that microorganisms during the stage of "physiological youth" (Walker, Winslow et al. 1933, 1939) exhibit an increased sensitivity to temperature, H^+ ions, phenol etc. (Sherman and Albus 1923) together with an increased metabolic activity. This sensitivity enables us to distinguish clearly between microorganisms from the beginning of the exponential phase and those from the latter part of this phase, as well as from the stationary phase. It has been shown experimentally that microorganisms cultivated in a continuous culture approach in sensitivity those from the beginning of the exponential phase. They are very much more sensitive than microorganisms obtained by means of a common static cultivation, and their sensitivity decreases in further stages even if it remains well above the level of static cultures. At the same time it was interesting to note that the culture always contained a certain number of resistant microorganisms. These experiments again supported the view that with continuous flow techniques such cultures are developed which in their physiological characteristics resemble microorganisms from the beginning of the exponential phase.

Further studies were carried out using an aerobic sporulating bacillus from the group *B. subtilis* (Ševčík (1952), Málek et al. (1953)). These experiments showed above all, that under the conditions of an aerated continuous culture the microorganisms studied formed more robust rods or fibers that reproduced vegetatively without sporulation. Ševčík observed that the capacity to form these robust rods was preserved by the bacilli even in the first static subculture. When there was a sufficient source of nitrogen present, together with a limited source of carbon (glucose), so that all carbon was utilised during the first stage, during the next stages sporulation, as a rule took place. Aside from

sporulation, we have studied the content of RNA and DNA, and the metabolic activity. We observed that during the stage when these robust fibers started to be formed, the content of RNA increased nearly to values at the beginning of the exponential phase in a static culture, and remained there for several days. During the second and third stage, after an analogous initial phase, a decrease could be observed to values lower than in the first stage, and an increase started only when sporulation set in. The metabolic activity, as measured by the comsumption of O_2 per mg of dry weight per hour, did not produce unambiguous results, probably because a non-uniform cellular development takes place. This inequality of individual cells in the population was a new, important finding; even cells in individual fibers behaved differently. There were fully live cells directly adjacent to dying ones. The same could be observed in the setting-in of sporulation in individual cells of chains and fibers. We are of the opinion that this inequality of individual cells in the population during a continuous culture constitutes a fact that must be taken into account much more seriously.

Further work concerning the physiology of microorganisms in continuous fermentation was carried out using the yeast *Saccharomyces cerevisiae*, baker's type. Because these experiments are to be discussed in another report of this symposium (Beran), I shall limit myself only to the conclusions. As is well-known, the growth of this yeast is a manifestation of mixed metabolism. During cultivation a certain amount of alcohol is always formed, qualitatively dependent on the rate of flow. If a high yield of yeast is desired it is necessary to prevent as much as possible the formation of alcohol, which can be done only by limiting the carbohydrate source. Furthermore, the fermentation is connected with certain characteristics which are of importance for the quality of the yeast, e. g. raising power in dough, and durability. We are dealing then with a complicated system, in which the physiological state of the culture is of importance for practical applications.

We carried out our experiments — for practical reasons using molasses — at different rates of flow. We worked most often at a rate corresponding either to a three-hour generation time — which was found to be optimal for our experimental purposes — or a four-hour generation time which is most often used in yeast manufacturing plants.

We tried to find an answer to the following questions: In which physiological phase is a continuous flow culture in respect to its development in a batch culture? To what extent do aerobic systems undergo changes caused by utilization of sugars, ethanol and acetic acid, as well as anaerobic systems responsible for anaerobic fermentation of glucose and maltose? For practical reasons we also studied the autolytic rate of these cultures and the relation of data obtained to the quality of produced yeast in the raising of dough.

On the basis of the RNA content, $Q_{O_2}^{air}$ values on ethanol and acetic acid,

and $Q^{N}_{CO_2}$ values on maltose, we reached the conclusion that at a flow rate corresponding to a 3—4 hour generation time, we obtain a continuous flow culture corresponding to the terminal part of the exponential phase of the growth curve.

The values of $Q^{ir}_{O_2}$ on maltose, glucose, ethanol and acetic acid did not change for the whole duration of the experiment (120 hours) and did not depend on the rate of flow. On the other hand $Q^{N}_{CO_2}$ values on glucose were dependent on the rate of flow. At a rate corresponding to the calculated 2,6-hour generation time, the $Q^{N}_{CO_2}$ value did not change, at a rate corresponding to a three-hour generation time it fell, but remained at a rather high value. At a four-hour generation time, however, a sharp drop could be observed during the 96 hours of cultivation. We consider it important that for the entire duration of experiments the CO_2 from maltose retains an adaptive character under anaerobic conditions, which fact is in keeping with the physiological state toward the end of the exponential phase of the growth curve in static fermentations. We feel that this phenomenon can be of practical value. The autolytic rate did not exhibit any characteristic changes during incubation. On the other hand we observed a change in shape of the yeast cells, manifested by an elongation without changes in volume, in the course of a short-term continuous cultivation.

From these experiments it can be concluded that yeasts grown in continuous cultures do not change in the course of the cultivation as to their aerobic systems, and are in good physiological condition, corresponding to the terminal stage of the exponential phase of the growth curve.

In orientation experiments we have shown that by a suitable modification of the multi-stage construction, it is possible to reach a continuous formation of such evolutionarily complex metabolites as penicilin (Řičica, Málek 1955).

In conclusion to these experiments, to a certain extent orientational in character, it can be said that the question of physiological state of microorganisms in continuous cultures is a very important one, which deserves considerable attention. With bacteria which undergo, in static cultures, a morphologically demonstrable development (*Azotobacter*, sporulating bacilli) it was shown that even in a continuous culture they can undergo an analogous development when suitable conditions are preserved, particularly at slower rates of flow. The multi-stage continuous culture has proved to be a convenient method not only for the study of the physiological state of continuous flow cultures, but also for other questions. It will be necessary to develop it both technically and experimentally to such perfection as has been reached by the one-stage batch method of the chemostat or turbidostat type.

SUMMARY

1. A review of results obtained in developing the continuous culture method is given.

2. From an analysis of the world literature it has been concluded that not enough attention is being given to the question of the physiological state of microorganisms cultivated under the conditions of continuous flow culture, although it is an important question both theoretically and practically.

3. The following important questions arise:

a) Does the continuous flow culture optimally ensure all the necessary conditions of growth and development of microbial cultures?

b) Is it possible to draw a parallel between the physiological state of microorganism in a continuous culture and their development in static cultures?

c) In what range of flow rates does the physiological state of the culture remain constant, and corresponds to the optimal state existing, as a rule, at the beginning of static culture development?

4. We have mentioned possibilities that exist for the study of physiological state in a multi-stage continuous flow culture, and have mentioned results obtained by its application to the study of the development of *Azotobacter* cultures, to the determination of sensitivity toward temperature and disinfectants with *Escherichia coli* and *Salmonella enteritidis*, to the study of aerobic bacillus sporulation, and to the production of penicillin. We have reached the conclusion that the physiological state of continuous flow cultures of bacteria corresponds, under our conditions, to static cultures at the beginning of the exponential phase. We have called attention to the practical importance of the study of the physiological state of baker's yeast grown in continuous culture; in this case cultures have been compared rather with the terminal part of the static exponential curve on the basis of their RNA content and of the analysis of aerobic as well as anaerobic fermentation.

5. It is concluded that it is necessary to pay more attention to the physiological state of continuous flow cultures, particularly from the methodological and experimental point of view.

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GENETIC AND PHYSIOLOGICAL STUDIES WITH THE CHEMOSTAT*)

Aaron Novick

It is exciting to participate in this Symposium on the Continuous Cultivation of Microorganisms because it provides a unique opportunity for the exchange of ideas and information workers from many countries. Such exchanges, besides personally rewarding, should help increase the rate of development and the applications of our science.

The introduction of new experimental techniques has played an important role in the history of many sciences. The introduction of agar medium for the cultivation of bacteria on a fixed surface made possible the development of modern microbiology. Not only did it permit the convenient isolation of pure cultures of bacteria, but it also permitted the simultaneous examination of many different bacteria in a single dish. The great rewards resulting from the introduction of the agar plate offer encouragement to examine other techniques for the study of micro-organisms. It is hoped that this Symposium will call wide spread attention to the advantages of continuous culture methods for growing bacteria. These methods permit experimentation in areas hitherto not conveniently accessible. This paper will attempt to illustrate some of these advantages in the study of bacterial genetics and physiology.

I would like in this paper to review the work done at the University of Chicago mostly by Professor Leo Szilard and myself on the development and application of techniques of continuous culture. After a discussion of the principles and techniques employed I will illustrate the kinds of experiments that can be performed with such methods.

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PRINCIPLES AND TECHNIQUES

Although microbiologists have employed continuous flow methods for the culturing of bacteria for some years (Novick, 1955) the useful application of such methods was made possible by the development of an understanding of the principles underlying such techniques, the theory being described simultaneously by Monod (1950) and by Novick and Szilard (1950a, b).

Typically a continuous culture apparatus consists of a growth tube in which a growing bacterial population is maintained at constant size by continuous dilution. The dilution is accomplished by the admission to the growth tube of sterile nutrient liquid and the removal of bacterial suspension from the growth tube by means of an overflow that is set to keep the volume in the growth tube constant. In such a system, the number, N, of bacteria per ml will change with time at a rate, $\frac{dN}{dt}$, given by

$$\frac{dN}{dt} = \alpha N - \frac{w}{V} N \qquad (1)$$

where α is the growth rate constant, w is the flow rate (ml/hr), and V is the growth volume (ml). The generation time, τ, is equal to $\frac{1}{\alpha}$. For the size of the population in the growth tube to remain constant, $\frac{dN}{dt}$ must equal zero, i. e., α must equal w/V.

Equality of growth rate and dilution rate can be established in two fundamentally different ways. In one case the bacteria grow at a fixed rate which is determined by the choice of medium and temperature. The dilution rate is adjusted to equal this growth rate by employing some mechanism, e. g. a light source and photocell, to observe the population size and to increase the flow whenever the population density exceeds a chosen value, N_o, and to decrease the flow when the density is less than N_o. As a result, the dilution rate will equal the bacterial growth rate, and measurement of the dilution rate gives the value of the growth rate constant. In such a case the growth rate constant is probably determined by the low concentration of some substance made by the bacterium at the lowest rate relative to the other substances made by the cell. Since the growth rate here is dependent on the concentration of a nutrilite supplied by synthesis within the cell the bacteria under these conditions can be said to be under internal control (Novick, 1955).

Such an apparatus was constructed and successfully operated in Chicago by Fox and Szilard (1955), and by a number of other workers elsewhere. All of these internally controlled systems, however, have shown a serious technical handicap. After the system has been in operation for a time, a large number of growing bacteria are found to adhere to the walls of the growth tube. This

"wall growth", being inefficiently diluted by the flow system, increases in size and begins to supply large numbers of bacteria to the medium. As a result there is an apparent dramatic increase in growth rate because the number of bacteria in the growth tube is increased, not only by the reproduction of the bacteria suspended in the liquid, but also by the additional supply sloughed off from the bacteria growing on the walls. Despite elaborate measures taken

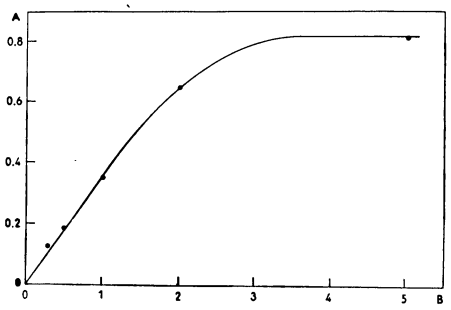

Fig. 1. Growth rate of a tryptophan-requiring strain of *E. coli* (B/l,t) as a function of the concentration of tryptophan.
A — Growth rate, α (hr^{-1}); B — Tryptophan concentration (γ/l).

to prevent wall growth, it has not been eliminated for long. Thus internally controlled systems can be operated for only relatively short periods of time.

A second method for establishing the equality of dilution rate and growth rate is found in the principle employed in the chemostat (Novick, Szilard, 1950a, b) and in the bactogen (1950). The present discussion refers only to the chemostat and experiments performed with it. All of the remarks concerning principles of operation hold equally for the bactogen. These systems, based on a principle of external control, operate in the following way. The dilution rate is set at some value less than the maximum growth rate, and the nutrient liquid is composed of a large excess of all required nutrilites but one, called the controlling growth factor. The density of bacteria increases with time as long as the growth rate exceeds the dilution rate. However, an increase in number of bacteria means a descrease in the concentration of the controlling growth factor in the growth tube. As the concentration of the controlling growth

factor continues to fall, it eventually will cause a decrease in the bacterial growth rate. It can be shown that the growth rate will fall until it becomes exactly equal to the dilution rate. As a result, the density of bacteria in the growth tube remains constant from then on.

An example of the dependence of growth rate on the concentration of a required growth factor can be seen in Figure 1. Here we seet that, for a tryptophan requiring mutant (B/1,t) of the B strain of *E. coli*, at higher concentrations of tryptophan the growth rate is independent of the concentration of tryptophan in the nutrient medium and at lower concentrations the growth rate depends on the concentration of tryptophan. One can expect, therefore, that in the chemostat or bactogen the rising density of bacteria must eventually reduce the concentration of tryptophan in the growth tube to such low values as to cause a reduction in the bacterial growth rate.

Since the bacterial growth rate in the chemostat is a function of the concentration of the controlling growth factor in the growth tube, equation 1 should be rewritten as

$$\frac{dN}{dt} = \alpha(c)\, N - \frac{w}{V} N \tag{2}$$

In the steady state that results, we have

$$\frac{dN}{dt} = 0 \quad \text{and} \quad \alpha(c) = \frac{w}{V} \tag{3}$$

The system is evidently self-stabilizing since any perturbation, (e. g., too high or low a value of N) causes a change which reduces the perturbation.

Morcover, it should be noted that since the growth rate constant $\alpha(c)$ is a function only of $\frac{w}{V}$, the dilution rate, the concentration of the controlling growth factor in the growth tube in the steady state is determined only by the dilution rate. That is, the concentration of the controlling growth factor in the growth tube in the steady-state is independent of its concentration in the incoming nutrient.

The density, N, of bacteria maintained in the growth tube in the steady state is given by

$$N = \frac{a - c}{Q} \tag{4}$$

where a is the input concentration of the controlling growth factor and Q is the yield constant (the amount of the factor required for one bacterium). This equation assumes that the yield constant, Q, is independent of c, the concentration of the controlling growth factor in the medium. Often c is found to be small compared to a, and N is well approximated by $\frac{a}{Q}$.

In these systems, therefore, the bacterial growth rate is determined by the concentration c of an externally supplied nutrient. For this reason the chemostat can be said to employ external control. For the controlling growth factor, any one of a wide variety of required nutrient can be used.

An interesting question concerns the range of growth rates which can be employed with the chemostat. Obviously the maximum growth rate is that observed at high concentration of the controlling factor. Little information, however, is available regarding how slowly bacteria may be grown. Experiments with a tryptophan-requiring mutant (B/1,t) of *E. coli* indicate that a lower limit is reached at generation time of 15 hours at 37°C. Lower growth rates at this temperature do not seem possible since at lower dilution rates the population seems not to grow a certain fraction of the time. Probably bacteria cease growing and go into the lag phase, or sporulate if they can, whenever the growth rate is less than some limiting value. If the dilution rate is too low, the growth tube contains growing bacteria part of the time and non-growing bacteria part of the time. Nevertheless, it is important to note that there is a wide range over which bacteria can regulate their growth rates. Apparently they are able to regulate all of their metabolic processes in this range so that they do not make a large excess of any amino acid (Novick, Szilard, 1954).

APPLICATIONS

Although the present discussion is concerned with the application of continuous culture methods only for experimental purposes, it should be noted that such methods offer great technological promise. Clearly, a continuous process has a higher production rate than does a batch process since it operates all of the time at maximum population levels. Another advantage, which should prove to be highly important, is that a continuous variation in the physiological state can be produced by variation of the growth rate. Quite likely there are many technologically important products made by microorganisms (e. g., antibiotics, amino-acids, vitamins, etc.,) which will be made in some cases at higher rates at lower growth rates. In this event, both the rate of production and the concentration of the product can be increased by selection of the growth rate optimum for these purposes.

For experimental purposes, the principle advantage of an apparatus such as the chemostat is that a population of bacteria of chosen fixed size can be grown under constant conditions for many generations at a selected growth rate. Not only are the bacteria growing in some well-defined state, but also the concentrations of all chemical substances remain constant. Even very low concentrations of a chemical substance can be maintained constant for long periods of time.

The first group of studies presented below takes advantages of the fact that the chemostat permits the study of a population for many generations, and includes studies on rates of mutation from observations of the accumulation of mutants over many generations, on "evolutionary" changes in bacterial populations, and on the slow adaptation of a bacterial population at low concentrations of a specific enzyme inducer. The second group of studies is based on the fact that the chemostat permits experiments at exceedingly low, yet constant, concentrations of a nutrilite. Here the experiments are concerned with the study of the regulation by bacteria of the rate at which certain amino acids are made.

MUTATION STUDIES

The chemostat offers a method for the accurate measurement of mutation rates in bacteria, whereas the usual methods encounter statistical difficulties leading to serious lack of precision. The results of a series of studies (Novick, Szilard, 1950b; 1951; Novick, 1956; Lee, 1953) of spontaneous and induced mutations in the B strain of *E. coli* are summarized below.

The principle employed to measure mutation rates with the chemostat is based on the fact that under certain conditions the fraction of mutants in a population, or the number per ml in the chemostat, rises linearly with a slope that is determined by the mutation rate. If the number per ml in the chemostat of a given mutant is m, if back mutations can be neglected, and if m is small compared to N, the total number of bacteria per ml, the rate of change in m with time is given by

$$\frac{dm}{dt} = \lambda\alpha N - m(\alpha - \alpha_m) \tag{5}$$

where α_m is the growth rate of the mutant, α the growth rate of the wild type equals the flow rate, and λ is the rate of mutation expressed per bacterium per generation. If $\alpha = \alpha_m$ then

$$\frac{dm}{dt} = \lambda\alpha N \tag{6}$$

and

$$m = m_0 + \lambda\alpha N t \tag{7}$$

Therefore, under the assumptions made, the number per ml of a given mutant rises linearly with time with a slope equal to $\lambda\alpha N$, thus permitting an accurate measure of λ. Conversely, whenever a linear rise in the number of a given mutant per ml is observed, it may be concluded that the assumptions above are valid and that the slope gives the correct value of the mutation rate. It is obvious that this method is not disturbed by such things as "plate mutations" (mutations which occur during the plating of an aliquot of the population to measure m); since the plate mutants are constant in number they do not affect the slope of the rise in number of mutants. Likewise, it is certain selection for or against the mutant plays no role since the linear rise shows that $\alpha = \alpha_m$ within experimental error.

If α should not equal α_m, i. e., should there be selection for or against the mutant, no linear rise will be found. Should $\alpha_m > \alpha$, the number of mutants will rise exponentially and eventually displace the wild-type organisms. Should $\alpha_m < \alpha$, the number of mutants will rise toward a limiting value given by

$$m = \frac{\lambda N}{1 - \frac{\alpha_m}{\alpha}} \tag{8}$$

This method of measuring mutation rates was used for several mutations in the B strain of *E. coli*, where linear rises were observed for mutations to resistance to phage T5 and to phage T6. It was found for these mutations that over a wide range of generation times the rate of mutation is a constant per unit time, i. e., proportional to the generation time (Novick, Szilard, 1950b; Lee, 1953). If the mutation rate per hour is given by μ, then $\lambda = \mu\tau$. Moreover, it was observed that the rate of mutation depends on the controlling growth factor used. For instance, control with an amino acid gives higher rates than does control with the source of energy, or nitrogen, or phosphorus.

Since the chemostat can be used for accurate measurement of mutation rates, a research program was undertaken to examine the mutagenicity of a wide range of chemical substances. It was hoped that the precision of the method would permit us to test the mutagenicity of compounds that might be only slightly mutagenic but yet would have more specific chemical properties than most of the reagents and conditions customarily employed. A survey was made of a wide range of chemical (Novick, Szilard, 1951; Novick, 1956).

A large number of purines and purine analogs were found to be mutagenic, the methylated xanthines being the most active. Caffeine, (trimethylxanthine) at a concentration of 150 mg/l raises the rate of mutation about 12-fold to T5 resistance and three fold to T6 resistance. Even the normally occuring base adenine is slightly mutagenic, although none of the pyrimidines or pyrimidine analogs studies were found to be effective.

Certain of the purine ribosides were found to be strongly anti-mutagenic, i. e., they antagonized the mutagenicity of the purine mutagens. Fairly low concentrations of adenosine completely suppress the effect of much higher concentrations of caffeine. In addition, it was found that these nucleosides reduce the spontaneous rate of mutation to about one third of the usual value. None of these anti-mutagens, however, have any effect on mutagenesis by

ultra-violet light or gamma radiation. Curiously enough, the desoxyribosides are much less effective anti-mutagens than the ribosides.

By providing a method for the accurate measurement of rates of mutation, the chemostat establishes a beginning for the study of the biochemistry of mutation. It is not yet understood how caffeine and adenosine produce their contrary effects; but perhaps further examination with the chemostat of compounds connected with nucleic acid metabolism, in the light of increased knowledge of this metabolism, will provide an insight into these problems and thereby furnish a better understading of the functioning of the genetic substance.

BACTERIAL EVOLUTION

When a chemostat is operated for many generations the initial strain of bacteria eventually becomes displaced by a strain which can grow more rapidly than the first strain at the low concentrations of the controlling growth factor which occur in the growth tube of the chemostat. This transition can be detected by observing the change in number of a given mutant the (T5-resistant, for example). When the initial strain is displaced by the faster growing strain, all of the T5-resistant mutants that had been accumulating in the initial strain are also displaced. This produces a discontinuity in the linear rise of T5-resistant mutants in the initial population. Once the faster strain is established in the growth tube and if the rate of mutation to T5-resistance is the same as in the initial strain, a linear rise in the number of T5-resistant mutants should again be observed. The total population of bacteria is unchanged by such a transition from a slower to a faster growing strain, as long as the quantity of tryptophan (Q) required per bacterium remains unchanged. Therefore, observation of the number of T5-resistant mutants provides a way of detecting such population changes when observation of the total population give no indication.

Operation of a chemostat for more than five hundred generations led to the detection of some ten or eleven transitions from slower-growing strains to faster strains. In each case the advantage in growth rate is observed only at low concentrations of the controlling growth factor. At high concentrations there is no such difference in growth rates; if anything, the later strains grow more slowly.

ENZYME INDUCTION

There are some enzymes, called inducible enzymes, which are made by bacteria only in the presence of certain specific inducers in the medium. For example, *E. coli* bacteria form β-galactosidase only when certain galactosides

are present in the medium. The kinetics of formation of this enzyme are readily observable with the chemostat since it can be used for experimentation under constant conditions for extended periods of time.

No difficulty was encountered with conventional methods in studying the kinetics of induction at high concentrations of inducer where, within a very short time following the addition of inducer to the medium, all of the bacteria form enzyme at maximum rate. At low concentrations of inducer, however, the rate of enzyme formation per bacterium rises slowly for many generations. Explanation of the nature of this rise was made possible by using the chemostat (Novick, Weiner, 1957).

Typically an induction experiment is performed with the chemostat by adding inducer in the desired concentration to the growth tube and to the reservoir at a specified time. From then on, of course, the concentration of inducer in the growth tube is automatically maintained at the desired value.

It was found that at low concentrations of inducer, starting from the time of addition of inducer, there is a linear rise in the rate at which β-galactosidase is formed, the slope of the rise depending on the concentration of inducer. The rise in the rate of enzyme synthesis continues up to some limiting value. This value, called intermediate saturation, for lower values of the concentrations of inducer, is less than the maximum found at high concentrations; but it becomes equal to the maximum value at higher values of the inducer concentration.

An explanation of these linear rises was found in the discovery that at low concentrations of inducer the bacterial population is heterogeneous with respect to the rate at which individual bacteria form β-galactosidase. In fact, the population consists of two kinds of cells, uninduced bacteria which make essentially no enzyme and fully induced bacteria which make enzyme at the maximum rate. At these and at higher concentrations of inducer all of the progeny of induced cells are also induced. The linear rise in rate of synthesis of β-galactosidase results from a linear increase in the fraction of the population in the induced state, the transition from the uninduced to the induced state occurring at a constant rate.

We can see here a resemblance between enzyme induction and mutation. In both cases transitions occur from one state to another at constant rate; and in both cases, selection being negligible, there is a linear rise in the fraction of the population in the altered state. The resemblance to mutation was made more striking when it was shown that the phenomenon of intermediate saturation resembles the phenomenon of selection equilibrium in mutation. Selection equilibrium occurs when the mutant type grows more slowly than the wild type; as a result, the fraction of mutants in the population rises to a constant value. Likewise, in the case of enzyme induction, intermediate saturation represents a steady state where a constant fraction of the population is induced because of the fact that induced bacteria grow more slowly than uninduced.

The existence at low concentrations of inducer of two possible states of induction (uninduced or fully induced) and of the inheritability of the state of induction can be explained on the basis of the presence in the bacteria of an inducible specific transport mechanism, called "permease" by its discoverers (Rickenberg et al., 1956) for the concentration of inducer inside the bacteria. The transition from the inunduced state to the induced could result from the chance appearance in a bacterium of a treshold level of permease. Upon cell division both daughters would obtain sufficient permease to remain induced. The growth rate difference between uninduced and induced bacteria could be explained, among other ways, by the extra work the concentrating cell must perform.

The use of the chemostat in physiological experimentation led to the discovery of the effect of carbon dioxide on the induction process. It was observed that the rate of rise of induced cells in the population increases at higher bacterial densities, and it could be shown that this result from the higher concentration of carbon dioxide in the medium at higher bacterial densities. The mechanism of action of carbon dioxide is still unknown, but the discovery of the effect followed directly from the use of the chemostat. Moreover, the effect of rising carbon dioxide concentration with rising bacterial density obscures in conventional growth apparatus the simple kinetics observable at constant bacterial density in the chemostat.

REGULATORY MECHANISMS

The chemostat is particularly useful for the study of the regulation of the rate at which a bacterium synthesizes an amino acid. Apparantly, the presence of an amino acid in the medium suppresses the synthesis by the bacteria of this amino acid. This suppression seems to occur even at very low concentrations of the amino acid, thereby making it difficult to investigate with conventional methods the relationship between the concentration of the amino acid and its rate of synthesis. However, such studies become easily possible with the chemostat because one can maintain a low concentration of an amino acid at a constant value in the presence of a large bacterial population.

In one case (Novick, Szilard, 1954) a study was made of the formation of a compound (now thought to be indol-3-glycerol phosphate; Yanofsky, 1957) presumde to be a tryptophan precursor, by a *coli* mutant (B/1,t) unable to synthesize tryptophan. This compound is not usually formed by these bacteria when they are grown in the presence of tryptophan, but is made only when the bacteria are grown at very low concentrations of tryptophan, such as occur at long generation times with tryptophan control in the chemostat. It was observed that at generation times longer than three hours the rate of production per hour is at a constant maximum value. As a result, the concentration of the

compound in the medium is proportional to the generation time in the range of three to twelve hours. At twelve-hours generation time the concentration of the compound in the growth is over one hundred times that in the incoming nutrient liquid. At generation times shorter than three hours there is a decrease in the rate of production of the compound. It could be shown that this decrease is caused by the higher tryptophan concentrations in the medium at shorter generation times. Moreover, it was possible to demonstrate that, when the bacteria are making this compound at the maximum rate, suddenly raising the tryptophan concentration leads to an immediate cessation in the rate of compound production. It was also observed that if the bacteria that are producing the compound are suddenly deprived of all tryptophan (stopping the flow of incoming nutrient in the chemostat) so that protein synthesis stops, production of the compound continues at the maximum rate for at least four hours.

The regulation of the rate of synthesis of an amino acid was further illustrated by a series of experiments designed to examine the effect of exogenously supplied arginine on the rate at which bacteria make arginine. In these experiments two strains of bacteria were employed, one unable to synthesize tryptophan (B/1,t) and one unable to synthesize arginine (D84/6). Since the first is resistant to phage T1 and the second to phage T6, the numbers of either in a mixture could be measured by plating with phage T1 or with phage T6.

For convenience, the concentrations of arginine and tryptophan in the medium are sometimes given in units proportional to the relative amount of each of these amino acids in the bacterial protein. One unit of concentration of tryptophan is taken as 500 γ/l, a concentration in the incoming medium which supports a density of $2{\cdot}5 \times 10^8$ of the B/1,t bacteria per ml in the chemostat. Since the ratio of arginine to tryptophan in these bacteria is 4·6, one unit of concentration of arginine equals 2300 γ/l. This concentration of arginine in the incoming nutrient in a chemostat would support a density of $2{\cdot}5 \times 10^8$ of the D84/6 bacteria per ml.

The first experiment demonstrated that not only can B/1,t bacteria assimilate arginine at low concentrations of arginine but also that these bacteria can take up arginine at concentrations too low to support the arginine-requiring strain, D84/6, at a high growth rate. In this experiment a chemostat containing one unit of tryptophan and one-fifth unit of arginine in the incoming nutrient was inoculated with both the B/1,t and D84/6 strains. If each consumed only its required amino acid and none of that required by the other strain, the population density would be $3{\cdot}0 \times 10^8$ ($1{\cdot}2 \times 2{\cdot}5 \times 10^8$). However, if one strain consumed the amino acid required by the other, the density would be less; and if one strain made an excess of the amino acid and secreted it, the density would be higher.

When an experiment of this kind was performed at a generation time of two

hours, it was observed that the density became constant at 2.5×10^8 and that the D84/6 bacteria did not remain in the growth tube. This shows that the B/1,t bacteria assimilate arginine so efficiently that the concentration of argi-

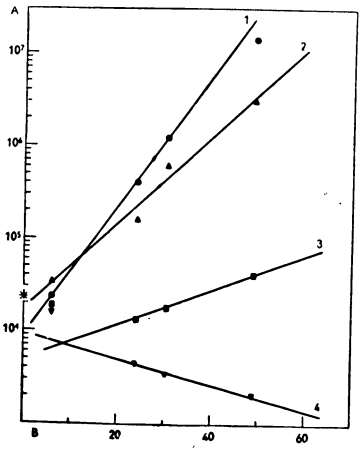

Fig. 2. The strain B/1,t was grown at a generation time of eight hours at a density of 2.5×10^8 with tryptophan control in four chemostats. Each had in addition the indicated concentration of arginine in the incoming nutrient, representing the amount given in parentheses of the arginine in this number of B/1,t bacteria. At zero time the chemostats were inoculated with the arginine requiring strain (D84/6), and the number of these bacteria were measured at various later time by plating with excess phage T6. $\tau = 8$ hrs.
A — Arginineless bacteria per cc; B — Hours; 1 — 1150 γ/l arginine (0·5); 2 — 690 γ/l arginine (0·3); 3 — 230 γ/l arginine (0·1); 4 — 0 γ/l arginine (0).

nine in the growth tube falls below that required to maintain the D84/6 bacteria growing at a generation time of two hours. The B/1,t bacteria obtain one-fifth of their arginine from the medium and synthesize four-fifths. In order to do this they need to assimilate arginine from the medium only one-fifth as fast as the D84/6 bacteria, since the D84/6 bacteria must secure all of their arginine from the medium. Thus if the B/1,t bacteria take up arginine at a given concentration more rapidly than one fifth the rate that the D84/6 bacteria take it up, the concentration of arginine will fall below that required for the same growth rate by the D84/6 bacteria. In conclusion, this experiment demonstrates not only that bacteria can efficiently assimilate arginine at low concentrations, but also that a low concentration of arginine in the medium is sufficient to reduce by twenty per cent the rate at which the bacteria make arginine.

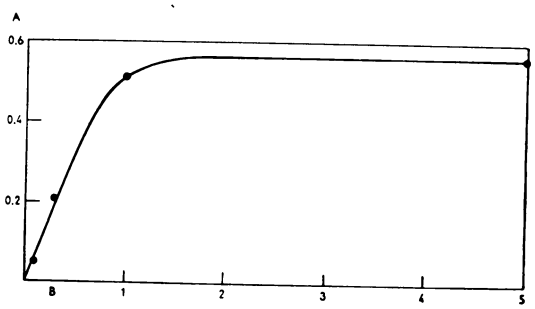

Fig. 3. Growth rate of an arginine-requiring strain of *E. coli*, (D84/6) as a function of the concentration of arginine.
A — Growth rate ($hr.^{-1}$); B — Arginine concentration (γ/l).

When experiments of this kind were done at longer generation times it was found that the D84/6 bacteria would remain in the growth tube, the total density of B/1,t approaching 3.0×10^8. The fact that the D84/6 is able to compete successfully with the B/1,t bacteria for arginine at longer generation times can be understood in the following way. The steady state concentration of arginine established by the B/1,t bacteria is that which allows the bacteria to make eighty per cent of their arginine per generation. At longer generation times the rate per hour at which arginine is made by the B/1,t bacteria decreases, and quite possibly this decrease in the rate of arginine synthesis corresponds to a rise in the concentration of arginine in the medium. Furthermore, at longer generation times a lower concentration of arginine is sufficient to support growth of D84/6 bacteria.

Experiments like that above can be used to establish the relationship between the rate of synthesis of arginine by the bacteria and the concentration of argi-

nine in the medium. The results of a series of experiments demonstrating such an analysis are given in Figure 2. Here a series of chemostats was set up to as to contain in the incoming nutrient the following concentrations of tryptophan and arginine. Each chemostat contained one unit of tryptophan, but the arginine content varied as follows: Case A, 0.5 units of arginine; Case B, 0.3 units;

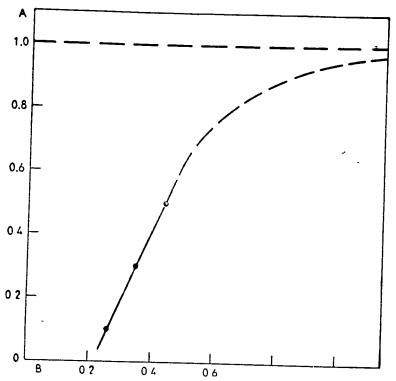

Fig. 4. The relationship between the fraction of arginine taken from the medium and the concentration of arginine in the medium.
A — Fraction of arginine content taken from outside; B — Arginine concentration (γ/l).

Case C, 0.1 units and Case D, no arginine. These four chemostats were initially inoculated with the B/1,t strain of bacteria, and they were permitted to run at a generation time of eight hours until a steady state was reached. In all cases the concentration of arginine in the growth tube fell so low as to be chemically undetectable. It can be concluded that in Case A the bacteria make fifty per cent of their arginine requirement and take up fifty per cent from the medium, that in Case B they make seventy per cent and take up thirty per cent, that in Case C they make ninety per cent and take up ten per cent, and that in Case D they make one hundred per cent of their arginine requirement.

The next step is to determine in each case how the concentration of arginine in the medium corresponds to the rate of synthesis. The concentrations of

arginine, being too low for chemical measurement, were measured microbiologically. This was done by observing the effect of these concentrations on the growth rate of an arginine-requiring strain. A number of DS4/6 bacteria, very small in comparison with the number of B/1,t, was added directly to the growth tube of each of the four chemostats once the steady state was reached. By plating with excess phage T6, the number of DS4/6 bacteria could be measured as a function of time, and it is these numbers that are given in the semi-log plot in Figure 2. From the rate of rise, or fall, of the number of DS4/6 bacteria per ml, the growth rate can be calculated, using the relationship

$$\frac{1}{n}\frac{dn}{dt} = \alpha - \frac{w}{V} \quad \text{or} \quad \alpha = \frac{1}{n}\frac{dn}{dt} + \frac{w}{V} \tag{9}$$

where α is the growth rate of the DS4/6 bacteria, $\frac{1}{n}\frac{dn}{dt}$ the slope of the rise or fall in the semi-log plot, and $\frac{w}{V}$ the dilution rate. The observed growth rate of the DS4/6 strain can be used to obtain the concentration of arginine in the medium, given the relationship shown in Figure 3 between the growth rate of DS4/6 and the concentration of arginine established in independent growth studies. In this way the results shows in Figure 2 were used to ascertain for the B/1,t strain the relationship shown in Figure 4 between the rate of arginine uptake (or synthesis) and the concentration of arginine in the medium.

SUMMARY

Continuous culture methods offer many advantages to the microbiologist. The chemostat can be used to maintain a population of microorganisms growing indefinitely under constant conditions. The size of the population, the growth rate, and the controlling growth factor can be selected within wide ranges according to the experimentor's requirements.

The chemostat is especially useful in two kinds of experiments, where attack by conventional methods is not feasible. For instance, the study of the accumulation of mutants in a population demands an environment where growth may be observed over many generations under constant conditions. The second kind of experiment involves an environment in which a metabolite, that is being rapidly consumed, must be kept at a low constant concentration in the medium. Applications of both kinds of experiment have been illustrated above.

It is expected that the chemostat will be used to obtain optimum production of industrially important biochemicals produced by microorganisms. Because it permits a wide variation of growth rate and choice of controlling growth factor, the chemostat can be used to determine the dependence of the rate

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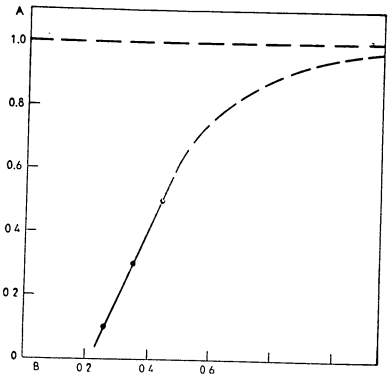

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It is expected that the chemostat will be used to obtain optimum production of industrially important biochemicals produced by microorganisms. Because it permits a wide variation of growth rate and choice of controlling growth factor, the chemostat can be used to determine the dependence of the rate

at which a given product is made on changes in these variables. Once the optimum conditions are established, the chemostat can be used for the continuous synthesis of the product at a high rate.

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CONTINUOUS CULTURE OF MICROORGANISMS; SOME THEORETICAL ASPECTS

D. Herbert

I. THEORETICAL

A simple mathematical theory of continuous culture can be derived (Herbert, Elsworth, Telling, 1956) from two basic features of bacterial growth which were first established in batch culture experiments (Monod 1942, 1950). In the simplest case, when bacteria are growing in a medium containing a single carbon substrate (e. g. glucose-ammonia-salts media), these can be stated as follows:

1. The growth-rate is a constant fraction, Y, of the rate of utilization of substrate:

$$\frac{dx}{dt} = -Y\frac{ds}{dt} \tag{1}$$

where x = concentration of bacteria, s = concentration of substrate, t = time and Y is called the yield constant. Over any finite period of growth:

$$\frac{\text{weight of bacteria formed}}{\text{weight of substrate used}} = Y \tag{2}$$

2. The specific growth-rate (μ) is a function of the concentration of limiting substrate, being proportional to the substrate concentration when this is low but reaching a limiting saturation value at high substrate concentrations according to the equation:

$$\frac{1}{x}\frac{dx}{dt} = \mu = \mu_m\left(\frac{s}{K_s + s}\right) \tag{3}$$

where μ_m is the growth rate constant (i. e. the maximum possible value of μ in the medium used) and K_s is a saturation constant numerically equal to the substrate concentration at which $\mu = \mu_m/2$. (This is similar to the Michaelis-Menten equation for the effect of substrate concentration on the velocity of enzyme action.)

From equations (1) and (3) a mathematical theory can be derived which allows quantitative prediction of the behaviour of a continuous culture. The type of apparatus considered is the "Chemostat"; bacteria are grown in a culture vessel or "fermenter" into which sterile growth medium is metered at a steady flow-rate (f) and from which bacterial culture emerges at the same rate, a constant-level device keeping the volume (v) of culture in the fermenter constant.

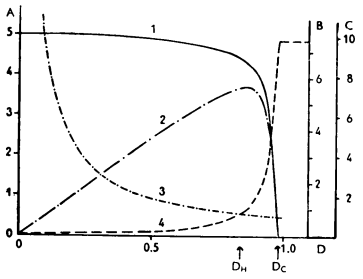

Fig. 1. Steady state relationships in continuous culture (theoretical). The steady-state values of substrate concentration and output at different dilution rates are calculated for an organism with the following growth constants: $\mu_m = 1{\cdot}0$ hr.$^{-1}$, $Y = 0{\cdot}5$ and $K_s = 0{\cdot}2$ g/l.; and a substrate concentration in the inflowing medium of $s_R = 10$ g/l.
A — Output of bacteria, Dx (g/l/hr); steady state bacterial concentration, x (g/l). B — Doubling time, t_d (hr.). C — Steady-state substrate concentration, s (g/l). D — Dilution rate, D (hr^{-1}). Curves: 1 — bacterial concentration; 2 — output of bacteria; 3 — doubling time; 4 — substrate concentration.

The culture in the fermenter is vigorously stirred and aerated, and temperature and pH are automatically controlled.

Residence-times in such a culture vessel will be determined not by the absolute values of the flow-rate and culture volume but by their ratio which we call the dilution rate, D, defined as $D = f/v$, i. e. the number of complete volume-changes per hour. The mean residence-time of an organism in the culture vessel is evidently equal to $1/D$.

Equations can be derived which completely predict the behaviour of such a system at different flow-rates, medium concentrations, etc. The most important prediction of the theory is that a continuous culture is an inherently stable system. For any set dilution rate, the culture automatically adjusts itself to a steady state in which the concentrations of micro-organisms and nutrients in the culture remain constant indefinitely, so long as the composition and

flow-rate of the incoming medium remain unaltered. In such a steady state, the growth-rate of the organisms (μ) must be equal to the dilution rate (D). The stability of the system is due to the fact that it is essentially substrate-controlled. A chemostat is in fact a device for controlling the growth rate through control of the steady-state substrate concentration; each dilution rate fixes the substrate concentration at that value which makes μ equal to D.

In Fig. 1, theoretical curves are drawn from the basic differential equations of continuous culture, showing how the steady-state concentrations of bacteria and substrate in the culture may be expected to vary when the dilution rate is varied, the inflowing substrate concentration (s_R) being held constant. It will be seen that the existence of an infinite number of steady states is predicted, with dilution rates ranging from almost zero to a maximum value corresponding to the maximum possible growth-rate in the medium used (μ_m); above this "critical" dilution rate, "wash-out" of the culture occurs if the growth rate is further increased. It will also be seen that over most of the possible range of flow-rates, the steady-state concentration of substrate in the fermenter is very low (i. e. the substrate is almost completely consumed); only at dilution rates close to the critical does unused substrate appear in the culture. Fig. 1 also shows the theoretical output of bacteria (i. e. g. cells/litre/hour) as a function of dilution rate; it will be seen that this curve goes through a maximum value at a unique flowrate which is the "optimum" for production of cells or products. The above features of a continuous culture are of obvious importance from the practical standpoint.

II. EXPERIMENTAL

Descriptions will be given of laboratory and pilot plant types of continuous culture apparatus used at M. R. E., Porton. These have been operated successfully with a number of different bacteria and recently with yeast (*Torula utilis*) and moulds. In each case, systematic studies were made to see how the behaviour of the organism in continuous culture compared with the theoretical predictions. The technique was to set up a steady state at a fixed flow-rate and allow the apparatus to run for some days, during which samples were analysed for total and viable cell counts, dry weights of organisms, glucose and other constituents of the culture supernatant etc; analyses for nucleic acids and other cell constituents were also made on the cells. The flow rate was then changed and the determinations repeated at a new steady state; the aim was to cover as wide a range of flow-rates as possible. A typical run usually lasts for 2—3 months; no difficulty is now found in maintaining sterility for such periods and some runs have lasted as long as 200 days. Usually the respiratory activity of the growing cells was determined by continuous analysis

and recording of the oxygen and carbon dioxide content of the exit gas from the fermenter.

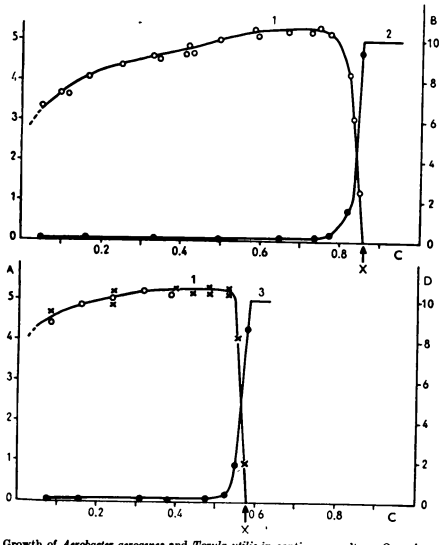

Fig. 2. Growth of *Aerobacter aerogenes* and *Torula utilis* in continuous culture. Organisms were grown with aeration at a number of different flow-rates; dry weight of cells and concentration of substrate determined after at least 2—3 days of steady-state growth at each flow-rate.
A — Cell concentration (mg dry weight/ml). B — Glycerol concentration (mg/ml). C — Dilution rate (hr^{-1}). D — Glucose concentration (mg/ml). X — Wash-out point. Curves: 1 — cell concentration; 2 — glycerol concentration; 3 — glucose concentration.

In general, results agreed quite well with theoretical predictions; in particular, the stability of a continuous culture over a wide range of flow-rates was confirmed with all organisms tested. This self-adjusting property makes a continuous culture very easy to operate; it is neccessary only to set and maintain constant the flow-rate, when the system will regulate itself.

Fig. 2 shows some illustrative quantitative data, obtained with *Aerobacter aerogenes* and with *Torula utilis*; similar results were found with other bacteria. It will be seen that (a) steady states were obtained over a wide range of dilution rates; the lowest ($D = 0.05\ hr^{-1}$) correspond to a cell division time of c. 14 hours. It is remarkable that bacteria and yeasts will continue to divide at this very low rate almost indefinitely; counts showed that the cultures were >90%

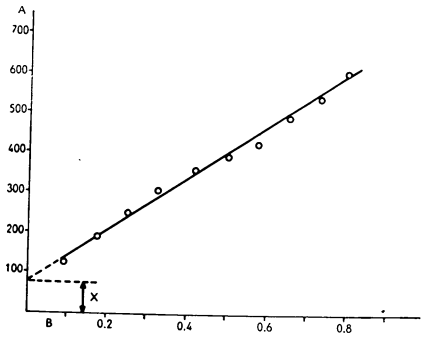

Fig. 3. Respiration of *Aerobacter aerogenes* growing in continuous culture. The organism was grown with aeration at a number of different flow-rates in a glycerol-NH_3-salts medium. Oxygen uptake of steady-state cultures determined with recording paramagnetic oxygen analyzer and expressed as Q_{O_2} ($\mu l\ O_2$/mg cells/hour).
A — Q_{O_2}, of cells (μl/mg dry wt./hr). B — Dilution rate (hr^{-1}). X — Endogenous respiration.

viable. (b) The "wash-out" points occurred at dilution rates of $0.585\ hr^{-1}$ for *Torula utilis* and $0.85\ hr^{-1}$ for *Aerobacter aerogenes*, corresponding to division times of 71 minutes and 49 minutes respectively; these values agreed well with measurements of the growth rates during exponential growth in batch culture. (c) Over most of the range of dilution rates the substrate was almost completely utilized, being so low as to be almost impossible to estimate; near the "wash-out" point, however, the substrate concentration rose abruptly as the cell concentration fell.

In the above respects, agreement with the simple theory is good. With both organisms, however (and with others we have examined), there is a noticeable discrepancy at low flow-rates, the yield of organisms being less than at high

flow-rates. In other words, the "yield constant" (Y) is not truly a constant, but decreases at low growth rates.

The most probable explanation of this is that in addition to the anabolic metabolism of the organisms (conversion of substrate to cell-substance), they have also a constant endogenous metabolism, by which cell-substance is oxidized to CO_2. At low growth-rates, when the residence-time of the bacteria in the fermenter is increased, this basal metabolism becomes proportionally more important, compared with the anabolic metabolism. Evidence for this has come from studies of the respiration at different growth-rates (Fig. 3).

In this experiment, which was made with *Aerobacter aerogenes*, the respiratory activity of the cells (Q_{O_2}) is seen to be a linear function of the growth-rate; however, the straight line does not pass through the origin but extrapolates back to a Q_{O_2} of about 65 at zero growth rate. Exactly similar results are obtained if Q_{CO_2} values are similarly plotted; moreover a larger proportion of the substrate-carbon used is converted to CO_2 and a smaller proportion to cell-carbon, when the growth-rate is low.

All the above facts support (though they do not definitely prove) the idea of a constant endogenous metabolism, independent of the growth rate. If an extra term for the endogenous metabolism is incorporated into the continuous culture equations, the "theoretical" curves now resemble those found experimentally (Fig. 2), rather than Fig. 1.

2. EFFECT OF GROWTH RATE ON CELL COMPOSITION AND MORPHOLOGY

During experiments of the type described above, cells grown in the steady state at different growth rates were analyzed for total protein, ribonucleic acid (RNA) and desoxyribonucleic acid (DNA). The mean cell mass (i. e. the average dry weight of a single cell) was also determined as the ratio of the dry weight of cells/ml. to the total count/ml. Results for *Aerobacter aerogenes* ares shown in Fig. 4 (similar results have been obtained with *Bacillus megaterium* and *Staphylococcus aureus*).

It will be seen that rapidly-dividing cells have a much greater dry weight (and microscopically are much larger) than slowly-dividing cells. In addition, the RNA content of the cells increases markedly with growth-rate, while the DNA-content decreases somewhat.

The writer prefers not to speculate at present on the meaning of these rather striking effects. It may be pointed out, however, that if the extreme ends of the curve are considered, the cells grown at the maximum rate correspond in chemistry and morphology to "logarithmic phase" cells in a batch culture, while those grown at the slowest rate correspond very closely to "resting cells".

The advantage of continuous culture techniques is that both these extreme types of cell, and an infinite number of intermediate graduations between them, may be isolated at will for study and grown indefinitely under steady-state

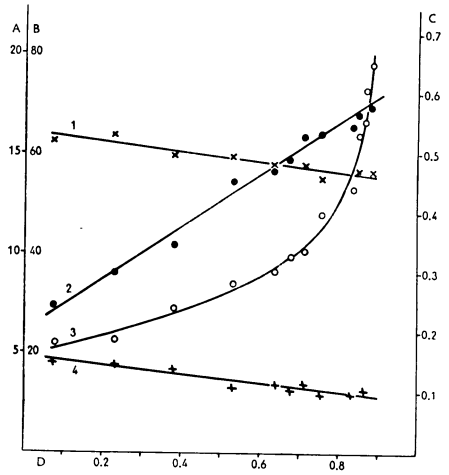

Fig. 4. Effect of growth rate on mean cell mass ond nucleic acid content of *Aerobacter aerogenes*. The organism was grown in a glycerol-NH_3-salts medium in continuous culture apparatus at a number of different flow-rates and the cells annlyzed after at least 2—3 days of steady-state growth.
A — % of RNA or DNA. B — % Protein. C — Mean cell mass (picograms). D — Dilution rate (= division rate) — hours^{-1}.
Curves: 1 — % protein; 2 — % RNA; 3 — mean cell mass; 4 — % DNA.

conditions, simply by appropriate adjustment of the flow-rate. This may serve as an illustration that continuous culture techniques are not only of considerable industrial importance, but also can be a powerful research tool.

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THE CONDITIONS OF GROWTH OF MICROORGANISMS IN CONTINUOUS FLOW CULTURES

N. D. JERUSALIMSKIJ

The method of cultivating microorganisms in continuous flow culture is receiving ever increasing application both in research work and industry. There exist three basic modifications of this method, not to mention various intermediates.

The first one in a cyclic-flow system in which fresh medium is continually added into the apparatus for the growth of the culture (the cultivator), while at the same time an equal volume of liquid medium is removed from it; microbial cells remain inside the apparatus all the time and multiply.

The number of living cells in 1 ml of medium gradually increases until it reaches the maximum level possible under the given conditions of the medium. Thus this method appears to be cyclic but not uninterrupted. It is applied industrially for the growth of yeast.

The second and the third methods are truly continuous. In the second method microorganisms grow in a fixed state on the surface of a solid phase while the liquid medium continually flows by. Some of the cells are washed away by the flow of the liquid and therefore the number of cells on the surface of the solid phase remains more or less constant even in the course of rather long experiments. A typical example of this method is represented by the so called fast method of vinegar production (Schnellessigverfahren) introduced in Germany at the beginning of the last century.

In the third type of cultivation cells develop in a liquid medium. The amount of incoming fresh medium is equal to the amount of cell-containing culture liquid removed from the apparatus. If the rate of washing away of cells equals the rate of their multiplication the density of the cell population inside the apparatus remains constant.

During the last few years this last modification of the method has received wide application in laboratory research work. It exists again in several minor modifications which differ in the type of regulation of fresh medium delivery,

of stirring the culture and of aeration. In some of them the medium is delivered towards the bottom of the apparatus while the excess liquid escapes on top; in others the flow of the liquid is reversed. Sometimes the apparatus consists of one "cultivator" only, in some cases, however, several of them are connected like a battery (the multistage, continuous culture). As long ago as in 1915 S. V. Lebedev proposed a similar battery consisting of several units for alcohol production. In the laboratory of one of the organizers of this symposium, Prof. Málek, the multi-stage culture is being fruitfully applied in the study of various problems.

In our laboratory we have studied the conditions governing bacterial growth when the first method and partly the second and third method are used. We used glass flasks of 25 to 250 ml volume according to K. I. Draganov (1957) as well as those according to S. V. Lebedev (1936). The rate of flow of the medium was regulated by changing the hydrostatic pressure and, in addition, by means of a screw clamp. The object of out study was mainly *Propionibacterium shermanii* (the work was carried out by N. M. Neronova) and *Clostridium acetobutylicum* (the work was carried out by G. V. Pinaeva).

Monod (1950) and Novick and Szilard (1950) derived mathematical formulae for the growth of culture during single-stage continuous cultivation. In other papers (e. g. Maxon, 1955) formulae are presented also for multi-stage cultivation. We should like to present the following universal formula characterizing the density of population in any culture system:

$$X = X_1 e^{(c-r)t} + \frac{r}{c-r} X_0[e^{(c-r)t} - 1] \quad (1)$$

The rate of growth of population in a cultivator is given by the following equation:

$$\frac{dx}{dt} = cX + rX_0 - rX \quad (2)$$

In the given formulae X and X_1 are the density of population (the number of cells per 1 ml) at the beginning and at the end of period t respectively; X_0 is the density of population in the liquid entering the cultivator; e = 2.718, i. e. the base of natural logarithms; $r = \frac{F}{V}$ is the rate of dilution of the culture, i. e. the ratio between the rate of flow of the medium (F ml/hour) and the volume of the cultivator (V ml); $c = \frac{dm}{dt}\frac{1}{m} = \frac{d(\ln m)}{dt}$ and indicates the relative rate of growth of microorganisms, i. e. the increase of one unit of living matter (m) per unit of time.

The rate of growth of microorganisms depends on the composition of the nutrient medium. When the nutrient contained in the medium in minimum

concentration is used up, the rate is slowed down. On this principle the regulation of growth of a culture in "Chemostat" (Novick and Szilard) and in "Bactogen" (Monod) is based. The accumulation of metabolic products can be another reason for the slowing down of growth; particularly, for example, of acids lowering the pH to undesirable values. In their turn, processes of utilization of nutrients and of accumulation of metabolic products are connected with the density of population. Consequently the rate of growth (c) depends also on the density of population (X): With increasing density the rate of growth decreases. Simultaneously the rate of increase of density of population decreases $\left(\frac{dX}{dt}\right)$ as follows from equation (2). When in this equation ($cX + rX_0$) becomes equal to rX, then $\frac{dX}{dt}$ is equal to zero. This means that the density of population does not increase any more and remains constant. The existing equilibrium is expressed by the following equation:

$$\frac{c}{r} = \frac{X - X_0}{X} \quad (3)$$

If, then, the rate of dilution (r) is purposely changed, the equilibrium is affected. The density of population (X) and the rate of growth (c) start to change in one or the other direction until an equilibrium — at a different level to be sure — is reached. Thus the growth of a continuous flow culture is actually a self-regulating process.

The medium entering the first cultivator contains no bacteria ($X_0 = 0$). In this case equations (1) and (2) may be simplified to:

$$X = X_1 \cdot e^{(c-r)t} \quad (4)$$

$$\frac{dX}{dt} = cX - rX \quad (5)$$

In the first cultivator equilibrium between the dilution and growth of the culture is established when

$$\frac{c}{r} = 1 \quad (6)$$

It follows that at equilibrium (when the density of population remains constant) the rate of growth of microorganisms in the first cultivator (c) is equal to the rate of dilution (r).

A study of continuous cultures of any microorganisms should start with measurement of the maximum rate of growth attainable under the given experimental conditions. This measurement should be carried out in the first cultivator.

In doing this we gradually increase the rate of flow of the medium and thus the rate of dilution. The highest rate of dilution at which the density of population still manages to remain constant represents the maximum rate of growth (c_0) : $r = c_0$ [cf. eq. (6)].

Let us give an example of the measurement of the maximum rate of growth in the case of *Propionibacterium shermanii* (Tab. 1).

Table 1

Time in hours	Rate of dilution $\left(r = \frac{F}{V}\right)$	Density of population in 10^6/ml (X)
0		274
16		280
18		253
20	0·38—0·42	
22		240
24		230
40		229
		302
46		177
48	0·60—0·66	154
50		150
64		165
66		157
68		166
70		160
116		15
118	1·0	13
120		11
121		12
136	0·9	22
138	1·0	20
140	1·2	9

As follows from Tab. 1 in the given case the limiting rate of dilution is approximately equal to 1·0. Consequently the maximum rate of growth is $c_0 = 1$. The generation time (the time for a two-fold increase of the living matter) is then:

$$t_1 = \frac{\ln 2}{c_0} = 0{\cdot}69 \text{ hours}$$

In the case if a continuous culture of *Cl. acetobutylicum* on maize mash with sugar added the maximum growth rate c_0 was equal to 0·8 and the generation time was correspondingly 0·87 hr. Under suitable conditions a continuous culture can proceed for any length of time. In our experiments, for instance, a culture of *Cl. acetobutylicum* multiplied at a constant rate for 200 days.

The density of population in the cultivator lay between 350 to 450×10^6 cells/ml. The rate of delivery of medium to the cultivator (F) was 20—23 ml/hr. while the volume of the cultivator was $V = 33$ ml. It follows hence that the rate of dilution (r) and the growth rate (c) was equal to 0·6—0·7. Proceeding from the relation between the growth rate and the generation time it is not difficult to deduce that in the course of the mentioned 200 days 4,360 generations were formed.

A battery consisting of several cultivating units can be used in order to obtain a denser cell population. But the density of population (X) is not the only criterion of the qualities of a given system of cultivation. With this in mind it is necessary to take into account the yield of living matter per hour:

$$\text{yield of living matter} = XF \tag{7}$$

Finally it is also necessary to know the productivity value, i. e. the amount of living matter produced per hour per unit of volume of cultivator:

$$\text{productivity} = \frac{XF}{V} = rX \tag{8}$$

Tab. 2 shows the density of population values of *Propionibact. shermanii* in various systems of cultivation; in Tab. 3 the values of productivity of different systems are presented.

At a low flow rate (F — 10 ml/hr.) the maximum density of population is reached when the volume of the second cultivator is equal to 150 ml (Tab. 2).

Table 2

Flow rate of medium F in ml/hr.	Mean population of cells of *Propionibact. shermanii* in cultivators — in 10^6/ml. Volume of cultivator in ml			
	first 25	Pth		
		70	150	240
10	730	2000	5390	5380
15	520	1170	3460	5050
25	28	395	3800	5100

At higher flow rates (15 and 25 ml/hr.) the increase of volume of the Pth cultivator from 150 to 240 ml causes a supplementary increase of the density of population.

As far as productivity is concerned (Tab. 3) at a flow rate $F = 10$—15 ml/hr. it reaches its maximum value already in the first cultivator. Therefore at such a flow rate it is necessary to connect another cultivator if it is required to obtain a higher concentration of cells in 1 ml.

At a flow rate of 25 ml/hr. the highest degree of productivity is reached when the volume of the P[th] incubator is 150 ml (Tab. 3). The density of population is at that time also sufficiently high. Therefore the given variant is the optimal one of all 12 studied in this experiment. In the second place lies the variant with a volume of the second cultivator equal to 240 ml and with a flow rate of 25 ml/hr.

Table 3

Flow rate of medium F in ml/hr.	Productivity of a system of cultivators expressed as the number of cells (in 10^6) produced per hour per 1 ml of volume of cultivators $\frac{XF}{V_1}$ and $\frac{XF}{V_1+V_2}$ Volume of cultivator in ml			
	first	P[th]		
	25	70	150	240
10	292	210	308	203
15	312	185	296	286
25	28	104	543	481

In order to determine in advance the optimal number of cultivator units, the relation of their volumes and the rate of delivery of medium it is necessary to know the law according to which the growth rate (c) changes. As mentioned above c is the reciprocal function of the density of population X.

As the first approximation it is possible to accept the following relationship between these values:

$$\frac{c_0 - c}{X - X_m} = \frac{c_0}{M - X_m} = \text{const.} \qquad (9)$$

In this equation c_0 is the maximum rate attainable in a given medium, X_m is the density of population which, when exceeded, causes the growth rate c to decrease, M is the limiting density of population which, when reached, stops the growth of the culture.

For each strain of microorganisms at a given composition of the medium the values of c_0, X_m and M are constant.

Providing the density of population is not too high the equation (9) satisfactorily corresponds to the obtained results (Tab. 4).

It we substitute the value of c from equation (9) in equations (3) and (6) we get formulae by means of which we can calculate in advance the density of microbial population in various types of cultivation.

Examples of such calculations are shown in Tab. 5. We assumed that the volumes of all "cultivators" in the battery are equal ($V_1 = V_2 = V_3$ etc.); the maximum growth rate $c_0 = 1{\cdot}0$ and $\frac{X_m}{M} = 0{\cdot}95$.

The highest productivity is reached — if single-stage culture is employed — when the flow rate is equal to 0·4—0·6 (Tab. 5). But then the density of population represents only 43 to 62% of the maximum value. It it is desired to have

Table 4

Density of population X in 10^6/ml	Growth rate c	Ratio $\frac{c_0 - c}{X - X_m}$
13*)	1·0**)	—
143	0·68	2·5
152	0·62	2·7
159	0·61	2·7
169	0·57	2·8
230	0·42	2·7
247	0·40	2·6
277	0·35	2·5
371	0·25	2·2
447	0·26	1·7
805	0·07	1·2

*) The value 13×10^6 is taken for X_m;
**) The value 1·0 is taken for c_0.

Table 5

Ratio of flow rate to volume of 1st cultivator $\frac{F}{V}$	Ratio of density of population to maximum density $\frac{X}{M}$ Cultivators				Productivity in arbitrary units at various numbers of cultivators in battery Number of cultivators			
	1st	2nd	3rd	4th	1	2	3	4
0·1	0·91	0·99	1·0	1·0	0·09	0·05	0·03	0·02
0·2	0·81	0·97	0·99	1·0	0·16	0·10	0·07	0·05
0·3	0·72	0·93	0·99	1·0	0·21	0·14	0·10	0·07
0·4	0·62	0·89	0·97	0·99	0·25	0·18	0·13	0·10
0·5	0·53	0·80	0·94	0·98	0·26	0·21	0·16	0·12
0·6	0·43	0·76	0·91	0·97	0·26	0·23	0·18	0·14
0·7	0·34	0·67	0·86	0·94	0·23	0·23	0·20	0·16
0·8	0·24	0·56	0·79	0·90	0·19	0·23	0·21	0·18
0·9	0·15	0·43	0·69	0·84	0·13	0·19	0·21	0·19
1·0	0·05	0·24	0·51	0·72	0·05	0·12	0·17	0·18

a density of population higher than 0·9, then it is necessary to use a battery of 2 or 3 cultivator units. In this case it would be uneconomical to use only one cultivator because of low productivity. Batteries consisting of 4 or more cultivator units are also uneconomical, but they have the advantage of enabling the simultaneous study of cultures at different stages of development.

At a flow rate of 25 ml/hr. the highest degree of productivity is reached when the volume of the Pth incubator is 150 ml (Tab. 3). The density of population is at that time also sufficiently high. Therefore the given variant is the optimal one of all 12 studied in this experiment. In the second place lies the variant with a volume of the second cultivator equal to 240 ml and with a flow rate of 25 ml/hr.

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As the first approximation it is possible to accept the following relationship between these values:

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For each strain of microorganisms at a given composition of the medium the values of c_0, X_m and M are constant.

Providing the density of population is not too high the equation (9) satisfactorily corresponds to the obtained results (Tab. 4).

It we substitute the value of c from equation (9) in equations (3) and (6) we get formulae by means of which we can calculate in advance the density of microbial population in various types of cultivation.

Examples of such calculations are shown in Tab. 5. We assumed that the volumes of all "cultivators" in the battery are equal ($V_1 = V_2 = V_3$ etc.); the maximum growth rate $c_0 = 1{\cdot}0$ and $\frac{X_m}{M} = 0{\cdot}95$.

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a density of population higher than 0·9, then it is necessary to use a battery of 2 or 3 cultivator units. In this case it would be uneconomical to use only one cultivator because of low productivity. Batteries consisting of 4 or more cultivator units are also uneconomical, but they have the advantage of enabling the simultaneous study of cultures at different stages of development.

It is possible to make calculations in a similar way also in the case of batteries with other ratios of cultivator volumes and at different microorganism growth rates.

It is necessary, however, to point out that at high densities of microbial population the growth rate equation (9) is no more valid and therefore the above-mentioned calculations are incorrect. In dense populations in continuous flow cultures only a part of the total number of cells continues to multiply while the rest gradually lose their living activity. In the end an equilibrium between the multiplication and loss of activity of the cells is established.

In our experiments the culture of *Cl. acetobutylicum* grew in collodion sacs submerged in the continuous flow medium. The density of living cells remained constant the whole time at a level of 1000—1200 $\times$ 10^6/ml, while the total density of cells increased continually along a straight line until it reached about 7000 $\times$ 10^6/ml or more. Similar data for *E. coli* can be found in literature.

The cultivation in collodion sacs in a flow medium can be compared with the first of the three types of cultivation described in the first paragraphs of this article. As stated there, in the second type of cultivation microorganisms grow on the surface of a solid phase along which liquid medium flows continuously. It has been mentioned in literature that the walls of cultivators are sometimes covered with a film of bacteria. This is considered as undesirable as it affects experimental findings.

We tried to add glass wool to a cultivating flask containing *Propionibact. shermanii*. At the beginning bacteria grew mostly on the surface of the wool and then spread through the liquid medium, where they started to multiply. The cell concentration in the escaping liquid reached a constant value. For instance, in one of the experiments their density in 1 ml remained within the limits of 3000 to 4000 $\times$ 10^6 in the course of 19 hours. The dilution rate in this experiment was equal to 1.0. Consequently the medium in the cultivator was actually renewed 19 times. At the same flow rate in a cultivator without any solid phase the density did not exceed 20 to 30 $\times$ 10^6/ml. Thus it appears that the presence of a solid phase considerably increases the productivity of the cultivator. Unfortunately the bacterial film is easily torn away from the surface of the solid phase and the continuity of the process is thus broken. In addition the amount of cellular material on the surface of the solid phase cannot be counted and it is impossible to determine the growth rate.

In the submitted paper we are dealing only with the problem of growth of bacterial mass in continuous flow media. But the method of continuous flow cultures can be conveniently applied to a number if different tasks, e. g. the obtaining of fermentation products, the study of metabolism and phenomena of mutability. In particular in our laboratory continuous flow cultures of *Propionibact. shermanii* were used to prepare vitamin B_{12}. Under the conditions

of continuous vegetative reproduction the culture of *Cl. acetobutylicum* was artificially adapted to higher concentrations of butylalkohol. A knowledge of the laws governing the growth of continuous flow cultures helps to draw a boundary between accumulation of individual resistant types on one hand, and the massive adaptive capacity of cells on the other hand. In our experiments with adaptation to butylalcohol this last phenomenon seems to play an important role.

A STUDY OF THE PROCESS OF DEVELOPMENT OF MICROORGANISMS BY THE CONTINUOUS FLOW AND EXCHANGE OF MEDIA METHOD

N. D. Jerusalimskij

There exist two basic points of view concerning the causes of development and ageing of microorganisms. Whilst some authors attribute a decisive role to intracellular processes, others maintain that neither progressive development nor ageing would be possible without corresponding changes of external conditions.

It is rather difficult to differentiate between the effects of inner and external factors of development on usual media, which are continually changing due to influence of the activities of microorganisms. Thus special cultivation methods have to be used, among others also methods of continuous flow and exchange of media. While the first method enables microorganisms to be maintained for long periods under stable media conditions, the second method makes it possible to change the medium as required and to regulate its composition.

Studies regarding this problem have been carried out in our laboratory (E. A. Rukina and G. V. Pinaeva) with spore-forming bacteria: anaerobic (*Cl. saccharobutyricum* and *Cl. acetobutylicum*), aerobic (*Bac. megatherium*) and facultative aerobes (*Bac. acetoethylicus*). Spore-forming bacteria are advantageous because with them the developmental cycle can be more clearly demonstrated than with asporogenous bacteria. The sporulation process is accompanied by extensive intracellular transformations with lysis of a considerable portion of the cytoplasm. It may be assumed that only stable hereditary properties can be transmitted either through spores or through reproductive cells, but not reversibly adaptive changes, or changes due to ageing of the cells, both occurring in the course of the individual life of the cell. On the other hand, during multiplication by vegetative division of cells, these changes are entirely transmitted to daughter cells. We have demonstrated these differences on the synthesis of adaptive enzymes required for the fermentation of xylose by *Cl. acetobutylicum*.

After inoculation of the culture from a glucose medium on xylose a lag-phase can be observed, related to the synthesis of these enzymes. During the following transfers by vegetative cells the culture grows on xylose medium without a lag phase, the capacity for the formation of adaptive enzymes being preserved. However, after sporulation this capacity is lost, as may be judged from the fact that the lag-phase again reappears.

Also changes of size are not transferred through spores. There are data in literature showing that as the result of prolonged vegetative multiplication spore-forming bacteria weaken and degenerate, but after sporulation their activity is restored. The studied culture of *Cl. acetobutylicum* degenerated after several dozen transfers.

However, such degeneration due to ageing is not unavoidable. We cultivated *Cl. acetobutylicum* in a continuous flow of stable and suitable medium for some 200 days. During that period more than 4000 vegetative generations were formed, corresponding to 400—600 transfers on normal, non-exchanged media. In spite of this prolonged vegetative multiplication the bacteria fully maintained their capacity of normally fermenting and of sporulation. Therefore the degeneration of a culture under normal conditions of cultivation can be explained by unsuitable changes of the media.

Under conditions of continuous cultivation in the presence of increasing amounts of butanol a culture of *Cl. acetobutylicum* changed, becoming more resistant to this toxic substance. The original culture withstood only 0·8% of butanol: after 21 days of growth in the presence of 0·6% butanol, it withstood up to 1% of butanol. This increased resistance to butanol was unstable and ceased after the cells had changed to spores, and these proliferated in the absence of butanol. In the course of further adaption the resistance to butanol increased still further and became constant. In the course of 200 days the bacteria were able to withstand more than 2·5% of butanol and this level of resistance was then transferred through the spores to further generations. Thus the resistance began to show a hereditary character.

It follows that the same bacterial culture may under certain conditions weaken and degenerate, while under different conditions it may remain unchanged or may even change its hereditary character.

The process of sporulation is also possible only under defined conditions of the medium, or, more exactly, under defined changes of these conditions. This problem was studied in detail in *Cl. saccharobutyricum*.

The culture was grown in collodium sacs, immersed in different media which could be exchanged for other media when required. The following variant proved optimal: at first bacteria were grown for several hours on a rich medium containing yeast hydrolysate; the medium was then exchanged for phosphate buffer containing 0·3—0·5% of glucose and 0·2% of acetate, and finally, this second solution was exchanged for tap water. Under these conditions about

75% of vegetative cells were transformed into pro-spores (clostridia), and 75% of the latter transformed into mature spores. The spore concentration amounted to 600×10^6 per ml medium. Such a high concentration of spores could not be obtained with unexchanged media, natural substrates included.

With the use of other variants spore formation proceeds less intensively, e. g. when the second medium (glucose-acetate-phosphate buffer) is enriched either by the addition of a source of nitrogen or when acetate is omitted, or when the culture is transferred directly from the rich medium to water with omission of the second medium. On cultivation of the culture in collodium sacs with a continuous flow of a rich medium practically no spores are formed; the total number of cells reaches, however, an unusually high number, i. e. 7000×10^6 cells per ml medium. It should be mentioned that of the above number of cells only $100-1200 \times 10^6$ cells per ml are viable.

The spore formation in *Cl. acetobutylicum*, grown in collodium sacs, is also stimulated by a lack of nitrogeneous substances. Butanol in a concentration of 0·3% exerts an additional favourable effect.

Thus the above experiments confirm the view that spore formation is, generally speaking, correlated with a lack of nutrients and to some extent also with the accumulation of products of metabolism in the medium.

It is clear that on media poor in nutrients spore formation will proceed more intensively than on rich media. On agar media, where in the vicinity of the cells a lack of nutrient substances may easily occur, spore formation proceeds more rapidly than in liquid media. This spore formation is still further increased by placing underneath the layer of nutrient agar a layer of agar without nutrients; the nutritive components then gradually diffuse into this layer from the one placed above.

Thus, a 5-day culture of *Bac. megatherium* on a synthetic medium with agar contained 50% spores when cultivated in the presence on inorganic nitrogen, 15% spores on a medium containing also hydrolysate of casein and only 5% spores on a meat-peptone medium.

When the same media were underlayered by a solution of agar in water only, the number of spores was increased to 70%, 70% and 10% respectively.

The culture of *Cl. saccharobutyricum* forms spores intensively on a rich nutrient agar, which is underlayered by an agar solution in water. On a rich nutrient agar this culture, however, forms only prospores (clostridial forms), which consequently lyse due to the absence of conditions required for the conversion into mature spores.

The developmental process is irreversible. This conclusion is borne out by the fact that clostridial forms may either change into mature spores or die off, but cannot revert to the vegetative mode of multiplication. This can also be demonstrated by following the changes of the number of cells of a developing culture of *Cl. saccharobutyricum* using two methods: direct counting of cells

and counting of colonies on Petri dishes. When the culture consists of young vegetative cells, both methods give identical results. When the vegetative cells begin to lose their viability or change to clostridial forms, the number of colonies is considerably smaller than the total number of cells. At a further stage unviable vegetative cells lyse and disappear; the total number of the population correspondingly decreases. However, the number of colonies grown on Petri dishes somewhat increases. This observation may be explained as follows: clostridia, themselves unable to multiply, are converted to spores which, after being transferred on to agar, can germinate and form colonies.

The above mentioned experiments were carried out with populations of different history: some of them divided, others converted to spores, and still others died off. It is known that the progeny of even one cell may greatly differ within a short period of time. The formation of these differences may depend first on the fact that individual cells may develop under non-identical microzonal conditions of the medium; secondly, in the course of the division two physiologically different cells with different properties may form. Some authors suggest that differences in the qualities of cells appear to be accidental and not of determined character. Notably the appearance of spontaneous mutations is explained in this way. Others maintain that such qualitative differences are a determined and necessary phenomenon, arising from unequal cellular division. According to this view of two just divided cells one always appears to be the mother cell, the other the daughter cell. (Kolbmüller, Bisset, Pennington, Málek, Strešinskij and others).

In order to be able to follow the development and ageing of individual cells a special microvessel was used. This consists of a flat flask with two tubes attached for the inflow and outflow of the medium. The vessel is placed on the microscope stage. The vessel is closed by a glass cover on the lower surface of which bacterial spores are placed; the spores are fixed to the surface of the glass cover by a very thin layer of cellulose in the same way as is in the case of electron microscopy. The vessel is filled to the top with nutrient medium which may be changed, if required, for a medium of the same or different composition.

Using these vessels, cultures of *Cl. saccharobutyricum* and *Bac. acetoethylicus* were studied. The experiments confirmed that bacterial spores are heterogeneous. This follows from their morphology, resistance to heat and rate of germination in the microvessel. Differences in the vegetative cells grown from these spores were also found. Not seldom was it found that of two vicinal cells one changed into a prospore, while the other remained in the vegetative state and later lysed. Differences in the fate of the cells might possibly be explained by the assumption that some of them were mother-cells, while others were daughter-cells. Cells in groups convert more easily to spores than isolated cells. It appears to be clear that the microzonal conditions surrounding the cells are not identical and also affect the development of the cells.

Simultaneously with this unexpected individual variability of the cells, determined changes of the whole mass were found, depending on the composition of the nutrient medium. Thus, when filling the microvessel with a rich nutrient medium, vegetative multiplication occurred until the whole microscopic field was practically full of cells; under these conditions no spore formation was observed.

If, however, at the correct time the rich nutrient medium is exchanged for a medium without nitrogeneous substances the majority of cells are converted to prospores (clostridia). If now the vessel is again filled with a rich nutrient medium, the remaining vegetative cells start to multiply again, whilst the clostridia die off and undergoe lysis. On the other hand, after sufficiently long maintenance on nitrogen-free medium and its subsequent exchange for water, the undamaged vegetative cells weaken and undergo lysis whilst clostridia convert to mature spores. Under appropriate conditions these spores may again germinate and change to vegetative cells if the vessel is once again filled with the rich nutrient medium. In this way the developmental cycle of the bacteria can be repeated.

The results of the experiments described above show that the physiological inequality of cells, due to inequality of division, does not determinate their further fate. Together with the physiological state of growth of the microorganisms the conditions of the external medium play an important role. Neither changes of the life cycle nor the weakening and death of cells appear to be processes occurring spontaneously and autonomously.

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GLYCOGEN FORMATION IN CONTINUOUS CULTURE OF *ESCHERICHIA COLI B*

T. Holme

Many industrial fermentations are based on specific nutritional deficiencies (Foster, 1949, Underkofler and Hickey, 1954). These processes represent a type of fermentation which depends on the existence of an inverse relationship between the growth rate of a microorganism and the synthetic rate of a certain product of its metabolism. Thus, an essential substance in a nutrient medium may be supplied in a concentration which limits the growth of a microorganism at a point, where optimal conditions for the synthesis of a desired product still are maintained. This production may proceed for a long time after growth has stopped.

It seems not to be generally realized, however, that a fermentation of this type may be well suited for a chemostatic continuous process. In the experiments to be presented here, a system of the above-mentioned type, namely glycogen synthesis in *Escherichia coli*, has been investigated with the continuous culture technique. In addition, the formation of some extracellular substances in the continuous cultures has been studied. The continuous culture system used was based on the principles given by Monod (1950) and Novick and Szilard (1950).

GLYCOGEN METABOLISM IN *ESCHERICHIA COLI*

Escherichia coli contains an alkali-stable polysaccharide which belongs to the class of glycogens (Palmstierna, 1956). In rapidly growing cells the amount of glycogen usually will not exceed 2% of the dry weight. However, a rapid accumulation of glycogen occurs in cells which are subjected to nitrogen starvation (Holme and Palmstierna, 1956). Figure 1 illustrates an experiment, where cells of *E. coli B* were inoculated into a synthetic medium which contained a limiting concentration of the nitrogen source. After an initial lag and a short multiplication period, exhaustion of the limiting factor prevented

further synthesis of nitrigenous compounds. During this period of constant population density, glycogen accumulated in the cells.

In similar experiments it could be shown, that during this period of constant population density (measured as bacterial nitrogen per unit volume of culture)

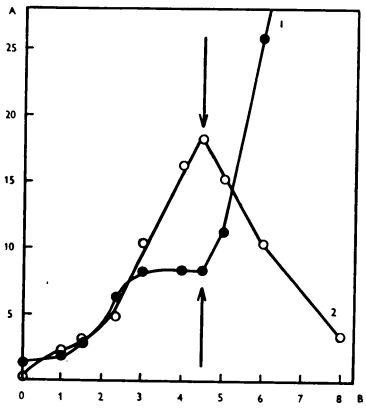

Fig. 1. Glycogen accumulation and utilization during growth in a culture of *E. coli B*. Cells were inoculated into a nitrogen-deficient medium with sodium lactate as the carbon source and ammonium chloride as the sole nitrogen source. The initial concentration of ammonium nitrogen was 10·5 mg per litre. At the moment indicated by the arrows, ammonium chloride was added in excess.

A — Protein N or glycogen (mg per litre of culture); B — Time (hours); Curves: 1 — Protein N; 2 — Glycogen.

glycogen reached a final value of 20—25% of the dry weight of cells. The accumulated glycogen was responsible for at least 90% of the increase in the dry weight of cells during this period.

In the experiment recorded in Figure 1, an amount of the nitrogen source was added immediately before the maximum content of glycogen was attained, to give the same concentration of this factor as in the complete medium. The addition caused an immediate resumption of growth. During this growth period the glycogen decreased rapidly.

CONTINUOUS CULTURE EXPERIMENTS

EQUIPMENT

A culture vessel consisting of a 2-litre Erlenmeyer flask was given a working volume of 1 litre by means of a small exit tube on the wall for removing the emergent bacterial culture. Mixing and aeration was achieved by placing the flask on a rotary shaker and letting the air stream enter the culture fluid through a sintered glass disc as close to the wall of the flask as possible. The neck of the flask was sealed with a rubber stopper, which was perforated by glass tubes for the supply of air and fresh medium and for inoculation. Effluent air left the flask only through the exit tube mentioned, making the transfer of the emergent bacterial suspension to a cooling flask a matter of seconds. A hose pressure pump was used for the continuous supply of fresh medium.

MEDIA

Two media were employed: one with sodium lactate (Friedlein, 1928) and the other with glucose (Hook et al., 1946) as the carbon source. Ammonium chloride was the sole nitrogen source in both of the media. This nutrilite was used as the limiting factor, and supplied in concentrations ranging between 50—200 mg per litre in different experiments, corresponding to a nitrogen content of 13—52 mg per litre.

RESULTS

In nitrogen-limited continuous cultures of *E. coli B* steady-state growth was established at dilution rates of 0·13—0·94 hr^{-1}. (Holme, 1957 a and b). The glycogen and nitrogen content of the cells and the dry weight were determined under these steady-state growth conditions. The results are summarized in Figure 2, where the amount of glycogen formed in one hour by a continuously growing population corresponding to 50 mg bacterial nitrogen is recorded. It is seen that the amount of glycogen formed in one hour increased when the dilution rate was reduced. This means that there exists a negative correlation between the rate of glycogen synthesis and the rate of synthesis of nitrogenous compounds in the cells. The production of glycogen in the continuous cultures could thus be increased about 3-fold in the lactate medium and about 1·5-fold in the glucose medium by a reduction in the dilution rate from 0·8 to 0·2 hr^{-1}. The glycogen content of the cells increased in the same interval from 2—3% to about 20% of the dry weight of cells.

Figure 2 also shows that rapidly growing cells have higher glycogen content in a glucose medium than they do in a lactate medium.

As a rough method of estimating the "overflow metabolism" the ultra-violet absorption of the cell-free culture medium was determined. The absorption increased considerably with decreasing dilution rate. This absorption in the ultraviolet region mainly had the character of an unspecific end absorption. It did not depend on accumulation of nucleic acids in the culture fluid, but

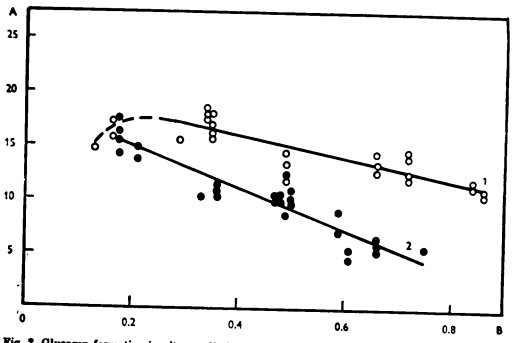

Fig. 2. Glycogen formation in nitrogen-limited continuous cultures of *E. coli B*. The values are recalculated on basis of a population density corresponding to 50 mg bacterial nitrogen per litre of culture. The culture volume was 1 litre.

A.— Glycogen (mg/hr.); B — Dilution rate (hr^{-1}); Curves: 1 — Carbon source: glucose; 2 — Carbon source: lactate.

was probably the result of an accumulation of a variety of compounds, the nature of which is unknown. These compounds were dialyzable to at least 90% as measured by the ultra-violet absorption.

The culture fluid in these nitrogen-limited cultures contained high levels of keto-acids, as determined by the method of Friedmann and Haugen (1943). Paper chromatography of 2,4-dinitrophenylhydrazones of the keto-acids according to Cavallini et al. (1954) revealed that the main portion of the isolated compounds consisted of α-ketoglutaric acid. Small amounts of pyruvic acid were also present. The formation of these substances has only been studied in culture grown with lactate as the carbon source.

In the upper graph of Figure 3 is shown the formation of keto acids, calculated as α-ketoglutaric acid, in the continuous cultures. It can be seen, that the rate of synthesis of these substances seems to be constant at dilution rates ranging between 0·2 and 0·7 hr^{-1}, decreasing at dilution rates lower than 0·2 hr^{-1}. A similar calculation may be made on basis of the determinations of the ultraviolet absorption of the culture fluid at different dilution rates.

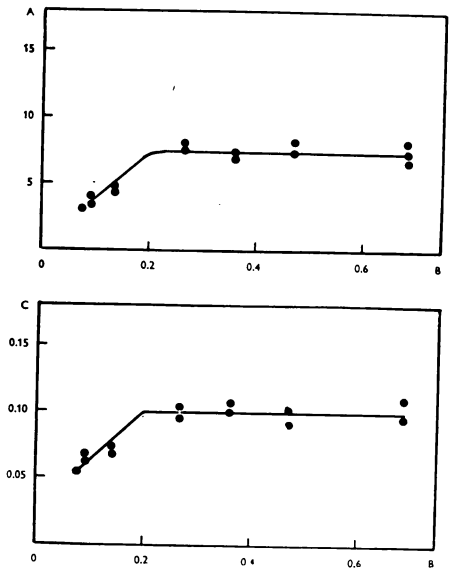

Fig. 3. Formation of extra-cellular substances in nitrogen limited continuous cultures of *E. coli B*. Population density corresponding to 50 mg bacterial nitrogen per litre of culture. The culture volume was 1 litre. Upper graph: keto acids calculated as α-ketoglutaric acid. Lower graph: substances causing absorption in the ultra-violet region

A — Keto acids (mg per hr); B — Dilution rate (hr^{-1}); C — Optical density (at 265 mμ).

If it is assumed that the absorption is caused by a variety of overflow intermediates in a constant proportion, and that the optical density is proportional to the concentration of the metabolic products, the optical density at a given wave-length may be taken as a rough estimate of the concentration of these products.

The values obtained if the optical density at 265 mμ is taken as a basis for the calculations are recorded in the lower graph of Figure 3. The similarity between the two curves presented in Figure 3 suggests that this type of production is not confined to the keto-acids but may also be true for other overflow intermediates. At the observed concentration levels, α-ketoglutaric acid did not contribute to the absorption at 265 mμ to any measurable degree.

DISCUSSION

In general, this discussion will be limited to the experimental system presented in this paper, but it may be justifiable to speak of this system in rather general terms, so that applications to other systems present themselves more easily. The intracellular glycogen and extracellular materials formed by *E. coli* in nitrogen-limited cultures will be referred to as "nitrogen-free" substances.

It appears that the formation of nitrogen-free substances in nitrogen-limited cultures of *E. coli* proceeds without being inhibited by the limitation. These compounds may be considered as "overflow metabolites" accumulating because of the reduced rate of the synthesis of nitrogenous compounds in the cells.

Since under conditions of nitrogen starvation the production of the nitrogen-free substances is directly proportional to the cell material producing them, a maximal productivity in the continuous culture might be expected at population densities approximating the maximal obtained in non-limited media.

However, two general limitations must be considered. Firstly, if the carbon source in a nutrient medium is not in great excess, too much of the available carbon may be used for the synthesis of cell material, and consequently too little left for an optimal product formation. Secondly, at population densities near the maximal in non-limited media, the synthesis of the nitrogen-free substances is strongly inhibited, even if the carbon source remains in excess. Unknown factors, presumably metabolic products formed during the synthesis of cell material, may be responsible for this inhibition. Deficiency in additional factors, such as oxygen, may also be responsible for the inhibition if too large population densities are used.

This means, that the nitrogen limitation provides an effective tool for limiting the production of cell material at the highest population density, where optimal conditions for the synthesis of a desired product still are maintained. From an experimental point of view, the continuous culture technique seems

to be well suited for studies on the influence of various environmental factors, especially the nutritional factors, on product formation.

It is seen in Figure 3, that the output of the extracellular substances decreases rapidly at dilution rates below 0·2 hr^{-1}. The explanation may be, that there is a certain minimal growth rate, i. e. a minimal rate of renewal of cellular material, which is necessary to maintain maximum activity of certain enzyme systems. This minimal growth rate may vary for different microorganisms.

At dilution rates between 0·2 and 0·7 hr^{-1}. in the continuous cultures the rate of glycogen synthesis increases significantly with decreasing dilution rates (Figure 2). The rate of synthesis of the extracellular materials seems to be independent of the dilution rate in the same interval (Figure 3). This means that these extracellular materials are poured out to the culture fluid at a constant rate, irrespective of the growth rate of the organisms. This may depend on an inhibition of their synthesis because of the accumulation of this new, low-molecular weight end product. When a high molecular compound, such as glycogen, is the end product, its influence on the equilibrium of the reaction responsible for its synthesis is very limited, and it may thus attain high concentration before the rate of its formation is inhibited.

In Figure 2 it is seen that there is a difference in the rate of glycogen formation depending on the nature of the carbon source. This difference is greatest at the high dilution rates, diminishing with decreasing dilution rates. If the straight lines obtained from the values of glycogen output represented in Figure 2 are extrapolated to zero, a value of the synthetic rate is obtained, comparable with the initial rate of glycogen synthesis in batch cultures under conditions of complete nitrogen starvation. It may be concluded that the maximal rate of glycogen synthesis is the same irrespective of whether the glycogen is synthetized from lactate or glucose.

A continuous fermantation has certain advantages over the corresponding batch process, e. g. reduced size of unit at the same production rate, and greater uniformity of the product. The conditions for the synthesis of nitrogen-free substances in chemostatic continuous cultures of *Escherichia coli* now seem well established but it should perhaps be pointed out that before general conclusions can be drawn, investigations of other systems must of course be carried out. In this connection seems particularly important to study if the concentration of a given substance formed in a chemostatic continuous culture may attain the same values as in a batch culture. At maximum output in the continuous cultures of *E. coli* the concentration of the nitrogen-free substances seemed to be somewhat smaller than that obtained in the batch cultures. However, the advantage of having a system in equilibrium, where optimal conditions for the formation of a given product have been established, should be obvious.

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CONTINUOUS CULTURE TECHNIQUES

J. Řičica

In the course of development of culture techniques enabling us to study the life activity of microorganisms ever increasing stress is being laid on the perfect culture technique that can make use of all possibilities afforded by the progress of improvement of apparatus and equipment and thus fulfil to the utmost the requirements dictated by the present progress of microbiology. Every type of microbiological work requires a corresponding culture technique, be it a diagnostical work, a genetic, selection or growth study, or an investigation of physiological and biochemical problems, or the synthesis and transformation of some product. The reproducibility of work on biological material is undergoing improvement owing to mechanization and automatization of culture apparatus. Its accuracy has been further increased by introducing physical and physico-chemical aspects and by applying them to culture techniques.

We shall omit the conventional static culture methods. An important improvement has been achieved here by the introduction of stirring. Movement ensures a better supply of nutrients to the cell. Organisms respond to it by intensified metabolism and growth. Therefore ingenious shaking machines have been developed based on the rotational or reciprocal principle. Due to disturbing of the liquid surface a more active gas absorption is achieved and thus growth and metabolism are further stimulated. To produce a similar effect tall and slender cylinders are used occasionally in which the stream of entering gas is dispersed by some sort of atomizer at the bottom of the vessel and passes upward in the form of tiny bubbles mixing with the liquid and saturating at the same time the medium with gas.

For some special purposes rotating drums have been constructued in which the liquid is stirred by rotating the whole vessel along its longitudinal axis either vertically or inclined to a certain angle. As the volume of medium is small in comparison with the whole vessel, the liquid can spread over the large surface of the vessel walls when rotating. Thus effective contact with the enter-

ing gas can be achieved; in most cases with oxygen from the air. An increase of partial pressure sometimes aids its dissolving.

For highly aerobic processes and in order to utilize better the space of the vessel fermentors have been developed in which stirrers are used dispersing the under pressure entering gas into minute bubbles. Due to the turbulent motion of the liquid in the whole volume oxygen is dissolved more easily and transported rapidly to the powerfully respiring cells of the aerobic microorganisms. The volume of passing gas is measured by flowmeters of various types.

Experiments have been carried out in which oxygen is supplied by electrolysis of the aqueous nutrient medium (Sadoff, Halvorson and Finn, 1956).

To determine the parameters controlling or reflecting the course of the process, culture vessels are supplemented with physicochemical analysers by means of which the content of O_2 and of CO_2 can be determined either in the escaping gas or directly in the culture medium. Also electronic equipment can be provided which automatically registrates and regulates the pH, the turbidity of cultures, flows of gases and liquids, temperature, etc., and controls further auxiliary apparatus.

Cultivation of microorganisms can proceed either in the form of a batch culture or continuously — in identical culture vessels, the only difference being that in the continuous type equipment is provided by means of which the nutrient medium is continuously added and withdrawn.

In batch culture the substrate is changed by the actively metabolizing microorganisms and the slightly modified medium thus affects reciprocally the microorganisms themselves. The rate of the whole process is determined by innumerable biochemical and biophysical reactions, limited first of all by the concentration of available nutrients in the substrate and by the size of the vessel. The process is a dynamic one in a statically closed and unchanging space and changes with time. It is extremely difficult under these conditions to study the individual reactions, particularly the irreversible ones, and to apply the laws of thermodynamics to them. As the nutrients are exhausted and metabolites accumulates the rate of the process is slowed down until it reaches the point when no free energy is transformed ($dF = 0$); then the process stops and an equilibrium is established. By applying this characteristic we can consider the batch process as a closed system.

The living cell, however, belongs fundamentally among open systems. If we wish to study the physiology of the fully developed cell and to follow the kinetics of one-step and of multi-step complicated reactions it is necessary to cultivate microorganisms under the conditions of open systems. These conditions are met only in continuous flow culture processes which range among the open systems.

The theory of dynamics and of yields of irreversible processes in open

chemical systems in a steady state, as determined by the reaction rate either for one or more reactors connected in series, has been well developed and studied and its results have aroused the great interest of many biologists.

So Pasynskij (1957) deals with the question of the application of classical thermodynamic equilibria of closed systems as well as of the physicochemical relationships of simple reactions of open chemical systems to the cell. He maintains that they cannot fully interpret the kinetics of chain reactions forming the basis of complicated physiological functions. Pasynskij states further that the simplest form of life (the biological open system) differs from the most complicated chemical reaction of an open system in having the capacity of self-preservation and auto-reproduction and in directing all the complicated chemical reactions to recreate the given system. He claims that it is impossible to design a chemical apparatus as a result of chemical changes in which a similar apparatus or its part would function. Pasynskij, however, does not stress sufficiently the fact that into a continuous culture vessel (a chemically open system) a living microbial cell (a biologically open system) can be introduced:

This type of open system is characterized by the fresh nutrient medium being added to the culture vessel and by removing at the same rate the medium modified by the metabolic activity of the organism, together usually with a part of the propagated organisms. The medium, the volume of which is kept constant, must be stirred thoroughly in order that the concentration of reacting factors (i. e. nutrients and cells) may be identical in the whole vessel. Considering the auto-reproductive capacity of microorganisms, the whole process can be compared to an auto-catalytic reaction proceeding at a constant rate. If the rate of inflow of fresh nutrients is equal to the total rate of their utilization the concentration of nutrients becomes stationary. If the specific growth rate is equal to the dilution rate and if both the concentration of nutrients and the dilution rate which must not exceed a certain critical value, remain constant, a steady state is established which is, under certain conditions, capable, of auto-regulation. This is a dynamic steady state and not an equilibrium because the transformation of free energy proceeds at a constant rate ($dF = \text{const.}$).

In a precisely defined steady state of any chosen quality the application of results from chemical open systems is facilitated and a mathematical interpretation of the kinetics of the individual reactions is simpler.

The application of the fully developed dynamics of propagation of microorganisms and of the formation of products is not only valuable for theoretical studies, but is of farreaching practical importance.

The continuous system has the following advantages as compared with the batch process:

1. The course of reactions can be studied in a certain chosen phase of the process under constant steady state conditions in which the time factor is eliminated. The influence of nutrients, temperature, pH, stirring, aeration and other factors on the course of reactions can be determined more easily and without complications due to the incessantly changing conditions.
2. The mathematical treatment of the course of the process is simpler.
3. The product is more homogeneous.
4. There is a greater possibility of automatizing the whole process and of regulating it by a suitable application of physicochemical methods.
5. The production is more economical due to two factors: the dimensions of the given apparatus can be decreased while the same production is preserved, the cultivation being more physiological and thus more efficient; the processing time can be decreased while the dimensions of the apparatus remain unchanged.
6. The cost of construction of a well-operating equipment is somewhat higher than in the case of the batch process, but as soon as the continuous process is in operation, the cost of production per unit weight of product is far lower.
7. The preparation of the medium and obtaining of the product are more efficient and more economical.

The idea of utilizing the continuous processes in various microbiological fields is not a new one. We have now very extensive literature on this subject which cannot be grasped in its whole. As early as at the beginning of this century the possibility of producing ethylalcohol continuously by means of fermentation was indicated. Later on a number of papers and patents appeared dealing with this fermentation process and it is actually the only process that has found wide application in industry. To a small extent the continuous production and baker's yeast has been used. Somewhat later also other branches of microbiology started to use continuous culture for experimental purposes. But since then the majority of continuous culture processes has been developed empirically and not always with full comprehension of the principles of open systems, and therefore the experimental results have not been applied significantly in practice. It may also be due to the fact that at that time the culture technique in industry had not reached its present level, particularly with respect to the ensuring of sterility, continuous preparation of the nutrient medium and automatization of the whole process.

It was only in the middle of this century (Monod, 1950; Novick and Szillard, 1950a, b) that the continuous methods were laid on a solid theoretical basis, particularly due to the mathematical treatment of the chief growth principles in open systems.

Further theoretical aspects were published by Adams and Hungate (1950), Gole (1953), Finn and Wilson (1954) and Northrop (1954); these authors,

however, did not stress sufficiently the mutual relationship between the substrate concentration, the rate of dilution and the specific growth rate. Novick (1954, 1955), Spicer (1955), Málek (1955) published extensive papers of a more general character denoted to the questions of continuous culture methods. Danckwerts (1954) treated the main principles of chemical flow systems and limited the possibilities of individual types of flow reactors for certain types of reactions. Some of his conclusions can be applied also to continuous flow cultivation. Saeman, Locke and Dickerman (1946), Maxon (1954), Katsume (1956) studied the problems of industrial continuous fermentations mostly with yeast organisms and Tamiya (1957) with algae.

The theory has been developed in a number of other papers. Herbert, Elsworth and Telling (1956) undertook a mathematical analysis of the kinetics of bacterial growth and changes in the concentration of microorganisms on the basis of a minimum number of very simple postulates. They treated again the steady state problem and the effect of various rates of dilution at various substrate concentrations in the inflowing medium. They supported their theoretical conclusions by practical results. Powell (1956) treated mathematically the relationship between the rate of growth and the percentage occurrence of cells of different ages and of different generation times in cultures growing under continuous flow conditions. He discussed the various factors leading to the establishment and preservation of a steady state. Pirt (1957) derived formulae for calculation of the oxygen intake in a continuous culture.

The perfection of the theory resulted in the production of new and better laboratory equipment of various types (Hedén, Holme and Malmgren, 1955; Fox, Szilard, 1955; Elsworth, Meakin, Pirt and Capell, 1956; Anderson, 1956; Perret, 1957).

The methods employing the continuously operating culture apparatus can be divided into four groups.

I. METHODS BASED ON THE PRINCIPLE OF CIRCULATION

The medium flowing out of the stock vessel and returning to it again circulates constantly through the cultivation vessel. Circulation of the liquid is effected by a pump or, with most types of apparatus, by an air lift, which at the same time aerates the liquid. Through the metabolic activity of the organisms the nutrient level decreases, the number of cells increases and metabolites accumulate. In spite of the fact that the apparatus operates continuously the process takes place batchwise and corresponds in character to the closed systems.

It is known in two basic types:

a) The organism grows on a fixed carrier and some of the free cells circulate with the liquid.

A typical example of this type is the production of vinegar in vinegar vats. The same principle was used by Hermann and Neuschul (1935) who studied the formation of gluconic acid by bacteria growing on wood shavings. Audus (1946), Lees (1949), Temple (1951) and Dubash (1956), who designed several types of perfusion apparatus studied the metabolism of microorganisms growing on soil particles by means of the percolation technique. Pasynskij and Nejmark (1952) caused the microorganisms to adhere to pieces of sintered glass in their circulation fermentor. Dimopoullosos and Pritham (1951) cultivated animal tissues fixed in a percolator. Darlington and Quastel (1953) used a double perfusion apparatus for studying the passage of various substances through the wall of a still living piece of intestine.

b) The organism grows dispersed in the medium. Lundgren and Jennison (1955) (Beesch, Shull, 1956) used the air lift pump principle in their laboratory fermentor and Ashton and Holgate (1951) patented a circulation equipment for the production of streptomycin. Tamiya (1957) mentions several methods of cultivation by circulation for the propagation of algae. Krauss and Thomas (1954) harvested algae by means of a continuously operating centrifuge and returned the supernatant to the cultivation trays. Boeckeler (1948) carefully separated alcohol from the circulating fermentation liquid in a stripping tower and returned the liquid into the fermentor together with non-damaged cells.

II. FEEDING METHODS

The substrate is fed at a certain rate, either periodically or continuously, to the growing culture. Only those types are of importance which start with a small volume of medium containing a certain concentration of microorganisms in the cultivation vessel and maintaining during the period of feeding a stationary concentration of cells and nutrients per unit of volume in harmony with the rate of growth. In this phase the rate of the process does not change in time and resembles the steady state of open systems but washing out factor is not represented here. Only the space and the amount of added nutrients change. As soon as the desired volume is reached feeding is stopped. From this moment on the cultivation process becomes a closed system changing with time in a constant space and ends like any other batch process.

The feeding types of cultivation are most widely employed in the fermentation industry. But most of the feeding schemes used to-day for yeast propagation have been designed on an empirical basis and do not always fully correspond to the specific growth rate.

The various feeding systems are in extensive use also for the propagation of a great mass of algae (Krauss, 1955; Tamiya, 1957). Olson and Johnson

(1948) fed slowly acid hydrolysed wheat mash to a culture of *A. aerogenes* producing 2,3-butylenglycol so that the sugar concentration in the medium was kept constant.

We know of experimental cases when the substrate was added periodically or continuously to a considerably grown culture. The inflow was started only when a certain phase of the cultivation process had been reached. These cases are, however, only modified examples of the batch system.

A similar type was used for the cultivation of fodder yeast by Tyler and Maske (1948) (Lee, 1949) who added a constant amount of nutrients into the recirculating liquid and thus combined the circulation and feeding systems. Davey and Johnson (1953) and Hosler and Johnson (1953) studied in their laboratory fermentor the effect of several saccharides on the production of penicillin by adding them periodically or continuously in certain constant amounts to a mold culture in which they kept the pH constant. Soltero and Johnson (1954) studied the formation of penicillin in shake flask fermentations to which they added glucose continuously at a constant rate. Jacob (1953) decreased the rate of growth of *E. coli* by adding a small amount of urease upon exhaustion of the nutrients, thus ensuring slow liberation of the ammonia from the urea, or by adding small doses of lactase, thus ensuring liberation of the glucose from the lactose.

III. SEMI-CONTINUOUS, PERIODIC METHODS

These processes are based on the principle of repeated and mutually dependent batch cultures placed in one or more vessels connected in series. The purpose of this process is long-term exploitation of the culture and its continuity is considered strictly from the industrial production point of view. According to the characteristics of the organism and the type of cultivation several modifications have been employed so far:

a) repeated use of a grown culture. When molds are grown in surface tray cultures the mycelium cakes are washed underneath with fresh substrate after the fermented medium has been removed. This type was used in the production of citric acid, but its application in surface fermentation of penicillin on trays or in a horizontal fermentor Abraham (1941), Haller (1950) (Brinberg, 1953) was not particularly successful in practice.

b) A part of the culture can be used as inoculum after the completion of fermentation. This type is widely employed in the fermentation industry where a part of the separated yeast cells are returned as the inoculum of a new fermentation process. Garibaldi and Feeney (1949) left a part of the culture in the fermentor on completion of the fermentation process as inoculum for a further fermentation process when producing subtilin. They repeated this

process several times, observing no decrease in the production of the antibiotic.

c) A part of the culture is removed at a given growth phase and the rest is supplemented with fresh substrate to the original volume. The removed part of the culture continues to ferment in one or several other flasks.

To meet the needs of the fermentation industry several modifications were developed, e. g. for the initial propagation of yeast (Castor and Stier, 1947), for yeast production (Meyer, 1929; Darányi, 1936; Píš, 1956), for gluconic acid production (Porges, Clark, Gastrock 1940), and for fodder yeast production (Leopold and Fencl, 1955). Wakaki, Ishida, Yamagushi, Mizuhara and Masuda (1952), Foster and McDaniel (1952), and the Schenley Ind. Co. (1953) tried to produce penicillin by a semi-continuous method and the Distillers Co. (Perlman et al., 1952) streptomycin. An analogous culture technique can be applied in the production of algae as shown by Tamyia (1957) in his general report.

IV. CONTINUOUS FLOW METHODS

The apparatus for continuous culture techniques consists usually of a reservoir of sterile nutrient medium, of a device effecting the inflow of the nutrient medium into the culture flask and an overflow device which ensures a constant volume of the culture in the growth tube by enabling the microbial suspension to escape. According to the type of process taking place in the described apparatus, the continuous flow techniques can be divided as follows:

1. HETEROCONTINUOUS METHODS

The nutrient medium flows continuously as a unified stream through the static space in which the microorganism grows. The composition of the medium in the flask changes along the direction of flow according to a certain rate gradient. If the conditions of flow rate are constant then the composition of the medium at a certain point of the culture space approaches a constant and time-independent value. The system as a whole does not change with time and is characterized by changes in the arrangement of spatial coordinates. In other words, it is spatially variant but temporally invariant.

Two modifications are applied:

a) The Organism Grows on a Fixed Carrier

Some examples belonging to this group form an exception as with them no mechanism for keeping the volume of culture constant is used. In order to observe cellular division under the microscope Harris and Powell (1951) constructed a small chamber in which bacteria grew on a cellophane membrane under which the nutrient medium constantly passed. Rosenberg (1956) observed under the microscope a lysis of a colony of *St. aureus* (LS_2) which grew on an agar plate covered with a cover glass. The medium was brought in and carried away by two strips of filter paper. Also Utenkov (1941) used agar slant as culture carrier in one of his modifications of a microgenerator. Savage and Florey (1950) studied the induced antagonism in a microculture of *B. proteus* which grew on a small metal spiral and was washed very slowly by the inflowing nutrient medium containing the suspension of the microbe against which the antagonism was to be formed. Kautsky and Kautsky (1951) cultivated bacteria, algae and *Rhodotorula* on a filter paper strip in the vertical position which was at its upper end continuously saturated with a substrate solution. Pasynskij and Nejmark (1952) used pieces of sintered glass as a carrier for organisms in a cylindrical flask. Northrop, Ashe and Morgan (1919) divided a cylindrical fermentor into several zones by means of perforated plates. On the corn cobs with which the individual zone were filled they grew *B. acetoethylicum* which fermented cane molasses flowing in the upward direction to acetone and ethylalcohol. Šarkov (1950) described a method of alcoholic fermentation of sulphite liquors in which yeasts grew in wood or metal baskets filled with cellulose fibres and submerged into the medium in the fermentor at a continuous flow of liquid. Kaljužnyj (1955) used yeast cells deposited on cellulose fibres to ferment sulphite waste liquors. Clifton (1943) tried to produce penicillin continuously in an modified vinegar vat. The mold grew on wood shavings in a tall and narrow glass cylinder. The stream of liquid and of air was directed downward. Swiss patent*) authors tried to use the method for industrial production of penicillin but without practical success. The mold was to grow on the surface of tubes, grids or various small objects washed by the substrate solution. Jeffreys (1948) underwashed continuously mold mats growing on the surface of a liquid and studied the production of mold enzymes. The same principle was used for the production of penicillin by Stice and Pratt (1946) who placed several converted trays one above the other into a cylindrical fermentor filled with medium. Under every tray they formed a bubble from the entering air. Thus the mold grew on the interface of a liquid and a gaseous phase. The flow of liquid was directed downward. Švachulová and Kuška (1956) described a simple apparatus arranged in such a way that *Mycobacterium tbc* could be cultivated in it on the surface of the nutrient medium undisturbed by the continuously passing liquid.

Moyer (1929) cultivated *Bact. aerogenes* and Moor (1945) cultivated some penicillia and yeasts under highly aerobic conditions in variously adjusted long glass tubes placed horizontally. Organisms grew on the lower side of the

*) Verfahren und Einrichtung zur Herstellung von Schimmelpilzprodukten, insbesondere Penicillin, Swiss Patent 279 098, 1948.

tubes in a slow current of liquid and flowed out from them into a collecting vessel, or else they were let out periodically from the sedimentation cells at the outflow end of the tube.

A typical example of the "piston flow" or of the "tubular flow" is the cultivation in which the liquid flows slowly inside a permeable cellophane tube and the organism grows on its external surface. Lewis and Lucas (1945) grew *P. notatum* first on the surface of a cellophane tube, but as the mold attacked the cellophane they used a porcelain tube, in some cases covered with a cellulose nitrate film. A ceramic cylinder was used for the same purpose by Beatty (1946) (Brinberg 1953). Harmsen and Kolff (1947) obtained considerable amounts of microbial mass on the same principle. They grew bacteria on the surface of variously prepared cellophane tubes.

b) The Organism Grows Diffusely Dispersed in an Unstirred Medium

This culture method has found wide application in the fermentation industry. Most methods are based on the empirical process of substrate utilization in a series of mutually connected fermentors. Long ago Lebedev (1915) (Lebedev, 1936) pointed to the possibility of fermentative production of ethylalcohol in this way; later on he developed it himself on an industrial scale. A similar type of process of fermentation of very varied substrate to ethylalcohol is described e. g. by Becze and Rosenblatt (1943), Altsheller, Mollet, Brown, Stark and Smith (1947), Savčenko (1957); to acetone, butylalcohol, ethylalcohol by Lagodkin (1939); to protein and fat yeast by Šarkov (1950). Saeman, Locke, Dickerman (1946) mentioned several types of industrial continuous fermentation processes of wood hydrolysates for alcohol and yeast production. The system of three fermentors of different sizes and in various combinations was patented by Sak (1932). Šarkov (1950) described also a continuous alcoholic fermentation process in which he increased the yield of alcohol per fermented sugar by returning a part of the separated yeast cells into the process.

The principle of vertical cylindrical fermentor divided by variously adjusted partitions into several zones was employed by Victorero (1948) and by Owen (1948) for continuous alcoholic fermentation. The former used the up-flow of mash, the latter the down-flow when yeast cells accumulate on the upper plates.

Further examples of continuous alcoholic fermentation were given by Šarkov (1950), Maxon (1954) Gaden (1956), Katsume (1956) and Ueda (1956).

Simple laboratory apparatus were described by Saenko (1950) who tried to adapt wine yeast to a higher concentration of alcohol in an unreliably operating mechanism, and by Rudakov (1936), and Verbina (1955) who studied the adaptation of yeast to antiseptics.

Čekan (1939) prepared bread leavening in a continuous multistage apparatus in which two different alcoholic fermentation and lactic acid fermentation took place.

Gotass et al. (1951), Oswald et al. (1953) (Tamiya, 1957) used specially adapted sewage lagoons and oxidation ponds which they named "symbiocon". They used them for continuous cultivation of algae together with aerobic bacteria without any supply of atmospheric oxygen. The bacteria utilized the oxygen liberated by algae during photosynthesis and oxidized the waste organic material. The algae in their turn photosynthesized organic matter from the CO_2 and NH_3 produced by the bacteria.

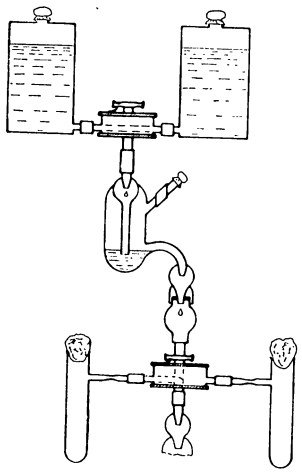

Fig. 1. Microgenerator; Utenkov (1941).

Also several bacteriological papers have appeared in which the possibility of maintaining constant culture conditions is discussed, but these same conditions are determined by the authors mostly empirically.

Utenkov, patented in 1922 (Utenkov, 1941) his microgenerator in which he studied two stages of development of microbes, the subcellular and the cellular one, and in his division theory he assumed even the existence of a sexual cycle. He presumed that each stage required special suitable conditions and arrived at the conclusion that the constancy of these specific conditions is ensured only by a flowing nutrient medium. In the course of later years Utenkov developed several types of microgenerators, one of which is shown in Fig. 1, as well as several culture techniques as a part of a broader method which he called "mikrogenerirovanie" (Utenkov, 1941, 1944). Felton and Dougherty (1924) constructed an auto-

matic transferring device in which they studied the conservation of the virulence and the influence of the pH on the virulence of one strain of *Pneumococcus* grown on skimmed milk. The new supply of food from the storage bottle was regulated by an electromagnetic clamp controlled by a time-relay so that active growth of organisms was made possible. The cell suspension flowed out of a U-shaped growth receptacle over on overflow siphon. Haddon (1928) (Novick, 1955) described a simple device for continuous culture of microorganisms.

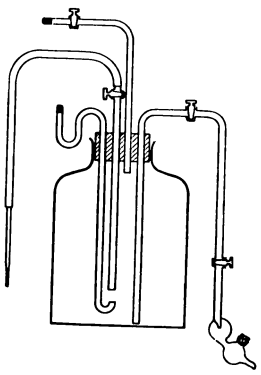

Fig. 2. Simple continuous culture apparatus; Málek (1943).

Rogers and Whittier (1930) proceeded from the analogy of a bacterial culture with a multicellular organism and in order to form analogous conditions they constructed a continuously operating apparatus in which they cultivated *E. coli* and *Str. lactis* either independently or in a mixed culture. The supply of nutrients was very slow so that the content of the culture flask was renewed once in 24—30 hours. They realized the importance of this method for industrial purposes and tried to produce lactic acid in open fermentors (Whittier and Rogers 1931).

The Rogers method was employed by Cleary, Beard and Clifton (1935) who limited growth by the concentration of various nutrients and tried to investigate the principle of the stationary phase at a very slow rate of flow of nutrients.

In the years 1934—1942 Málek (1955) formed a working hypothesis as to the indirect proportionality between the virulence and ease with which bacteria can propagate. He tried to solve this problem as well as that of the propagation, M-concentration and variability of bacteria using *E. coli* in a simple flow culture tube (Málek 1943) (Fig. 2).

Jordan and Jacobs (1944) proceeded from a criticism of papers concerning the M-concentration. Their starting point and comparison standard was the concept of static cultivation. Therefore they used a very slow rate of flow of substrate added automatically at a constant rate by means of a special pipette.

Barnes and Dewey (1947) described a simple laboratory device for continuous cultivation of *St. paradysenteriae*, and Duché and Neu (1950) for cultivation of *Dermatophyta*.

2. HOMOCONTINUOUS METHODS

The process takes place in a thoroughly stirred culture flask to which fresh substrate is continuously added at a fixed rate. In order to keep the volume of the culture constant the fermented medium flows out through an overflow together with an amount of propagated organisms. The medium composition is the same at all points of the culture space as well as in the overflow. Although a number of reactions take place here the system as a whole does not change; the fact that some reactions must have taken place is evident from the difference in the composition of the inflowing and outflowing medium. Under constant conditions of flow a complete stationary state of the culture is established which is both temporally and spatially invariant. The growth process can be compared to an autocatalytic reaction for which a thoroughly stirred and in certain cases also aerated flow fermentor is the most suitable one, a fact which is not sufficiently appreciated in cases when the fermentation liquid is not stirred sufficiently merely by passing air.

There are two types of culture methods enabling the achievement of a constant density of microorganisms.

a) Methods Based on the Principle of the Turbidistat

It is assumed that in a turbidistat microorganisms grow at their maximum growth rate while their density is kept at a certain selected value of a controlled flow rate.

The density of microorganisms in a turbidistat is observed directly in the culture flask by means of physical or physicochemical methods. Most widely used are photocells by means of which the intensity of light beam is measured after passing through the cell suspension. Sometimes also indirect measurement is employed, e. g. measuring the pH or concentration changes of some substance resulting from the changes in the number of microorganisms. The device used to measure the density automatically controls the rate of flow of the nutrient medium. The constancy of flow and the density depend on the sensitiveness of the detection and supply mechanism. If the organism is to grow at a maximal rate its density must be chosen within such limits that even a small change can be optically registered and at the same time that the nutrient capacity of the culture medium is not exceeded. The experimenter can change the density depending on external conditions, within certain limits, preserving at the same time the maximal growth rate which in a turbidistat is given by the chemical and physical quality of the medium.

Myers and Clark (1944) were among the first to employ the method of photoelectric control of density of unicellular algae. They studied the efficiency of inorganic and organic nutrient media for growth at a given rate of flow of the inflowing medium. They controlled the dilution of growing cultures by means of an overflow making it possible to change at will the volume of medium and thus also the dilution rate. Cook (1951) cultivated algae (*Chlorella pyrenoidosa*) on a laboratory scale in a tall glass cylinder, aerating the suspension by a mixture of air and CO_2, and studied the influence of intensity and time of illumination. He measured density in a side circuit through which part of the culture passed. The fresh medium supply was controlled automatically by a solenoid valve connected in the photoelectric circuit. He also proposed developing industrial production in long horizontal tanks into which the medium would enter through holes located at definite distances along the tank. Phillips and Myers (1954) described a small apparatus in which they measured the growth rate of unicellular algae as a function of intensity and itermittency of illumination under conditions of constant density of population and of constant volume. Krauss (1955) discussed the physical conditions and nutrition requirements in a large continuous production, Tamiya (1957) quoted in his report a number of further papers dealing with the propagation of a considerable amount of algae by means of this culture technique.

Bryson (1952, 1953) tried to obtain forms resistant to antibiotics in his turbidistatic selector. He measured the turbidity of the culture by means of a photoelectric system into which a device was also introduced which controlled automatically the supply of nutrient solution when the culture reached a certain constant turbidity. Also in this circuit a proportional-feed system was placed which made it possible to increase geometrically the concentration of the toxic substance in the medium in proportion to the propagation of cells. Graziosi (1956) described an automatically operating turbidistat in which he registered under certain conditions the rate of adaptation of *Micrococcus pyogenes* and *Proteus vulgaris* to the antibiotic novobiocin (Graziosi and Puntoni, 1957).

Northrop (1954) adapted his automatically operating turbidistat for use with a commercial-type photoelectric colorimeter in order to study the growth of a lysogenic strain of *B. megatherium*. Bacteria growing on the walls of the culture cell in which the measurements were carried out were removed by windshield wipers fixed to a stainless stirring rod which moved vertically, driven by a motor. In his turbidistat he registered changes of the growth rate under the influence of oxytetracycline and studied the rate of adaptation (Northrop, 1957a); he also endeavoured to treat mathematically the formation of resistant mutants (Northrop, 1957b).

Fox and Szilard (1955) constructed a turbidistatic cultivation device with a photoelectric system which they called a "breeder". They kept the density

of exponentially growing bacteria at a certain value by controlling the influx of fresh nutrient medium, and maintained the volume of the culture at a constant value by means of an overflow siphon. The operation of the breeder was controlled by a time-relay. Every 4 minutes stirring was stopped, the light source turned on, a density reading carried out, the light source turned off, stirring started again and the influx of the medium adjusted according to a pre-set density value. The whole operation did not last more than 30 seconds.

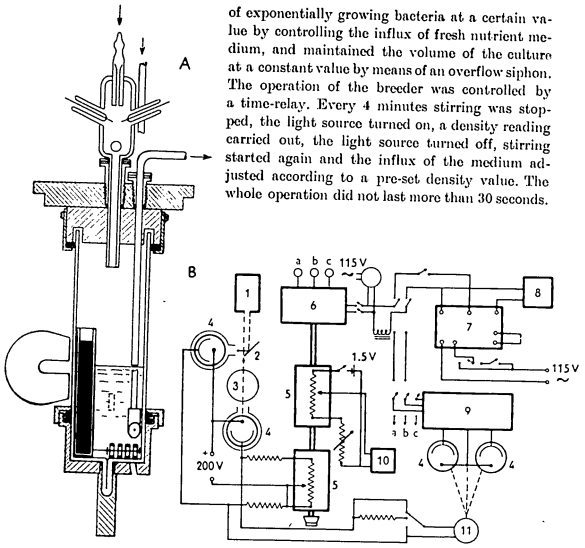

Fig. 3. Microbial auxanometer (diagrammatic); Anderson (1956).
A — Growth tube; B — Circuit of completo devicewith turbidity recording; 1 — light source; 2 — mirror; 3 — growth tube; 4 — phototube; 5 — helipot; 6 — balancing motor; 7 — relay; 8 — drop recorder; 9 — thyratron relay; 10 — turbidity recorder; 11 — galvanometer.

They broke up foam with a red-glowing platinum filament which was stretched across the flask above the surface of the liquid. Fox (1955) measured the rate of formation of mutations in a breeder and in a chemostat with two strains of *E. coli B* under a variety of steady state growth conditions.

Anderson (1953, 1956) described a turbidistatic device called a "microbial auxanometer" (Fig. 3) which, by means of a system of photocells and relays, registers automatically the course of the process and controls the process itself according to preselected values under turbidistatic and chemostatic

conditions. The inflow of the liquid into the culture flask is controlled automatically and registered by means of a magnetic valve and a drop counter. The culture flask rotates while the wipers, fixed magnetically in a certain position, clean the walls and stirr the culture.

b) Methods Based on the Principle of the Chemostat

In a chemostat the medium flows continuously at a given rate. The volume of the thoroughly stirred culture is kept constant by means of an overflow system. The growth of organisms is controlled by the concentration of substrate and each dilution or flow rate fixes the substrate concentration at a value required for the specific growth rate to be equal to the dilution rate. Under these conditions the specific growth rate is a function of the substrate concentration or rather of the concentration of an important nutrient which is often called the limiting or growth controlling factor. Of decisive importance are the quantitative chemical conditions from which the method derives its name. The chemostatic continuous cultivation of microorganisms makes it possible not only to select at will any growth rate with the density unchanged, but also to select different values of density under certain conditions.

Before the chemostatic conditions were mathematically analysed and the controlling influence of the concentration of the substrate on the growth of microorganisms was classified and stressed, the continuous processes were used in industry as well as sometimes in the laboratory rather with the aim of full utilization of fermentable sugars. It was the metabolic activity of the organism that was considered the leading component of the process of fermentation. The mutual dependence of the rate of growth, rate of flow and concentration of the substrate was not understood to its full extent. For this reason the production processes, even though appreciating the advantages of continuous processes, were based on empiric experience. The first traces concerning fermentation of this type appeared in the fermentation industry in the process of production of ethanol and yeast cells from various substrates containing saccharides. A minor part of the work, mainly of an experimental character, was concerned with the possibilities of full development of the dynamics of multiplication under continuous flow conditions and utilized or followed the influence of a purposefully chosen limiting factor on the growth or some other physiological function.

Hayduck (1923) patented a method of yeast cultivation with aeration and at a slow rate of flow of concentrated molasses in order to utilize also the produced alcohol. Imray (1928, 1929) directed the rate of inflow and outflow of wort according to the sugar exhaustion. The regulation was performed by means of lowering the inflow or by returning the separated yeast. He carried out aerated fermentations both in the presence and in the absence of alcohol.

Bührig (1929) produced yeast by the continuous-addition-withdrawal process. The diluted substrate flowed into the suspension of yeast cells and was withdrawn at an approximately identical rate into a reserve vessel where the ripening was completed. Both vessels were aerated. Harrison (1930) used three cylinders inserted into one another for the production of baker's yeast. The medium was continuously added to the central cylinder from which it flowed down through a number of overflow openings into the adjacent annulus with a lower level and then identically into the second annulus with the smallest volume of culture. All three stages were aerated thoroughly. Olsen (1930) used a series of mutually connected fermentors in which he kept a constant rate of flow. If the nutrients were not utilized in one fermentor he added another one. Bilford, Scalf, Stark and Kolachov (1942) used a continuous fermentation process for the production of ethylalcohol; this took place in one fermentor stirred by CO_2 in the course of a 5—7 hour cycle. Seidel (1943) patented two procedures for the production of yeasts and other microorganisms. In one case he used three intensively aerated wide fermentation vats arranged in a cascade. Yeasts separated from the last vat were returned to the first one. In the second case he divided concentrically a wide cylindrical vessel into a number of round compartments linked together alternately by overflows and openings near the bottom. The medium added continuously or periodically into the non-aerated centre, flowed through the openings at the bottom into the neighbouring compartment whence it was carried by air to the overflow and into the next nonaerated compartment from which the yeast suspension was withdrawn for further treatment. Unger, Stark, Scalf and Kolachov (1942) described a continuous aerobic propagation process of alcohol yeasts which were used only after separation as a starter in a proper alcoholic fermentation. Ruf, Stark, Smith and Allen (1948) were able to ferment both in the laboratory and in the pilot-plant acid-hydrolyzed grain mash in the course of a 12 hour cycle.

At that time a number of papers appeared studying fodder yeast production on molasses and on sulphite liquors, as shown by Lee (1949) in his general report. Harris, Hannan, Marquardt and Bubl (1948) cultivated *T. utilis* on wood hydrolyzates using a battery of interconnected fermentors. Later Harris, Saeman, Marquardt, Hannan and Rogers (1948) and Harris, Hannan and Marquardt (1948) used a fermentor of the Waldhof type in which they studied the problem of supplementation with inorganic salts when cultivating *T. utilis* on non-fermentable sugars remaining after the alcoholic fermentation of wood hydrolyzates.

Stier, Scalf and Brockman (1950) were able to obtain yeasts with constant fermentation properties after having grown them continuously under anaerobic conditions in their glass apparatus. They stirred the culture by means of passing nitrogen through it. Adams and Hungate (1950) tried to calculate the

characteristics for a yeast flow culture using the growth curve and the curve of sugar decrease obtained in a batch culture. Maxon and Johnson (1953) discussed the problems of oxygen transport and of material balance in a continuous fermentation process in one fermentor. A device measuring the pH of the outflowing liquid controlled automatically the addition of ammonia to the culture. Owen and Johnson (1955) designed a continuous shaker propagator for yeasts and bacteria. Schulze (1956) studied the influence of various concentrations of ammonium dihydrogen phosphate on the growth rate of yeasts cultivated continuously on sulhpite liquors from beechwood. Málek, Burger, Hejmová, Řičica, Fencl and Beran (1957) used a multi-stage apparatus to study hexose and pentose utilization by *T. utilis* and *Monilia murmanica* growing on non-diluted sulphite waste liquors. The organisms became adapted during the flow culture to the toxic substances contained in the liquor but no adaptation could be observed to those sugars to which the organisms had not been adapted previously. Borzani and Aquarone (1957) fermented continuously blackstrap molasses to ethylalcohol on a semi-production scale and studied the influence of the sugar concentration, feed rate, agitation speed and fermentor capacity. The application of penicillin to suppress contamination had a beneficient influence on the course of fermentation.

In the field of industrial production of antibiotics it was Donowick (1952) (Perlman et al., 1953) who tried to produce streptomycin in the continuous fermentation way, and Kolachov and Schneider (1952) who tried it with penicillin. Liebmann and Becze (1950) patented a method for the production of antibiotics and especially of penicillin; they start to add substrate at the time of maximum multiplication. The overflowing liquid was collected in another vessel where the final phase of fermentation took place.

Nowrey and Finn (1955) (Beesch, Shull, 1956) studied the continuous fermentation production of acetone and butylalcohol by *Cl. acetobutylicum* keeping a very low concentration of cells in the propagator. But a practically applicable continuous fermentation method for these products has not been discovered as yet. Protiva and Dyr (1958) and Dyr and Protiva (1958) used a glass apparatus with several flasks arranged in a cascade for the same purpose.

Ketchum and Redfield (1938) cultivated the marine diatom *Nitzschia closterium* under chemostatic conditions. The rate of growth of this photosynthetic organism changed according to the intensity of light and to the concentration of CO_2. Browning and Lockinger (1953) and Vávra (1958) constructed a simple apparatus for cultivation of the infusorium *Tetrahymena gelei* under stabilized chemical and physical conditions; Vávra also studied the morphology of *Euglena gracilis* (Klebs).

Most experimental and theoreitcal papers dealing with stabilized conditions under continuous flow of the medium have appeared in bacteriology, but only a small number have been treated from the point of view of practical application. The greatest attention has been given to genetic and growth problems and only recently has the study of metabolite production and of various enzymatic systems stepped into the foreground.

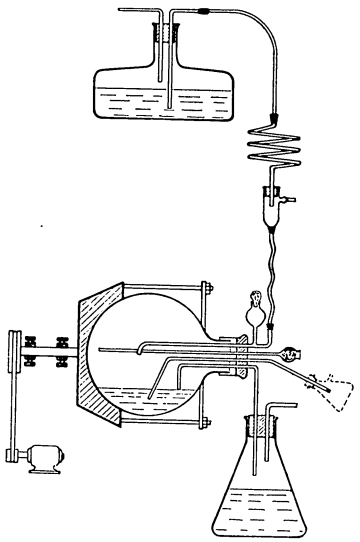

Fig. 4. Bactogen; Monod (1950).

Gerhardt (1946) designed a laboratory apparatus in which the addition of fresh medium and the withdrawal of the product were accomplished continuously and simultaneously. He used an aerated culture of *Brucella suis* at various generation times without selecting the limiting growth factor.

Málek and his co-workers used a cultivating device consisting of several aerated growth vessels arranged in a cascade connected by overflows. They

studied the problem of growth cycles of bacteria, particularly of *Azotobacter* (Málek 1952a, b; Macura, Kotková 1953), of the resistance (Málek, Vosyková, Wolf 1953) and the vegetative forms and spore formation of bacilli (Ševčík 1952; Málek, Chaloupka, Vosyková, Vinter, Wolf, Hlavatá 1953). Málek (1955) compiled his and his co-workers' results and laid the main stress on the importance of studying metabolism and growth conditions from the point of view of the optimal physiological state of the cultivated microorganism.

Monod (1950) studied the growth principles of bacteria and the role of the concentration of important substrate on the rate of growth. By his mathematical treatment of the principal steady-state factors a basis for the theory of continuous flow cultures was laid. He called his apparatus "Le Bactogène" (Fig. 4). The culture flask rotates along its longitudinal axis inclined by 3—4°. In the rotating flask the liquid spreads on the flask walls and thus the culture is stirred and aerated. The same principle was employed by Hofsten, Hofsten and Fries (1953) for cultivation of the *Ophiostoma multiannulatum* mold; they controlled the rate of growth by supplying new nutrients. Cohn and Torriani (1953) studied the kinetics of the formation of a certain protein which is related to beta-galactosidase while cultivating *E. coli* on maltose, lactose and galactose in a synthetic medium. Perret (1953) added small doses of penicillin to a culture of *B. cereus* growing in steady state at a constant density and measured the subsequent changes in the kinetics of penicillinase formation. He used the disappearance of ^{32}P to measure exactly the rate of dilution. Hedén, Holme and Malmgren (1955) modified somewhat Monod's bactogen and used it to study the possible relationship between the growth rate and nucleic acid content in *E. coli*. Rogers (1957) studied the suppression of hyaluronidase formation in *St. aurenus* cultivated in the bactogen. Perret (1957) described an autoregulating apparatus for continuous cultivation applying the rotating flask principle; its axis was inclined at an angle of 60° with the horizontal. He described the function of an auxiliary device which kept the rate of flow of medium and of air constant. In the apparatus described by Anderson (1953, 1956) a cylindrical vessel rotates along the vertical axis, the culture being stirred by magnetically fixed wipers. He equipped his apparatus with a very ingenious photoelectric attachment which automatically registers and regulates the cultivation process.

At the same time as Monod, Novick and Szilard (1950a, b) described their Chemostat (Fig. 5) and endeavoured to analyse mathematically the steady state conditions of culture. They treated systematically the influence of the concentration of various limiting factors, particularly of purines, on the rate of formation of mutants of *E. coli* resistant to phage in relation to the absolute time and presented a theoretical treatment of this process (Novick and Szilard 1950b, 1951, 1952, 1953, 1954). Using a chemostat Lee (1953) found that the rate of formation of resistant mutants is independent of the rate of growth.

The limiting factors were tryptophane and theophylline, which, under certain conditions, differed in their influence on the rate of formation of resistance toward phages T_s and T_e. Labrum (1953) (Novick, 1955) studied the relationship between the generation time and the time necessary for expression of induced mutations. Various chemostats for use in continuous culture of bacteria were described by Kubitschek (1954), Haan and Winkler (1955), and Rotman (1955). The sonic lysis of cells of *Azotobacter vinelandii* and of *E. coli* grown

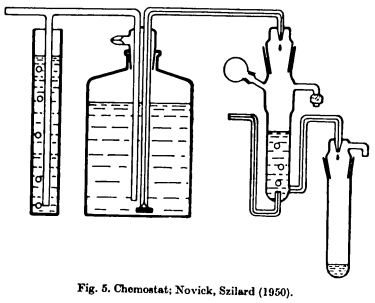

Fig. 5. Chemostat; Novick, Szilard (1950).

on synthetic media in the chemostat was studied by Rotman (1956), Formal, Baron and Spilman (1956) studied the influence of continuous culture on virulence and immunogenicity with mice of two strains of *Salmonella typhosa*. Karush, Iacocca and Harris (1956) described an apparatus which they used to keep a culture of haemolytic streptococcus of group A in a steady state at different rates of growth. They studied the influence of the pH, glucose and tryptophane as factors limiting growth. Holme (1957) carried out continuous cultivation of *E. coli* in an Erlenmeyer flask placed in a rotation shaker. He studied the relationship between the rates of synthesis of nitrogen-containing components and of no-nitrogen compounds such as glycogen, using the source of nitrogen as the limiting factor.

A technically acceptable and modernly equipped apparatus of larger type for use in bacterial culture was described by Elsworth and Meakin (1954) and Herbert, Elsworth and Telling (1956). A very advanced laboratory fermentor together with a reliably and constantly operating auxiliary equipment was constructed by Elsworth, Meakin, Pirt and Capell (1956) (Fig. 6). Their appara-

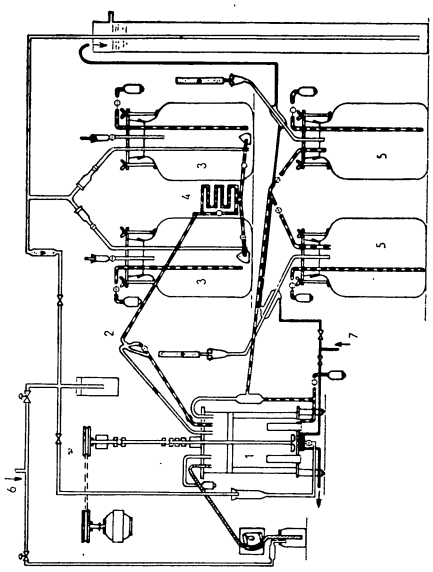

Fig. 6. Continuous culture apparatus (diagrammatic); Elsworth et al. (1956).
1 — culture vessel; 2 — medium line; 3 — supply reservoir; 4 — capillary resistance; 5 — harvesting bottle; 6 — air line; 7 — water line.

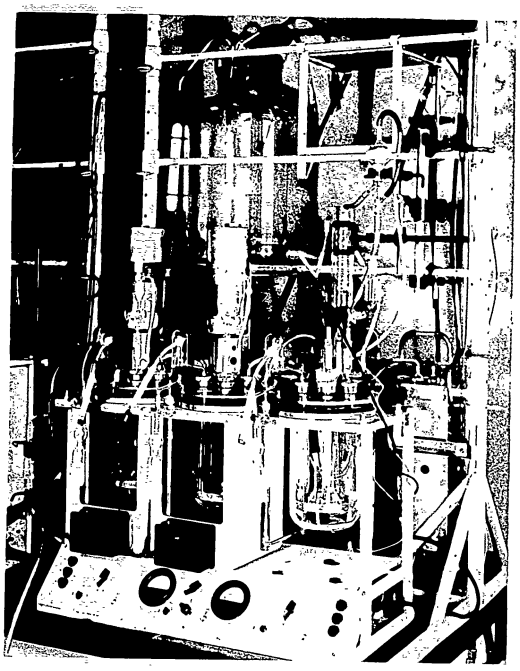

Fig. 7. Two litre scale continuous culture apparatus.
(Photo J. Fiala.)

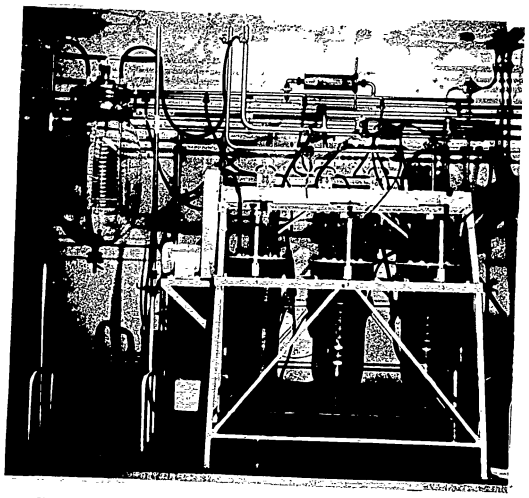

Fig. 8a. Twenty litre scale continuous culture apparatus. Preparation of inoculum.
(Photo Ing. J Hospodka)

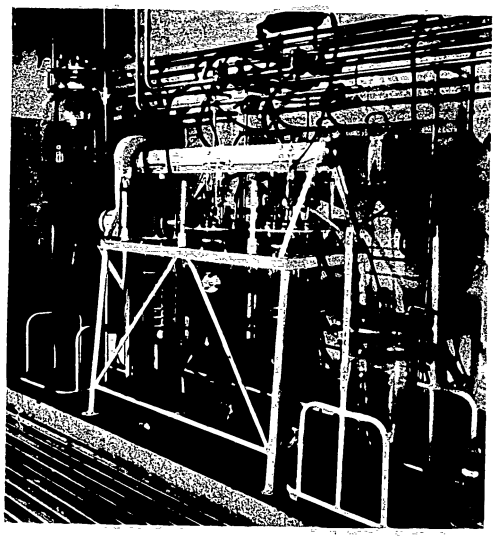

Fig. 8b. Twenty litre scale continuous culture apparatus Two stage baker's yeast continuous culture with unequal volume of liquid.
(Photo Ing J Hospodka)

tus was then equipped with an automatic pH registration and regulation device designed by Callow and Pirt (1956) who also developed a method for chemical sterilization of glass electrodes.

Fig. 7 shows a laboratory fermentation apparatus which can be used for three simultaneous batch fermentations or for three simultaneous continuous processes under indentical or different conditions. As fermentors are fixed on a common carrier it is possible to connect them in series for some special purposes (e. g. mixed fermentations). According to the rate of reaction taking place in the individual vessels the rate of dilution can be adjusted by changing the volume of culture. The feed rate of the substrate can be regulated by a vertical movement of the outflow end of the thermostatic resistance capillary tube with respect to the constant level in Mariotte's bottle. The amount of liquid passing through can be measured either by a flowmeter, by the number of drops, or by reading off the difference in the graduated storage bottle level. Both the liquid and the air are thoroughly mixed by a centrifugal glass stirrer (Ričica, Grünwald 1954). Any registration or regulation device can be connected with this apparatus.

Fig. 8 a and b show a glass flow apparatus with a working capacity of 20 litres which was used for continuous cultivation of *S. cerevisiae* on molasses. At first the inoculum was prepared by a batch process in the first fermentor. The continuous process took place in two other fermentors the second of which had a smaller operating capacity so that the period of delay corresponded to the time of complete utilization of the formed alcohol. The complex of problems presented by this process as well as the qualitative properties of baker's yeast prepared in this way are discussed by Beran in another report in this symposium.

AUXILIARY DEVICES

The device by means of which the substrate solution is continuously added to the culture flask must be simple, reliable and constant in operation as well as easily manageable. It must be easy to sterilize and keep sterile while operating. It should be made from corrosion-proof material.

Most authors use the principle of Mariotte's bottle. The rate of feed is regulated by a screw clamp, by an electromagnetic valve, or in most chemostatic cultivations by a thermstatic resistance capillary tube. The limiting factor is either the gas pressure above the level in the stock bottle or the difference between the level and the outflow end of the capillary tube. Elsworth et al. (1956) and Perret (1957) have recently described a very good apparatus of this type. Lundsted, Ash and Koslin (1950), Michaeli (1951), Maude (1952) and others used the principle of a rod or plunger being inserted into the tube in

order to regulate the flow. Geankoplis and Hixson (1952) constructed a glass needle valve used for adding small amounts of liquids under pressure. Jordan and Jacobs (1944) added the fresh nutrient medium by means of an automatic special pipette. Rosenberg (1956) added substrate to a microculture by drawing it in by means of a filter paper strip. Stier et al. (1950). displaced the liquid from the stock bottle by means of nitrogen. Soltero and Johnson (1954) used the gas evolved by electrolysis of water, the amount of gas being regulated by the intensity of the current.

Browning and Lockinger (1953) transferred small amounts of liquid into the cultivation flask by means of a small rotating motor-driven glass dipper. But its rotating parts could be kept sterile only with difficulty. The constant speed cam principle was employed by Andreev (another report in this symposium) and by Main, Cole, Bryant and Morris (1957); by raising and lowering the dosing flask the cam regulates the rate of flow. Sims and Jordan (1942), Savage and Florey (1950), Dale, Amsz, Ping Shu, Peppler and Rudert (1953), Karush et al. (1956), Formal et al. (1956) used variously adjusted piston pumps mostly adapted from syringes. As with most piston and membrane pumps the weak point is represented by valves and by the interrupted flow of the liquid a number of authors preferred pumps by means of which they could add continuously solutions even of suspension character. The rotary rubber tubing pumps proved to be the best ones (Weigl and Stallings 1950; Apolcin 1953; Maxon and Johnson 1953; Hosler and Johnson 1953; Málek 1955; and others); also the keyboard and hose pressure Sigmamotor pumps (Holme, 1957) proved to be very reliable and exact.

The volume of passing liquid is usually measured in graduated vessels, flowmeters (Herbert et al., 1956), or by automatic drop counters (Anderson, 1953, 1956). Catheron and Hainsworth (1956) presented a review of flow measurement methods mentioning a diaphragm with an electric differential transfer system to the electric registration device, a diaphragm with a pneumatic differential transfer system to the pneumatic registration device, a magnetic flowmeter with electronic registration, a controlled regulation valve with electronic registration etc. The rate of flow as measured by radioactive indicators was studied, for instance, by James (1951), who designed an induction flowmeter, and Perret (1953). In his review of various dosing mechanisms, particularly those for dosing of liquids, Henke (1955) considered also the problem of corrosion. Fuld and Dunn (1957) described an apparatus for continuous refraction index measurement by means of which they control sugar concentration during yeast propagation and simultaneously automatically control the pH, temperature and the anti-foaming agent. Bartholomew and Kozlov (1957) designed an apparatus for automatic control of the addition of the anti-foaming agent and of the nutrient medium to the battery of fermentors.

One of the most important problems of continuous fermentations is presented

by continuous preparation and sterilization of the substrate. A solution to this problem was sought, for instance, by Unger et al. (1942), Gallagher et al. (1942), Stark et al. (1943), Pfeifer and Vojnovich (1952), Whitmarsh (1954), Tomíšek (1956) as well as by others.

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AN IMPROVED CELL FOR MAINTAINING BACTERIAL CULTURES IN THE STEADY STATE

JOHN H. NORTHROP

If bacteria are grown in a constant volume of culture medium, the components of the medium, the growth rate, and the physiological state of the cells are constantly changing.

If the concentration of bacteria is kept constant, on the other hand, the composition of the culture medium and the growth rate and physiological state of the bacteria are also constant.

The bacteria may be kept at constant concentration by diluting the culture at a constant rate which is less than the maximum growth rate of the bacteria in that culture medium. Under these conditions, the concentration of bacteria will increase, and the growth rate decrease, until the latter exactly equals the dilution rate. The system is now in stable equilibrium. This principle has been used by Monod (1950) and Novick and Szilard (1950), Novick (1955), and Perret (1957) to maintain the culture in continuous growth. It has the advantage of experimental simplicity.

It has the disadvantage that it is not possible to maintain a culture at its maximum growth rate, since this would be an unstable equilibrium. In order to maintain the culture at its maximum growth rate, it is necessary to control the rate of flow of the culture medium in such a way that the cell concentration remains constant, no matter what the growth rate. Several types of apparatus for this purpose have been devised (Felton and Dougherty, 1924; Myers and Clark, 1944; Bryson, 1952; Anderson, 1953; Northrop, 1954). This method is more complicated experimentally, and is restricted to a range of cell concentration which may be determined by optical methods. It has an advantage in that it is possible to make automatic records of growth rate with considerable accuracy for long periods of time.

Under these conditions the growth of the organism in the cell is exactly compensated for by the addition of more culture medium so that

$$K = \underset{\text{growth rate}}{\frac{\mathrm{d}B}{\mathrm{d}t\,B_c}} = \underset{\text{dilution rate}}{\frac{\mathrm{d}V}{\mathrm{d}t\,V_c}} = W$$

$\mathrm{d}B$ is the number of organisms washed out of the cell in unit time, B_c is the number of organisms in the cell (a constant), V_c is the volume of the cell and $\mathrm{d}V$ is the overflow from the cell in unit time.

On integration

$$K = \frac{B}{B_c t} = \frac{V}{V_c t}$$

since when $t = 0$, $B = 0$ and $V = 0$.

The value of V may be determined by measuring the volume of culture medium which flows through the cell or calculated from the kymograph record which shows the fraction of the time during which the culture medium flowed into the cell. In this case

$$K = \frac{\text{ml. hr.}^{-1}}{V_c} \times \frac{\text{time of flow}}{\text{elapsed time}}$$

where ml. hr.$^{-1}$ is the ml. of culture medium which will flow per hour without interruption. If the drops per ml. of the culture medium from the capillary tip is known,

$$K = \frac{\text{drops culture medium min.}^{-1}}{\text{drops culture medium ml.}^{-1}} \times \frac{60}{V_c} \times \frac{\text{time of flow}}{\text{elapsed time}}$$

It may be noted that under these conditions the increase in bacteria (assuming that growth occurs only in the cell) is arithmetic, instead of logarithmic, as under usual conditions, since the increase in bacteria is removed as fast as it is formed. If this were not the case, the growth rate could not be balanced by an arithmetic dilution rate.

The time for the number of bacteria to double, therefore, is equal to the generation time 1/K, instead of $\frac{\ln 2}{K}$ as in growth at constant volume.

In case the culture contains 2 organisms growing at different rates, the ratio of the 2 is

$$\frac{M}{W} = \frac{M_0\, \mathrm{e}^{t(K_m - K_w)}}{W_0}$$

where $\frac{M_0}{W_0}$ is the ratio of the 2 cells at $t = 0$ and $\frac{M}{W}$ is the ratio at time t. K_m and K_w are the respective growth rate constants. The time required to reach various values of $\frac{M}{W}$ is (Northrop, 1954)

$$t = \frac{2{\cdot}3\left(\log \frac{M}{W} - \log \frac{M_0}{W_0}\right)}{K_m - K_w}$$

EXPERIMENTAL

The principal experimental difficulties are foaming and sticking of the organisms to the walls of the cell. For practical purposes this may be an advantage, as in the quick vinegar process, or the continuous acetone-ethyl alcohol fermentation (Northrop, Ashe, and Morgan, 1919). For theoretical purposes, however, it is necessary that the concentration of organisms in the overflow is the same as that in the cell, since this is assumed in deriving the equations. If the cell contents foam, the foam contains fewer bacteria per ml. than the bulk of the suspension (Northrop and Murphy, 1956). If the organisms stick to the walls, they interfere with any optical system and also cause an error in the growth rate determination. If these organisms differe qualitatively from those in suspension, analysis of the overflow may lead to entirely erroneous conclusions.

The cell shown in Fig. 1 has been found to overcome

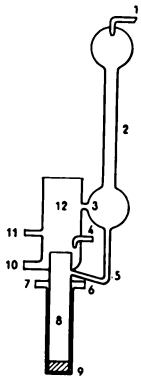

Fig. 1. Cell for maintaining bacterial cultures in the steady state. 1 — Culture medium inlet, 3 mm I. D. with fine tip; 2 — Tube, 10 mm I. D.; 3 — About 2 mm I. D. 4 — Lactic acid inlet, 3 mm I. D.; 5 — Tube, 3 mm I. D.; 6 — Water outlet, 5 mm I. D.; 7 — Water inlet, 5 mm I. D.; 8 — Vessel, 22 mm O. D., 85 mm high (up to the water outlet); 9 — Black tape, 15 mm; 10 — Overflow, 5 mm I. D.; 11 — Air, 3 mm I. D.; 12 — Vessel, 35 mm O. D.

both of these difficulties. The air does not bubble through the suspension so that there is little foam. The solution is kept saturated with air and the cell walls are kept clean by the rapid motion of the wipers.

The wipers are made from Lusteroid test tubes and are transparent so that they do not interfere seriously with the optical system. A 10 per cent solution of lactic acid, containing formaldehyde or any suitable disinfectant, is dropped continuously (except when a sample is taken) into the collar of the cell where it mixes with the overflow from the cell. This keeps the overflow clean and sterile, and prevents contaminating organisms from growing back up the overflow tube.

The rate of flow of the culture medium is controlled by a galvanometer connected to the photo-electric colorimeter in which the cell is placed. The galvanometer operates a photoelectric realy which in turn activates a solenoid controlling the flow of culture medium.

In case the growth in a large vessel is to be controlled, the overflow from the tank is allowed to flow through the cell. The flow of culture medium into the tank is then controlled in the same way as that of the cell.

In case pathogenic organisms are grown, the cell may be put under slight negative pressure, instead of positive. The rubber stopper and stirrer guard tube may be enclosed in a cylinder filled with strong disinfectant.

CONSTRUCTION OF THE APPARATUS

APPARATUS REQUIRED

Cell and wipers as in Fig. 1.
Klett-Summerson Industrial Colorimeter, Model 900.3.
Worner Photo-electric Relay, Model 5-100R.
Galvanometer, Leeds and Northrup, No. 2420, Coil P. I. 102 B. S.
Potter and Brumfield Relay, 6V-50-60 C (For kymograph pen).
General Electric Solenoid. CR9503-209C modified as in Fig. 2.
Westinghouse double contact projection lamp, 100W-PH/100T8/108.
Bodine speed reducer motor, NSE-12RH, 115 AC-DC, 1.1 amps., int. duty, 5000 RPM, 1/18 HP, with 10 : 1 reducing gear, with eccentric crank having about 1 cm stroke.
100 Ω resistance.
100 Ω var. resistance.
40 Ω var. resistance.
200 Ω var. resistance.
Bird kymograph (No. 70—060) with pen attached so as to drop 5 cm every revolution of the drum.
6 cm UF Pyrex filter.

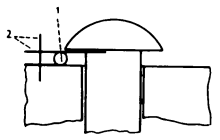

Fig. 2. Control of flow of culture medium by General Electric solenoid. 1 — Culture medium; 2 — Pins.

The Cell Wipers

A 15 × 110 mm lusteroid test tube is cut open lengthwise and the closed end cut off. It is cemented to a No. 16 stainless steel wire by means of a solution of lusteroid in acetone. The tube is cut about every 1·5 cm as shown in Fig. 3.

Electrical Connections

The wiring diagrams are shown in Fig. 4. It is advisable to have a constant voltage transformer in the lamp circuit, since the sensitivity varies somewhat with the light intensity.

The wires from the photo cells in the Klett-Summerson colorimeter are disconnected from the colorimeter galvanometer and connected to the Leeds and Northrup galvanometer. A 40 ohm variable resistance is put in parallel with the galvanometer.

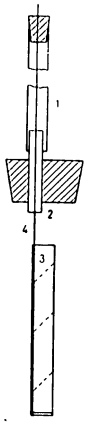

Fig. 3. Wiper for culture cell.
1 — Rubber tubing, 5 mm I. D.; 20 cm long; 2 — Glass tube; 3 — Lusteroid with cuts; 4 — 16 Stainless steel wire.

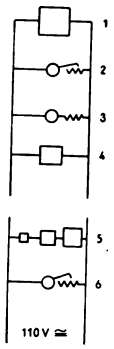

Fig. 4. Wiring diagram.
1 — 110 V constant voltage transformer; 2 — Colorimeter lamp and 100 Ω variable resistance; 3 — Galvanometer lamp and 100 Ω resistance; 4 — Photo-electric relay — operating circuit; 5 — Photo-electric relay circuit, solenoid, kymograph; per magnet; 6 — Wiper motor and 200 Ω variable resistance.

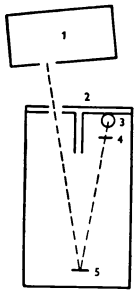

Fig. 5. Galvanometer light and photo-relay.
1 — Photo-electric relay; 2 — Screen; 3 — Lamp; 4 — Lens; 5 — Galvanometer Mirror.

The 100 W projection lamp is put in the galvanometer case in place of the usual lamp. 100 ohms resistance is put in series with the lamp. A screen is put around the lamp so that light from the lamp cannot strike the photo-cell of the relay (cf. Fig. 5).

The galvanometer, photo-relay, and light are adjusted so that, when the galvanometer is at rest, the image of the galvanometer lamp filament is projected through the opening in the screen and on the photo-relay tube so as to operate the relay.

Rubber to Glass Connections

Coat glass with rubber cement, wet rubber tubing with acetone and slip over glass.

Connection between Culture Medium Tube and Intake Tube of Cell

Cf. Fig. 6.

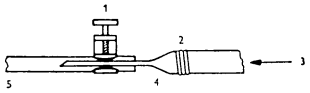

Fig. 6. Method of connecting tube from culture medium stock bottle to intake tube of cell. The connection is immersed in 50 per cent alcohol.
1 — Clamp; 2 — Wire binding; 3 — Culture medium; 4 — 21 Hypodermic needle; 5 — Rubber tubing, 3 mm I. D.

Operating Directions

Connect glass air filter and culture medium inlet tube to cell. A fine glass capillary tube to regulate the flow of culture medium is put in the culture medium intake line. The air intake tube is closed with a spring clip to prevent the filter from becoming wet when the cell is autoclaved. The lip of the cell, in which the rubber stopper will be inserted, is coated with rubber cement, and the cell and connections autoclaved for 1/2 hour. The cell stopper and wipers are inverted and immersed in a cylinder containing 5 per cent formaldehyde for 24 hours. The stopper and wipers are then inserted in the cell, and fastened in place with strong rubber bands. The apparatus is then assembled as in Fig. 7.

The culture medium is allowed to flow, and the motor regulated so as to operate the wipers as rapidly as possible, without foam. This is usually 200 to 500 strokes per minute. If this is not sufficient to prevent organisms sticking to the glass, a commutator may be put in the kymograph axle which will speed the motor up to 700 to 800 strokes per minute for 5 to 10 seconds every half hour.

The colorimeter is now adjusted so that the relay operates when the colorimeter dial reads in the range of 0 to .400, and the sensitivity of the system regulated by means of the intensity of the colorimeter lamp and the galvanometer damping resistance so that the relay operates in a range of about ± 5 divisions on the colorimeter scale. If the system is too sensitive, the time during

which the culture medium flows will be too short to read from the kymograph record. If it is not sensitive enough, the bacteria concentration will vary too much during the cycle.

The culture medium is allowed to flow for several hours, until all the formaldehyde has been washed out of the cell. The cell is then inoculated by injecting a few milliliters of culture through the rubber guard tube of the wiper, by means of a hypodermic syringe. The flow of culture medium is now stopped and the organisms allowed to grow up in the cell until the concentration is reached at which the culture is to be maintained. The colorimeter dial is adjusted so that the relay operates at this point. The system should now operate automatically so as to maintain the bacterial concentration at this level.

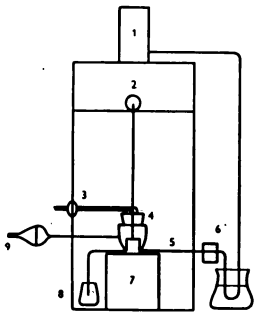

Fig. 7. Diagrammatic flow sheet.
1 — Culture medium; 2 — Motor; 3 — Clamp; 4 — Cell; 5 — Capillary; 6 — Solenoid; 7 — Colorimeter; 8 — Overflow; 9 — Air in.

In case a kymograph record is made*), it is necessary to adjust the sensitivity of the galvanometer, the flow rate of the culture medium, and the speed of the kymograph so that the time interval during which the culture medium flows into the cell may be accurately read from the record. The best condition is when the culture medium flows into the cell about half the time. This arrangement allows for quite wise changes in the growth rate. The faster the growth rate, the better the automatic control, since the optical density changes rapidly. Very slow growth rates $< 0{\cdot}1$ hr.$^{-1}$, cannot be controlled accurately since the change in optical density, and hence in current through the galvanometer is too slow for satisfactory operation.

EXPERIMENTAL RESULTS

The results of experiments in which the growth rate of *B. megatherium* cultures has been determined under various conditions have been reported previously (Northrop, 1954; Northrop and Murphy, 1956; Northrop, 1957).

*) The kymograph record may be made more regular by inserting a 5 minute time delay relay in the circuit which regulates the kymograph pen and the flow of culture medium.

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A STUDY OF THE METABOLISM OF MYCOBACTERIA IN A FLOW SYSTEM

J. Kuška

In the static culture of microbes the results are frequently influenced and even often distorted by a number of factors. Thus with this type of cultivation, for example, the effect of the progressive exhaustion of the nutrients in the medium, the accumulation of various metabolites during the growth phase and their action on the cultivated microbes all play a role. Similarly in the study of the sensitivity of different strains to various antibiotics or antituberculotics as in following the influence of various chemical substances the culture is also exposed to the effect of their metabolic products.

We can eliminate the influence of these factors by means of culture in a flowing media. This method of cultivation makes it possible to study the dynamics of the growth and metabolism of the bacterial cultures being studied under constant conditions of the culture medium. The problem of flow culture in microbiology has been most thoroughly worked out in industrial microbiology where, by using aeration and mixing under constant optimum conditions, the maximum metabolic activity of the culture is achieved.

A number of instruments for flow culture are described in literature. They are constructed for submerged cultures and their purpose is to attain the maximum multiplication of the culture or the maximum concentration of a certain metabolite with complete utilization of the culture medium.

We have endeavoured in our laboratory to set up a simple apparatus for the continuous surface culture of the *Mycobacterium tuberculosis* group which would make it possible to establish approximately such experimental conditions as would in their dynamics duplicate the dynamics of the processes in the organism.

The culture apparatus consists of a supply vessel containing the culture medium, the culture vessel (Fig. 1) and the receiver for collecting waste products.

To prevent the flushing of microbes from the apparatus the cap (3) is placed on the outlet tube (4), on which it is maintained in vertical position by bending the walls of the cap inward at two points. The nutrient medium drips from the drop counter into the tube which extends to the bottom of the culture flask, thus permitting us non-turbulent addition of nutrient medium to be attained. In this way complete diffusion of the newly added medium is ensured without disturbing the level of the medium in the vessel. Experimentally it has been ascertained that for a complete exchange of the nutrient medium in a vessel of 500 ccm volume it is necessary to feed one litre of fresh medium per 24 hours.

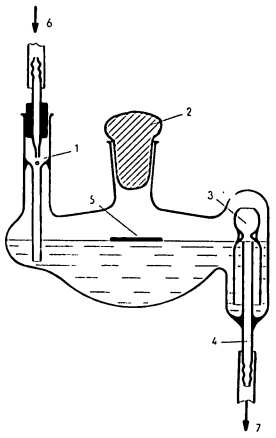

Fig. 1. Growth vessel.
1 — Drop counter; 2 — Cotton plug; 3 — Protecting cap; 4 — Overflow tube; 5 — Inoculum; 6 — Medium supply from reservoir; 7 — Overflow to waste storage bottle.

In this apparatus we followed the influence of fresh and auto-oxidised forms of l — ascorbic acid on $H_{37}Rv$ strains freshly isolated from lupus, BCG and *Mycobacterium smegmatis* on the surface film of these strains on Sauton's medium. The rate of exchange of medium was one litre per day, the concentration of vitamin C being 4 mg/ccm. Vitamin C was freshly added daily to the medium in the form of Celaskon (for injection). The auto-oxidised product was obtained by storing the medium with the same concentration of vitamin C in an incubator at 28 °C for a period of six days. Subcultures were cultivated for eight days on Sauton's media. For each experiment three preparations from the same subculture were simultaneously inoculated with a bacterial film, one with fresh vitamin C, the second with the auto-oxidised form, and the third without vitamin C. The cultures were then incubated for a period of three weeks. Only *Mycobacterium smegmatis* was cultured for four days. The effect of both forms of vitamin C was followed by weighing the increment of the bacterial mass. The decomposition of the vitamin C was followed by determin-

ing the decrease of its reducing activity in Sauton's medium and in a physiological solution by titration with 2,6-dichlorophenolindophenol and the decrease of the formation of hydrazones with 2,4-dinitrophenylhydrazine.

Both the fresh and auto-oxidised forms of vitamin C have an inhibiting action on the growth of these strains. With the strain from lupus and $H_{37}Rv$ the inhibition by both forms of vitamin C is, on the whole, the same, and in this series

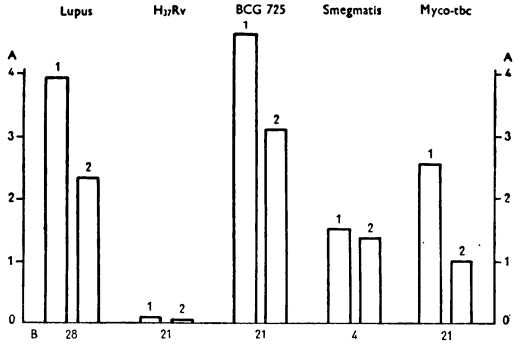

Fig. 2. Effect of medium exchange on growth of *Mycobacteria*.
A — Grams; B — Time of cultivation (days). 1 — Continuous flow culture; 2 — Static culture.

of experiments the increment of the bacterial mass is decreased by approximately half in comparison with the control experiments without vitamin C. With the BCG strain the auto-oxidised form of vitamin C inhibits to a greater extent. Neither constituent has an effect on the growth of the *Mycobacterium smegmatis* group.

The actual effect of the exchange of the nutrient medium (Fig. 2) on the growth of the lupus, $H_{37}Rv$ and BCG strains was favourable and with all strains increased the yield of the bacterial mass by approximately a third and with the 1476 strain by more than a half. The exchange of medium has no effect on the growth of *Mycobacterium smegmatis*.

The curves of decomposition of Celaskon determined by this technique do not differ in physiological solution and Sauton's medium. The curve of decrease of reducing activity (Fig. 3) in comparison with the curve of decrease of

hydrazone formation has a sharper fall because it does not indicate the dehydroascorbic acid and further decomposition products which can still form hydrazones.

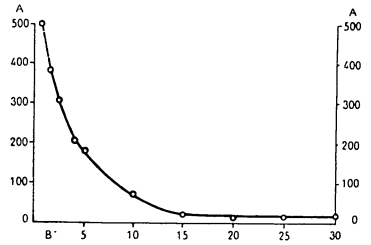

Fig. 3a. Formation of hydrazones.
A — mg %; B — Days.

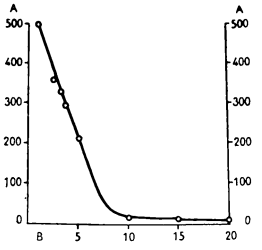

Fig. 3b. Reducing activity.
A — mg %; B — Days.

The auto-oxidised solution of vitamin C obtained by the above technique had approximately two-thirds less reducing activity and gave a correspondingly reduced formation of hydrazones.

The influence of INH was followed by the same cultivation technique with the *Mycobacterium tuberculosis* 1476 strain which is resistant to 100 gamma

INH/ml in a static culture. The strain was simultaneously inoculated from an eight day subculture on Sauton's medium into four flasks:

1st flask: INH — flow culture (INH — F)
2nd flask: INH — static culture (INH — S)
3rd flask: flow culture without INH (C — F)
4th flask: static culture without INH (C — S).

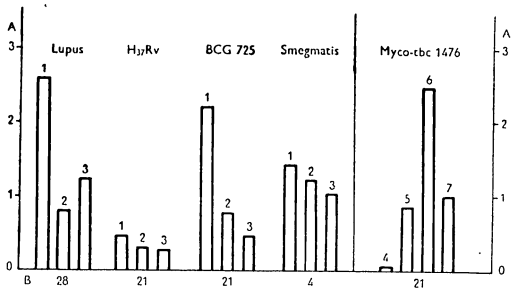

Fig. 4. Effect of vitamin C and INH on growth of *Mycobacteria*.
1 — Without vitamin C; 2 — With natural vitamin C; 3 — With auto-oxidised vitamin C; 4 — INH — P; 5 — INH — K; 6 — K — P; 7 — K — K; A — Grams; B — Time of cultivation (days).

The INH is added to sterile medium in the amount of 50 gamma INH/ml from the supply of sterile solution. The media are stored under refrigeration and before use are incubated for 24 hours at 38 °C. The flow rate of the nutrient media and the cultivation period as well as the evaluation of the growth of the cultures are carried out in the manner already described.

In a flow culture of the *Mycobacterium tuberculosis 1476* strain a significant suppression of the growth occurs at a concentration of 50 gamma INH/ml of culture. The static cultivation of the strain with the same INH content in comparison with the static cultivation without INH does not show a significant difference in the amount of the bacterial mass.

The rations of the increments of the individual strains and the action of the natural and the auto-oxidised form of vitamin C as well as the effect of INH on them can be seen in Fig. 4.

The values of the respiration quotients Q_{O_2} for the various cultures as well as the catalase test show that the properties of various cultures from different

culture environments will probably be identical. Similarly the rough qualitative determinations of fatty acids obtained by hydrolysis of the cultures show insignificant variations and probably only quantitative differences of the fatty acids occur here.

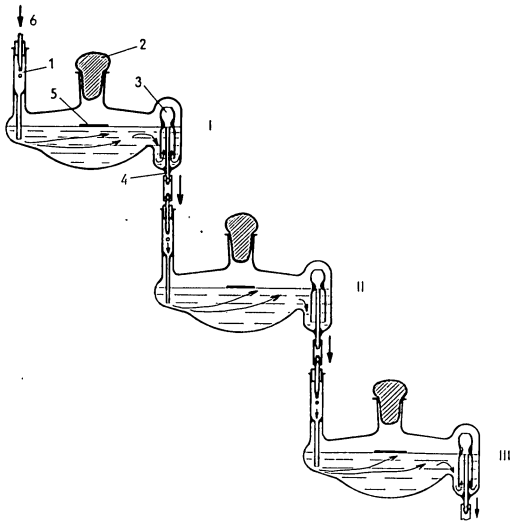

Fig. 5. Three stage continuous flow culture.
1 — 6 see Fig. 1; I — Ist stage; II — IInd stage; III — IIIrd stage.

As follows from these experiments, natural vitamin C, just like its auto-oxidation decomposition products of unknown composition, has approximately the same inhibiting effect on the growth of *Mycobacterium tuberculosis*, the human type of lupus and the $H_{37}Rv$ and BCG strains. These constituents lower the growth of the bacterial mass by a half in the case of these strains during three weeks of cultivation, while they have no effect on the growth

of *Mycobacterium smegmatis*. The exchange of Sauton's medium without vitamin C under the cited experimental conditions increases the growth of the bacterial mass by a third in comparison with the static control for the strains from human lupus, $H_{37}Rv$ and BCG; it has, however, no effect on the growth of *Mycobacterium smegmatis*.

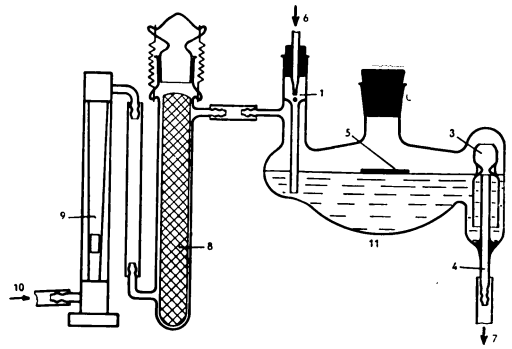

Fig. 6. Apparatus for continuous exchange of medium and gas.
1—7 — see Fig. 1; 8 — Cotton air filter; 9 — Gas flowmeter; 10 — Gas inlet; 11 — Culture vessel.

Massive suppression of the growth of the INH resistant *Mycobacterium tuberculosis* 1476 strain occurs in continuous culture with only half the dose of INH, i. e. 50 gamma INH/ml of media, while in the static culture with the same amount of INH no significant difference in the increase of the bacterial mass in comparison with that of the static culture without INH is observed.

The difference in the amount of bacterial mass of the *Mycobacterium tuberculosis* strain 1476 in the static and continuous culture also explains to a certain extent the differences between the values of the resistance found in vivo and in vitro, where the degree of resistance of the strains in vivo is as a rule smaller. We assume that in the flowing environment as well as in the organism the microbes are always attacked in their logarithmic growth phase by fresh materials, so that they appear more sensitive to a given environment.

Our equipment can also be used for studies of continuous utilization of the nutrient medium and the influence of metabolites on the culture, by using it as a unit in a cascade of flow culture units (Fig. 5).

With a sufficiently fast flow rate of the nutrient medium it is possible to study a given culture at different ages in a single experiment by using a cascade of flow units.

A modified type of apparatus (Fig. 6) is arranged so that in addition to constant conditions of the nutrient environment (liquid phase), it is possible also to set up constant conditions of the gaseous environment (gas phase) in the flowing culture. This method of cultivating *Mycobacteria* affords good possibilities for the study of their metabolism.

This cultivating procedure will be especially suitable for the study of the metabolism of atypical tuberculosis bacteria in comparison with the metabolism of the remaining types of tuberculosis *Mycobacteria* since, as shown by preliminary experiments, certain differences in the multiplication of the culture and in its metabolism can be expected among the various types.

In conclusion we can say that this apparatus for flow culture has proved very satisfactory in practice and affords good possibilities for wider use in the study of the metabolism of *Mycobacteria* and of other microbes growing on a surface. It can be stated that the goal which we set ourselves of approaching the dynamics of processes in the organism has been attained in the study of the dynamics of the cultivation process. This question has, however, still not been solved completely and depends on further development of the technique of flow culture. Most probably, as indicated by preliminary experiments, this question will be solved by the use of flow micro-culture with a solid substrate such as cellulose, fibrin, etc.

CONTINUOUS FLOW CULTIVATION OF BAKER'S YEAST ON BEET MOLASSES WORT

KAREL BERAN

Technologists have for a long time been interested in fermentation continuous flow processes by means of which it is possible to increase considerably the productivity of the apparatus and to introduce automatisation into the whole procedure. Interest has also been shown in this process for a long time for the production of baker's yeast as can be seen from the number of patents, especially in the years 1920—1930. Among others (Reiner 1879, cit. Irvin 1954; Hayduck 1923a, b), Sak was the first (1928, 1929, 1932) to study this process intensively. In addition to processes with one fermentation vessel processes using two vessels (Bühring 1929) and more vessels (Olsen 1930, Darányi 1936) were patented together with a special fermentation apparatus constructed for this purpose (Seidel 1943, Harrison 1930). Attention was also paid to alternation of molasses wort and wort prepared from starch raw material to eliminate degeneration of the yeast which occurred during these processes (Meyer 1929). Some of these processes are actually more of the semi-continuous type (Darányi 1936) and their modification is used also elsewhere (Stuchlík, whose work was discussed by Píš, 1956). As can be deduced from these patents procedures were worked out entirely on an empirical basis. It is difficult to decide which of the processes has the most advantages, but we believe that Bühring's procedure is the summit of these works (1929).

In addition to older patented literature possibilities of continuous flow fermentation have been considered more recently for baker's yeast in technological processes with fundamentally different aims, e. g. Harris et al. (1948), Unger et al. (1942) .The work of Adams and Hungate (1950) and Maxon and Johnson (1953) were concerned with the elements of continuous flow cultivation of *Saccharomyces cerevisiae*. Maxon presented the most complete review of continuous processes also from the technological point of view (1955). In general however, there are no systematic data regarding the bases of the process of continuous cultivation of baker's yeast.

The mathematical bases of flow cultivation elaborated in 1950 by Monod, Novick and Szillard (1953, cit. Novick 1955) Northrop (1954) and others were further developed by Herbert (1956) and Powell (1956) and form also the theoretical basis of continuous flow cultivation of baker's yeast without which further elaboration of basic problems is unthinkable. Also work on problems of the biology and physiology of microorganisms, carried out especially by Málek (1943, 1955) forms a basis for their deeper studium.

Basically it is possible to separate the flow processes that are of interest to technical microbiologists into two groups: 1.The production of biomass where growth is the result of pure aerobic metabolism. 2. The production of the required metabolites, possibly in connection with the growth of the given microorganism. In between these two groups we have fermentation of such a type that growth is the result of mixed metabolism, as is usually the case in the production of baker's yeast. The later type of fermentation process has its practical problems. From the practical point of view it is necessary for the process to occur at the maximum rate of flow with the maximum yields, as far as possible, in one operation. With regard to the yield it is thus necessary to exclude side metabolism and with regard to production only one fermentation vessel should be used if possible, and the most rapid growth, which according to theory, depends on the concentration of the substrate. We believe that the production of baker's yeast has a special position during these fermentations as it is connected with certain indication of quality, fermentation strength, dough tests and keeping properties.

If both types of metabolism appear during fermentation where growth is the result of mixed metabolism, then a steady concentration of cells is not the only function of the flow rate, as is the case in pure aerobic metabolism. Thus if in continuous flow cultivation of baker's yeast the feed of medium is so great that alcohol formation occurs, this metabolism results in lower yields and a new equilibrium is established with a lower cell concentration.

It is known that in cultivation of baker's yeast alcohol formation can be prevented only by decreasing the carbohydrate sources below a certain level (White 1954, Lemoigne et al. 1953). This means that during continuous flow cultivation the growth rate is considerably smaller than the possible maximum. The question arises, therefore, whether under such conditions there is a change in the physiological state of the yeast and what properties are acquired by it from a practical point of view. Obviously this limitation of carbohydrate sources can only be considered if the yeast has a sufficient supply of other factors necessary for growth. These are especially biotin, pantothenate, inositol, pyridoxine, and in parts also thiamine, trace elements and salts containing especially nitrogen and phosphorus. Aeration must also be sufficiently effective so that oxygen does not become a limiting factor.

A group of workers led by the academician, Málek (Dr. Říčica, Dr. Chaloupka,

Ing. Hospodka, Dr. Burger, Dr. Beran) worked on problems of the physiology of baker's yeast during continuous flow cultivation also in relation to its baking qualities. We used a laboratory and a semi-plant apparatus described in another paper presented in this symposium (Ričica 1958). Cultivation was carried out on molasses wort containing 1% and 3% of invert sugar. The molasess were cleared and enriched in the usual way. $MgSO_4$, $(NH_4)_2HPO_4$ and urea as the main nitrogen source were added. The nitrogen content, including the assimilative nitrogen of molasses, was adjusted so that the protein content of yeast was 50%. Experiments were started by feeding a small volume of yeast suspension in diluted molasses wort inoculated with dried yeast corresponding to the equilibrium flow state. The amount of word fed was calculated according to the chosen doubling time and the expected yield. When the full working volume of the fermentation was attained the flow of the wort corresponded to the rate determined for continuous flow cultivation.

SOME FUNDAMENTAL FACTORS IN CONTINUOUS FLOW CULTURE OF BAKER'S YEAST ON BEET MOLASSES

THE EFFECT OF ADDING BIOTIN

As has been mentioned above the growth of *S. cerevisae* yeast of the baker's type depends on various conditions and also on the presence of some growth factors. As we used beet molasses in our experiments it was necessary to determine first of all to what extent the necessary growth factors on which the growth of the baker's yeast used here depends are present in normal fortified molasses wort. Beet molasses diluted to contain 1% invert sugar was used enriched as described above. It was shown in preliminary experiments that the molasses used did not contain a sufficient amount of all factors. At first, when a rate corresponding to a doubling time of three hours was used, (i. e. with the fermentation vessel filled with two Litres the flow rate was 460 ml./hr.) the required equilibrium weight of yeast dry matter achieved by the feed method dropped rapidly and even when the feed of wort was decreased to only 290 ml./hr. equilibrium of the yeast dry matter was still not achieved.

It is known that beet molasses are to certain extent deficient in biotin (White 1954). We therefore first tested this growth factor. A rate of wort flow was used corresponding to the calculated three hour doubling time in a system of three tanks in series. Fig. 1 shows that the yeast dry matter (curve 3) decreases to 0·2 % during 54 cultivating hours. If after this period a medium enriched with biotin is used (10 μg/l) the dry matter again increases to the required equilibrium value of 0·5%. Changes in pH (curve 2) and the amount of alcohol formed (curve 4) follow this increase.

Without the addition of biotin the required equilibrium dry matter was also not achieved in the second and third fermentation tanks. The yeast dry matter was higher, but did not exceed 0·35% in the third tank. This is best apparent from the yields calculated from the amount of yeast gained from the

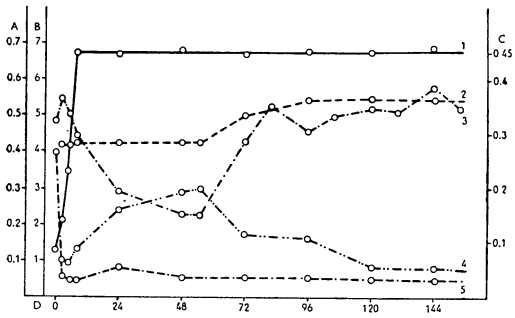

Fig. 1. Effect of adding biotin on the growth of baker's yeast in continuous flow cultivation on beet molasses wort containing 1% of invert sugar. First fermentation vessel.

A — % of yeast dry matter, alcohol and invert sugar; B — pH; C — flow rate of molasses wort in litres/hour; Curves: 1 — flow of wort; 2 — pH; 3 — % of dry matter; 4 — % of alcohol; 5 — % of invert sugar.

wort flowing from the third fermentation vessel. The yield was 41·7% in the 24th hour, 32·1% in the 48th hour and 32% in the 72nd hour. After the addition of biotin during hours 54 the yield was 48% in the 96th hour, 49·3% in the 120th hour and 54·8% in the 114th hour.

The above is not surprising as it is known that beet molasses have certain defeciency of biotin. During flow culture a steady state occur corresponding to this limiting factor. As biotin is not considered as a limiting factor in the production of yeast by the feed method from the point of view of technology, we considered it necessary to demonstrate the effect of biotin addition experimentally. Biotin must be considered as an important factor especially in view of the economy of the flow process. In further experiments wort enriched with biotin was compared with wort enriched also with other vitamins necessary for good growth of yeast of the *S. cerevisiae* group of the type, used in yeast technology: pyridoxine, Ca-panthothenate and inositol (200, 100 and

1000 µg/l of wort). Some trace elements were also added (Zn, Cu, B and Mn in amounts of 410, 25, 120 and 100 µg respectively). We found that there were no considerable differences in the results with wort enriched only with biotin and worts with an addition of the other substances.

White (1954) studied the need of baker's yeast of individual vitamins and found that in order to achieve maximum yields 1 µg biotin was necessary for every gram of yeast grown. We proved this for continuous flow cultivation of our yeasts. Using a biological test with yeast (Snell et al. 1940) in a modified medium according to Olson and Johnson (1949) it was found that the molasses wort used containing 1% invert sugar had a content of 2 µg biotin per 1 litre.

In view of the fact that the expected dry matter in our experiments was 0·5% per litre of wort a further 3 µg biotin were added per litre of wort. This content was found to be fully sufficient for the attainment of maximum yields. The proportion of 1 µg biotin per lg of grown dry matter was maintained in all further experiments.

Experiments were carried out in a system of three tanks with the same volume of culture fluid. We always attempted to use two stage fermentation in which the first tank was mainly intended for growth and the second for ripening of the yeast. Even though it may be assumed that the second tank should have a smaller volume than the first, this was not the case in our laboratory experiments. The rate of flow in all experiments in which biotin was added corresponded to a calculated three hour doubling time. We determined the dry matter, alcohol, invert sugar yield and Q_{O_2} on glucose*). An example of the basic fermentation curves is given in Figs. 2 and 3. Fig. 2 shows that the concentration of dry matter in the first vessel (curve 4) had the required steady state values of 0·45—0·5% after the initial drop. The concentration of alcohol was about 0·1 and 0·2% (curve 5). The first 8 hours belonged to the feed method the technique of which has been described above. The invert sugar was actually completely utilised in the first fermentation vessel and the values shown (curve 6) are mainly reducing non-sugar substances that have been demonstrated in molasses, (Erb and Zerban 1947). Chromatographically only very small amounts of glucose and fructose could be demonstrated. The pH value remained below 5, usually 4·5 (curve 3). The Q_{O_2} was about 110 or 100 (curve 2). The initial temporary decrease in dry matter was considered characteristic of flow cultures of yeast especially during fermentation with

*) When determining metabolic quotients the Warburg vessel contained 1 ml. of buffer solution and 0·5 of ml. yeast in physiological saline. The side arm contained 0·5 ml. of substrate, the funnel 0·2 ml. distilled water or 0·2 ml. of 10% KOH. When determining $Q^{S}_{CO_2}$ on glucose and maltose and Q_{O_2} on these sugars (when not indicated otherwise) the medium consisted of a mixture of phtalate buffer, pH 4·5, with a final concentration of 0·05 M and KH_2PO_4 with the same final concentration. When determining Q_{O_2} on glucose, ethanol and acetate a phosphate buffer pH 6 5 was used, the final concentration being 0·1M. The final concentrations of glucose and maltose were 0·5%. Concentrations of ethanol, acetate and glucose were chosen in such a way that 1 mg. carbon was present in the vessel.

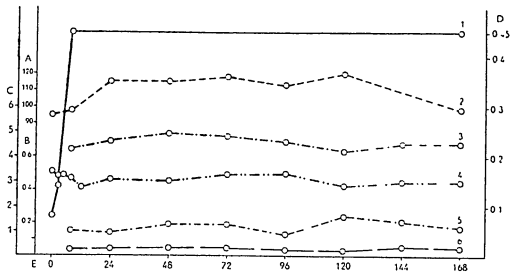

Fig. 2. Basic fermentation curves in continuous flow cultivation of baker's yeast on beet molasses wort containing 1% of invert sugar and fortified with 3 µg biotin in one litre of wort. First fermentation vessel.

A — Q_{O_2} on glucose; B — % of dry matter, alcohol and invert sugar; C — pH; D — flow rate of wort in litres/hour; E — time in hours. Curves: 1 — flow of wort; 2 — Q_{O_2}; 3 — pH; 4 — % of dry matter; 5 — % of alcohol; 6 — % of invert sugar.

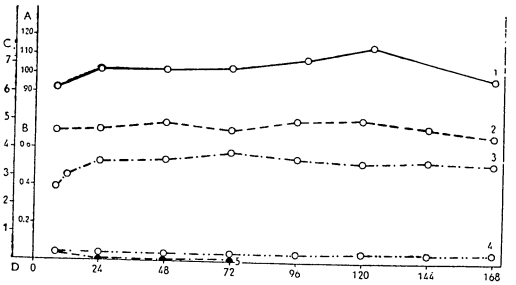

Fig. 3. Basic fermentation curves in continuous flow cultivation of baker's yeast on beet molasses wort containing 1% of invert sugar and fortified with 3 µg biotin in one litre of wort. Second fermentation vessel.

A — Q_{O_2} on glucose; B — % dry matter, alcohol and invert sugar; C — pH; D — time in hours. Curves: 1 — Q_{O_2}; 2 — pH; 3 — % dry matter; 4 — % invert sugar; 5 — % of alcohol.

1000 μg/l of wort). Some trace elements were also added (Zn, Cu, B and Mn in amounts of 410, 25, 120 and 100 μg respectively). We found that there were no considerable differences in the results with wort enriched only with biotin and worts with an addition of the other substances.

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Experiments were carried out in a system of three tanks with the same volume of culture fluid. We always attempted to use two stage fermentation in which the first tank was mainly intended for growth and the second for ripening of the yeast. Even though it may be assumed that the second tank should have a smaller volume than the first, this was not the case in our laboratory experiments. The rate of flow in all experiments in which biotin was added corresponded to a calculated three hour doubling time. We determined the dry matter, alcohol, invert sugar yield and Q_{O_2} on glucose*). An example of the basic fermentation curves is given in Figs. 2 and 3. Fig. 2 shows that the concentration of dry matter in the first vessel (curve 4) had the required steady state values of 0·45—0·5% after the initial drop. The concentration of alcohol was about 0·1 and 0·2% (curve 5). The first 8 hours belonged to the feed method the technique of which has been described above. The invert sugar was actually completely utilised in the first fermentation vessel and the values shown (curve 6) are mainly reducing non-sugar substances that have been demonstrated in molasses, (Erb and Zerban 1947). Chromatographically only very small amounts of glucose and fructose could be demonstrated. The pH value remained below 5, usually 4·5 (curve 3). The Q_{O_2} was about 110 or 100 (curve 2). The initial temporary decrease in dry matter was considered characteristic of flow cultures of yeast especially during fermentation with

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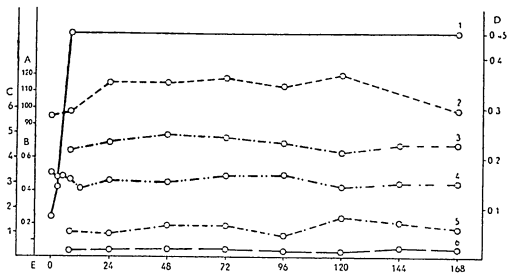

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A — Q_{O_2} on glucose; B — % of dry matter, alcohol and invert sugar; C — pH; D — flow rate of wort in litres/hour; E — time in hours. Curves: 1 — flow of wort; 2 — Q_{O_2}; 3 — pH; 4 — % of dry matter; 5 — % of alcohol; 6 — % of invert sugar.

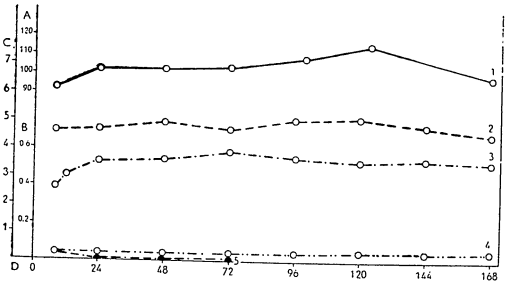

Fig. 3. Basic fermentation curves in continuous flow cultivation of baker's yeast on beet molasses wort containing 1% of invert sugar and fortified with 3 μg biotin in one litre of wort. Second fermentation vessel.

A — Q_{O_2} on glucose; B — % dry matter, alcohol and invert sugar; C — pH; D — time in hours. Curves: 1 — Q_{O_2}; 2 — pH; 3 — % dry matter; 4 — % invert sugar; 5 — % of alcohol.

more concentrated worts. This was obviously due to the fact that during the flow older cells were rapidly removed. These cells were grown in the feed phase and the rate of flow was not in agreement with their generation time. In the second fermentation vessel (Fig. 3) alcoohl was used for growth as can be

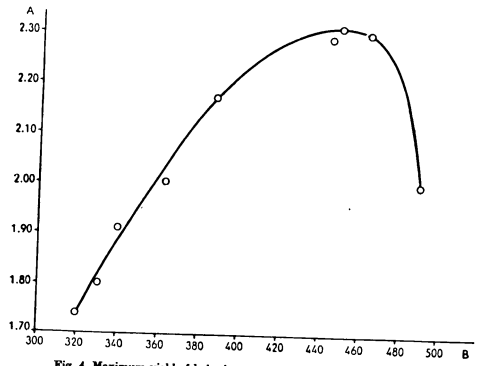

Fig. 4. Maximum yield of baker's yeast in continuous flow cultivation. A — g dry matter in one litre of wort/hour; B — flow of wort in l/hour.

deduced from its completely disappearance (curve 5) and from the increase of dry matter to 0·5—0·55% (curve 3). The content of residual reducing substances expressed as invert sugar remained practically unchanged (curve 4). The Q_{O_2} value was steady and lower by about 10 units in comparison with the first vessel. The pH value was somewhat higher in the second tank and remained nearer 5 (curve 2). The constancy of these conditions was verified in longer experiments lasting up to 14 days.

MAXIMUM PRODUCTIVITY

While further developing the theory of continuous flow cultures worked out by Monod (1950), Novick (1955) and others Herbert (1956) calculated the relation for such a dilution rate at which a given system has maximum productivity. Fig. 4 shows results of experiments in which maximum productivity was tested. As can be seen maximum productivity was attained at a rate of

flow of about 450—460 ml./hr which, under our conditions, corresponded to the calculated approximate three hour doubling time. This was the time chosen by us in previous experiments.

CONCERNING THE PHYSIOLOGICAL STATE AND QUALITY OF BAKER'S YEAST FROM CONTINUOUS FLOW CULTURES ON BEET MOLASSES

In all the experiments outlined above a very diluted molasses wort was used containing 1% of invert sugar and giving in our experiments the expected equilibrium yeast dry matter of 0·5%. As is known a yeast dry matter of 1·2 to 1·5% is usually obtained in the production of yeast by the feed method. We attemped, therefore to obtain such equilibrium dry weights and also tested some further aspects. We wanted to ascertain the phase of the physiological state of the flow culture with respect to the state of batch fermentation cultures. According to White (1954) the baking value of yeast depends on (1) the total activity of yeast in fermentation obligatory sugars, especially glucose, (2) the malto-zymase activity of yeast (i. e. the rate at which maltose is fermented), (3) the dispersibility of yeast and (4) the osmosensitivity with respect to the addition of salt to dough. Further should be mentioned (5) the dependence of the baking value on the keeping properties, i. e. the time during which yeast deteriorates under certain conditions. It seems of interest therefore to study changes in the aerobic systems of yeast (indicated by the respiration of different C sources) in flow cultures and also anaerobic systems responsible for the fermentation of glucose and maltose. We further studied changes in the rate of autolysis in flow cultures and the relation of some of the tests to the quality of the yeast obtained (especially to the dough test).

Saccharose is the main source of carbon in molasses in the production of yeast. The question arises, therefore how the yeast activity changes during flow culture with regard to maltose. As the maltose produced by the action of flour amylase is the final substrate in dough after the fermentation of saccharose and laevosine it may be expected that yeast having a high maltase activity ferments maltose more rapidly and is of higher baking strength than yeast with a low maltase activity. According to White (1954) the maltase activity in yeast depends on the ability of such baker's yeast to become adapted rapidly to the fermentation of maltose. Fig. 5 shows the fermentation of maltose and glucose by baker's yeast as reported by White (1954). The fermentation of glucose is more rapid than that of maltose and its course is linear. The rate of maltose fermentation is at first slow, but constantly increases as the yeast becomes adapted to maltose as a substrate. It must be added, however, that the problem of fermentation of maltose by *S. cerevisiae* is complicated by opinions concerning the mechanism of fermentation. The generally accepted

mechanism is that put forward by Gottschalk (1950) implicating hydrolytic enzyme maltase. Hestrin (1949) believed hovever, that direct fermentation occurs. This opinion has again been discussed recently (White 1956).

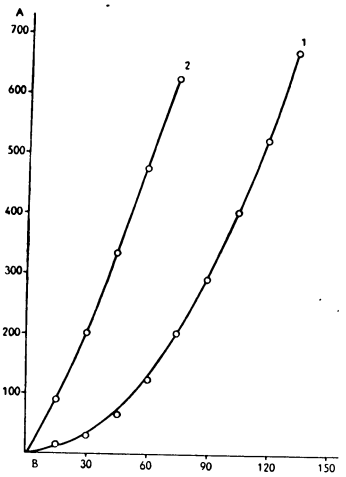

Fig. 5. The course of glucose and maltose fermentation by baker's yeast (White 1954). A — ml. CO_2/g. yeast; B — time in minutes. Curves: 1 — fermentation of maltose, 2 — fermentation of glucose.

THE PHYSIOLOGICAL STATE OF BAKER'S YEAST IN BATCH FERMENTATION ON BEET MOLASSES WITH THREE PERCENT INVERT SUGAR

If the total amount of culture medium is inoculated with a certain amount of yeast and if aeration is sufficient, the growth curve is divided in two phases in response to changes in the substrate, as alcohol becomes the substrate in the second phase (Lemoigne et al. 1953).

We studied changes in such cultures on molasses wort containing 3% of invert sugar and fortified with mineral salts and biotin. Fig. 6 shows the course of the basic fermentation curves, the changes in the ribonucleic acids content

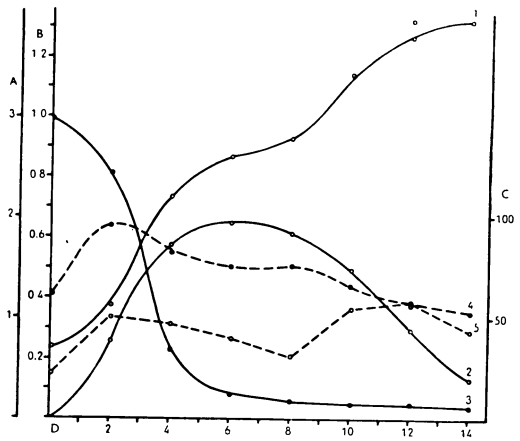

Fig. 6. Basic fermentation curves, RNA content and autolytic rate of yeast in batch cultivation on beet molasses wort containing 3% of invert sugar.

A — % invert sugar; B — % dry matter and alcohol; C — RNA in µg/mg dry matter and % of autolysed proteins in yeast. Curves: 1 — % of dry matter; 2 — % of alcohol; 3 — % of invert sugar; 4 — RNA; 5 — % of autolysed proteins. RNA — ribonucleic acids.

and the autolytic rate of yeast in such batch cultivation. It can be seen that a sudden change in growth intensity occurred (curve 1) during the 6th hour of cultivation, i. e. at the time when the substrate contained the maximum amount of alcohol (curve 2) and when the sugar had nearly disappeared (curve 3). The maximum in the ribonucleic acids content was reached (curve 4) during the 2nd cultivation hour (97 µg/mg) and during further cultivation there was a continuous decrease down to 54 µg/mg in the 14th hour of fermentation. The rate of autolysis also changed with the growth curve (curve 5). The method for determining the former was basically that of Vosti and Joslyn (1953).

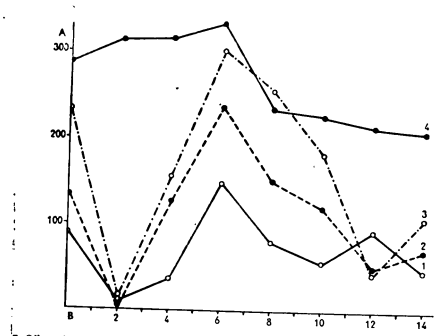

Fig. 7. $Q^{N}_{CO_2}$ of yeast on maltose and glucose in batch cultivation on beet molasses wort containing 3 % of invert sugar.

A — µl CO_2/hour/mg. dry matter; B — time in hours. Curves: 1, 2, 3 — $Q^{N}_{CO_2}$ on maltose during the 1st, 2nd and 3rd hours of manometric determination; 4 — $Q^{N}_{CO_2}$ on glucose.

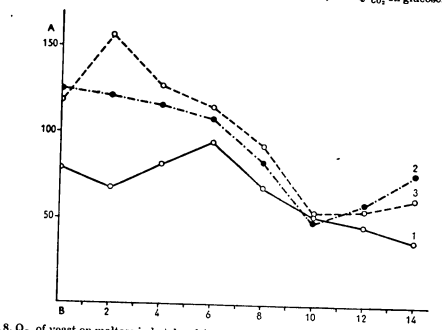

Fig. 8. Q_{O_2} of yeast on maltose in batch cultivation on beet molasses wort containing 3 % of invert sugar.

A — µl O_2/hour/mg dry matter; B — time in hours. Curves: 1, 2, 3 — Q_{O_2} on maltose during the 1st, 2nd and 3rd hours of manometric determination.

The rate increased to 50% during the 2nd hour of fermentation and dropped to 30% in the 8th hour, this being the beginning of the second growth phase. Then it rose again to 55 or 60%. As the presence of trehalose in yeast may be related to the fermentation rate and the dough test (Pollock and Holmstrom 1951), we also studied its content in yeast chromatographically. It was found that the first traces of trehalose appeared in yeast from the 6th fermentation hour, increased in the 8th hour and remained unchanged from the 10th hour up to the end of the cultivation process.

Fig. 7 shows the course of $Q^{N}_{CO_2}$ on maltose and glucose in relation to the same growth curve. The $Q^{N}_{CO_2}$ on glucose (curve 4) remained high up to the end of the first part of the growth curve (6th hour of cultivation), then dropped rapidly but still remained above 200. The $Q^{N}_{CO_2}$ on maltose for the first hour of manometric determination (curve 1) and for the 2nd and 3rd hours (curve 2 and 3) had a characteristic course. In yeast from the 2nd hour of fermentation it dropped to 10, but during the following hours it rose and reached the highest values at the end of the first phase of the growth curve, i. e. in the 6th hour of fermentation. The value for the 2nd and 3rd hours of manometric determination showed an increasing enzymatic system not only in the 6th hour of fermentation but also at the time when the end of the first part of the growth curve was reached. The value for Q_{O_2} on maltose have a somewhat different course (Fig. 8) according to the values in the first, 2nd and 3rd hour of manometric determination the increasing affinity to maltose appeared in the second hour of the experiment. In the 6th hour the Q_{O_2} after one hour's determination reached its maximum in the same way as the $Q^{N}_{CO_2}$. The results showed that fermentation of maltose was most rapid in yeast from the end of the first part of the growth curve and that the ability to increase the rate of fermentation was also maintained about that point. Fig. 9 confirms this in an experiment in which a lower inoculation yeast dry matter was used than in the above experiments. In this case the end of the first part of the growth curve was moved to the 8th cultivation hour. This time was also characterised by the maximum amount of alcohol and the almost complete disappearance of invert sugar, as shown above. Fig. 9 also shows that the most rapid fermentation of maltose occurred during the 8th hour of fermentation as judged from the $Q^{N}_{CO_2}$, i. e. at the end of the first part of the growth curve and increased fermentation of maltose appears in the 6th hour and was maintained even during the 10th hour of the experiment.

Changes in the activity of the aerobic systems in relation to the growth curve were determined manometrically by finding the Q_{O_2} on glucose, ethanol and acetate. This is shown in Fig. 10. The values for Q_{O_2} on all substrate dropped up to the 4th hour of cultivation and then rose rapidly, especially on ethanol and acetate (curves 2 and 3). The Q_{O_2} on ethanol reached 130 in the 10th hour of cultivation and thus was higher than the Q_{O_2} values on glucose. The Q_{O_2} on

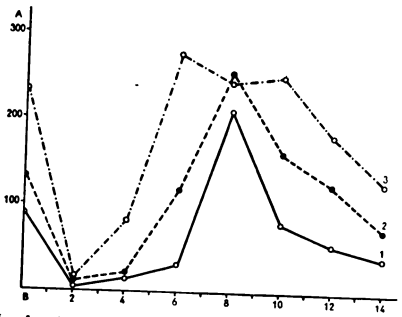

Fig. 9. $Q^{N}_{CO_2}$ of yeast on maltose in batch cultivation on beet molasses wort containing 3% of invert sugar. Lower inoculation dry matter.
A — µl CO_2/hour/mg dry matter; B — time in hours. Curves: 1, 2, 3 as in previous figures.

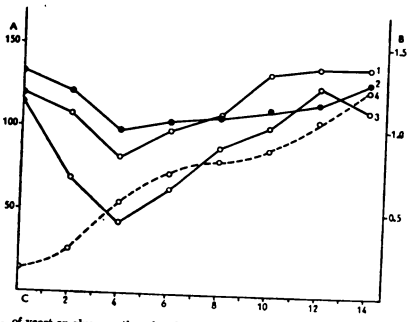

Fig. 10. Q_{O_2} of yeast on glucose, ethanol and acetate in batch cultivation of baker's yeast on beet molasses wort containing 3% of invert sugar.
A — µl CO_2/hour/mg. dry matter; B — % of dry matter; C — time in hours. Curves: 1 — Q_{O_2} on ethanol; 2 — on glucose; 3 — on acetate; 4 — % of dry matter. pH of buffer used = 6·5. Substrates used in equimolar proportions.

acetate is near that on glucose. These results were in agreement with those of Eaton and Klein (1954). The value of Q_{O_2} on glucose at the end of the first part of the growth curve was about 100.

THE PHYSIOLOGICAL STATE OF YEAST DURING CONTINUOUS FLOW CULTIVATION ON BEET MOLASSES WORT WITH THREE PERCENT INVERT SUGAR

These changes in quotients during batch cultivation were compared with values from similar tests in yeast from continuous flow cultivation under different flow conditions. Three values for rate of flow of medium were chosen: a rate of flow corresponding to the calculated doubling time of 2·6 hours, 3 hours according to the experimentally determined optimum time and finally 4 hours, i. e. the period most frequently used in yeast production when using the feed method. Beet molasses containing 3% of invert sugar fortified with salts and containing 15 µg of biotin per litre of wort were used. The molasses wort contained 3% of invert sugar. Fermentation occurred in two fermentation vessels in series. Their volume was 2 litres. The apparatus is described in another paper presented in this symposium (Ričica 1958).

Table 1
Basic cultivation values at different rates of flow of wort

Rate of flow corresponding to doubling time	Hr. of fermentation	Fermentation vessel 1				Fermentation vessel 2			
		Yeast dry matter %	Alcohol %	Invert sugar %	RNA*) µg/mg	Yeast dry matter %	Alcohol %	Invert sugar %	RNA*) µg/mg
4·0	0			2·90					
	24	1·43	0·16	0·16	69·9	1·45	0·00	0·15	63·0
	96	1·44	0·10	0·14	61·6	1·50	0·00	0 12	62·5
3·0	0			3·4					
	24	1·40	0·45	0·24	65·8	1·75	0 07	0·21	47·0
	96	1·44	0·45	0·23	68·8	1·82	0·10	0 21	50·7
2·6	0			3 35					
	24	1·08	0·73	0·15	86·7	1·57	0 14	0·13	59·5
	96	0·90	0·83	0·23	85·4	1·25	0·33	0·20	52·2

*) RNA = ribonucleic acids

Table 1 gives the characteristic values of fermentation from the first and second vessel. It can be seen that at a rate of flow corresponding to a doubling time of 4 hours all sugar was practically utilised in the first vessel mainly for growth, equilibrium weight of yeast dry matter was attained and alcohol was only formed to a small extent. With a rate of flow corresponding to 3 hours

doubling time a considerable amount of alcohol was still formed but cultivation was in a steady state as the equilibrium dry matter of 1·4—1·45% was maintained throughout cultivation. Alcohol was utilised for further growth in the

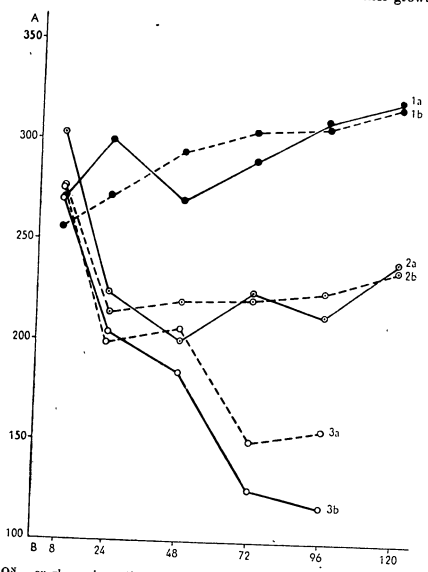

Fig. 11. $Q^{N}_{CO_2}$ on glucose in continuous flow cultivation of baker's yeast on beet molasses wort containing 3% of invert sugar.
A — µl CO_2/hour/mg. dry matter; B — time in hours. Curves: a — $Q^{N}_{CO_2}$ for the first hour of manometric determination; b — for the second hour; 1 — $Q^{N}_{CO_2}$ at a flow rate equal to a doubling time of 2·6 hours, 2 — doubling time of 3 hours; 3 — doubling time of 4 hours.

second vessel. With a rate of flow corresponding to a doubling time of 2·6 hours equilibrium dry matter was not attained, as is evident from its low value of (0·90% in hour 96) and alcohol was formed to such an extent that it was not

fully utilised even in the second vessel. All of the invert sugar was practically used up in the first fermentation vessel in all cases.

The ribonucleic acids content of yeast in the first vessel was nearly the same

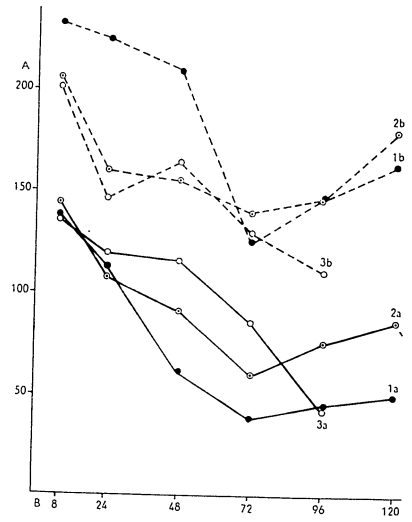

Fig. 12. $Q^{N}_{CO_2}$ on maltose in continuous flow cultivation of baker's yeast on beet molasses wort containing 3% of invert sugar. Marking as in previous figure.

at all rates of flow at about 62—70 µg/mg of yeast dry matter. In the second vessel the ribonucleic acids content of yeast decreased at rates of flow corresponding to 3 and 2·6 hours doubling time. This was evidently in agreement with changes in the ribonucleic acids content observed in a study of growth curves.

We studied further the metabolic quotients of yeast at the three rates of flow indicated. Fig. 11 gives the $Q^{N}_{CO_2}$ values on glucose. With a rate of flow

corresponding to a doubling time of 2·6 hours (curve 1) the yeast on the average slightly increases its fermentative aktivity during flow cultivation. With a rate of flow corresponding to 3 hours doubling time (curve 2) and 4 hours doubling time (curve 3) the ability of yeast to ferment glucose decreased relatively rapidly. With a rate of flow corresponding to a doubling time of 4 hours the $Q^N_{CO_2}$ of yeast from the 96th cultivation hour decreased to 118 and with a rate of flow corresponding to 3 hours doubling time it lay between 200 and 230 from the 24th hour of cultivation. It thus appears that the rate of flow considerably affects the rate of fermentation of glucose.

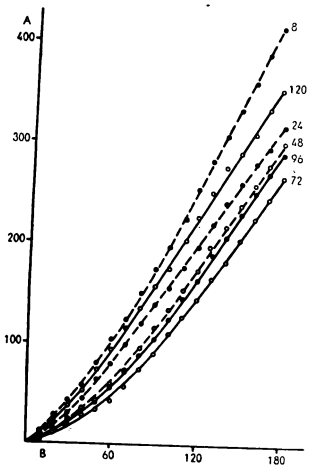

Fig. 13. Fermentation of maltose by baker's yeast from continuous flow cultivation at a rate flow equal to a doubling time of 3 hours.

A — µl CO_2/hour/mg. dry matter; B — time in minutes. The figures on the curves are for yeast of the corresponding fermentation time.

Fig. 12 shows the same relations when maltose is fermented. $Q^N_{CO_2}$ values for the yeast from continuous flow cultivation in the first hour of manometric determination decrease more rapidly with a rate of flow corresponding to 2·6 hours doubling time (curve 1) and more slowly with a rate of flow corresponding to 4 hours doubling time (curve 3). The curve for $Q^N_{CO_2}$ for the second hour of manometric determination shows that at the slowest rate of flow there is also an equal decrease in the ability to strengthen the enzymatic systems taking part in maltose fermentation. At higher rates of flow, however, the increased fermentation rate of maltose is maintained for the whole time of observation. Fig. 13 gives an example of the course of fermentation of maltose by yeast under anaerobic conditions at a rate of flow corresponding to a doublng time of three hours.

The course of maltose fermentation is shown directly on the curves of manometric determination. It can be seen that in all cases the ability to increase the activity of the system responsible for maltose fermentation is preserved. Similar curves could be demonstrated for yeast also from other experiments, especially with a rate of flow corresponding to a doubling time of 2·6 hours. We can thus conclude that at all rates of flow used the rate of maltose fermentation decreases for yeast in the first vessel. At slow rates of flow the ability to increase the activity of anearobic maltose fermentation decreases together with the rate of maltose fermentation, but at higher rates of flow the decrease ceases at certain values and the ability to speed up fermentation is preserved. Table 2 shows the $Q^N_{CO_2}$ for the 1st, 2nd and 3rd hours of manometric determination on maltose in yeast from the second fermentation vessel. The $Q^N_{CO_2}$ values are all lower than those from the first vessel and also the ability to increase the rate of fermentation is small, or absent, excepting only a rate of flow corresponding to 2·6 hours, doubling time. The endogenous $Q^N_{CO_2}$ which has low values was not taken into consideration in the above experiments. It only reaches a value of about 5 during the first hour of determination. The $Q^N_{CO_2}$ on glucose is also lower in the second vessel and is about 150 at the slowest rate of flow, 170 at the medium rate and 260 at the highest rate.

Table 2

$Q^N_{CO_2}$ values on maltose of yeast from the 2nd fermentation vessel at different flow rates

Rate of flow corresponding to doubling time	Hours of fermentation	$Q^N_{CO_2}$		
		1	2	3
4·0	23	35·3	50·7	58·3
	48	40·7	82·5	71·7
	72	68·1	73·8	85·6
	96	36·0	47·5	56·9
3·0	24	61·0	47·0	40·5
	48	47·2	49·7	55·3
	72	49·2	39·1	41·3
	96	48·8	44·3	43·3
	120	78·0	50·3	37·1
2 6	24	39·8	77·8	142·2
	48	46·2	120·0	177·0
	72	50·2	128·5	167·5
	96	—	—	—
	120	57·5	35·5	42·7

1, 2, 3 — Values for 1st, 2nd and 3rd hour of manometric determination. The endogenous values are not subtracted.

Thus both the fermentation rate of glucose and maltose and especially the ability to increase the activity of the system participating in the anaerobic fermentation of maltose are considerably decreased in the second vessel. It

will probably be necessary from a practical point of view to determine empirically the volume of the fermentation liquid in the second vessel in order to preserve as far as possible the physiological state of yeast grown in the first vessel.

In addition to the rate of glucose and maltose fermentation by yeast in the above experiments we also studied the rate of oxygen consumption by the same yeast in different substrates. The Q_{O_2} values on maltose, glucose, ethanol and acetate during the first hour of manometric determination at the rates of flow studied are given in table 3. The Q_{O_2} values on maltose and glucose from the first vessel are stable during cultivation and equal about 100. Only with a flow rate corresponding to a doubling time of 2·6 hours were there greater variations in Q_{O_2}, especially towards lower values. The Q_{O_2} values on ethanol were about the same at all rates of flow and equal to about 80. The same holds good for acetate with a value of about 50. In general at a rate of flow corresponding to a doubling time of 2·6 hours the values varied more in relation to the time of cultivation than is apparent from the table.

Table 3

Q_{O_2} values on maltose, glucose, ethanol and acetate of yeast from the 1st fermentation vessel at different rates of flow

Rate of flow corresponding to doubling time	Hour of fermentation	Q_{O_2}			
		Maltose	Glucose	Ethanol	Acetate
4·0	24	100·0	108·0	81·0	49·0
	96	101·0	90·0	74·0	52·0
3·0	24	90·0	100·5	76·5	56·0
	96	106·5	99·4	76·0	60·0
2·6	24	127·0	127·0	82·0	50·0
	96	107·3	104·0	86·0	47·7

The endogenous respiration values are not subtracted. The pH of the buffer on glucose was 6·5. The glucose concentration was equimolar to that of ethanol and acetate.

Fig. 14 gives examples of the course of oxygen consumption on maltose. They give a clearer picture of the oxygen consumption during respiration of maltose than the Q_{O_2} values. Curves are drawn for the oxygen consumption at different times of flow cultivation at a flow rate corresponding to a doubling time of three hours. This is the same yeast the curves for CO_2 evolution during maltose fermentation of which are shown in Fig. 13. It is evident that the oxygen consumption in yeast from the flow culture had, on the whole, a linear course. It must, of course, be pointed out that the values for endogenous respiration were not substrated, as they were small. The Q_{O_2} values on maltose,

glucose, ethanol and acetate for yeast from the 2nd vessel are given in table 4. As can be seen there was no significant difference between the Q_{O_2} values on glucose for yeast from the first and second vessels and the course was also

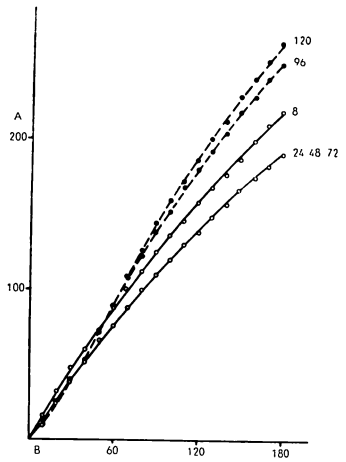

Fig. 14. Oxygen consumption of baker's yeast from continuous flow cultivation at a flow rate equal to a doubling time of 3 hours.

A — µl CO_2/hour/mg. dry matter; B — time in minutes. The figures on the curves are for yeast from the corresponding fermentation time.

the same. The Q_{O_2} values are lower on maltose than in the first vessel, but the course of changes remained the same. The rate of autolysis of yeast from both vessels was also determined at a rate of flow corresponding to a doubling time of three hours. This was stable during 120 hours of cultivation and was equal to 50—60% of autolysed proteins in the first and 60—70% in the second vessel. Trehalose could not be demonstrated in yeast from the first vessel. In yeast from the second vessel this oligosaccharide occurred irregularly and only in very small amounts.

Table 4

Q_{O_2} values on maltose, glucose, ethanol and acetate for yeast from the 2nd fermentation vessel at different flow rates

Rate of flow corresponding to doubling time	Hour of fermentation	Q_{O_2}			
		Maltose	Glucose	Ethanol	Acetate
4·0	24	73·0	91·0	87	57
	96	70·0	100·0	82	41
3·0	24	45·6	98·0	86	79
	96	57·0	80·0	71	43
2·6	24	72·5	100·5	93	45
	96	82·5	101·0	100	83

The endogenous respiration values are not subtracted. The pH of the buffer on glucose was 6·5. The glucose concentration was equimolar to that of ethanol and acetate.

Let us now compare the results obtained when studying the physiology of yeast from batch cultures and those from continuous flow cultures. The physiological state of yeast from batch cultures at the end of the first part of the growth curve is characterised by a high rate of glucose fermentation, a maximum rate of maltose fermentation accompanied, in addition, by a considerable ability to activate the enzymatic system participating in anaerobic fermentation of maltose. In addition, we find a certain decrease in the ribonucleic acid content in yeast as compared to the state of the culture at the beginning of the growth curve. The oxygen consumption on maltose is the highest in yeast from the end of the first phase of the growth curve ($Q_{O_2} = 100$) with only small or even no ability to increase the activity on maltose. The Q_{O_2} value on glucose is the same as that on maltose and does not show such characteristic changes as those for maltose. The Q_{O_2} values on ethanol have a growing tendency at the end of the first part of the growth curve.

When compared with that from the end of the first part of the growth curve in batch cultivation yeast obtained from continuous flow cultivation in the first vessel preserves its ability to increase maltose fermentation at higher rates of flow corresponding to the determined doubling time of three hours and also a high rate of glucose fermentation, especially at higher rates of flow. The values for ribonucleic acid indicate that the culture is not in the logarithmic phase during the first part of the growth curve in batch cultivation. Also the Q_{O_2} values on maltose and glucose indicate that the physiological state of yeast from flow cultivation corresponds to that of yeast at the end of the first part of the growth curve in batch cultivation. The Q_{O_2} values on maltose are more in agreement with this conclusion as they change more.

This is evident from observations on yeast cultivated in batch fermentation on one hand and from those on yeast from the second fermentation vessel in continuous flow cultures on the other. The Q_{O_2} values on ethanol and acetate are lower than would correspond to the state of the culture at the end of the first part of the growth curve in batch cultivation and indicate that the state of yeast corresponding to this part of the growth curve has not been exceeded. This is also the case as judged from the trehalose content of the yeast.

From a comparison of these results it is concluded that yeast from continuous flow cultivation is in a physiological state corresponding to the end of the first part of the growth curve of the batch fermentation process at higher rates of flow and more or less below this state at slower flow rates. This conclusion is also confirmed by further decrease of some values in yeast from the second vessel. This applies to $Q^N_{O_2}$ on maltose and glucose, the values of which decreases and which occasionally also decreases the ability to increase the affinity to maltose under anaerobic conditions. The same conclusion can be reached from decreased values of Q_{O_2} on maltose or, on the contrary, from increased Q_{O_2} on ethanol and acetate.

SEMIPILOT-PLANT EXPERIMENTS WITH CONTINUOUS FLOW CULTURES OF BAKER'S YEAST

Results obtained in small laboratory vessels were verified in vessels ten times as large. We used a two vessel system with a fermentation liquid capacity of 20 litres in the first and 10 litres in the second tank. The fermentation system is described in another paper in this symposium (Ričica 1958). Molasses wort containing 3% invert sugar was used fortified in the same way as in laboratory fermentation. The speed of the mixer was 500 r. p. m. in both vessels. The first was aerated with 10 lt air/min., the second with 5 l/min. The oxygen diffusion rate under these conditions was 110 mmol O_2/lt/hr in the first and 80 mmol O_2/lt/hr in the second vessel (measured by the sulphite method of Cooper, Fernstrom and Miller, 1944). The oxygen diffusion rate into the fermentation liquid was the same as in the laboratory experiments.

We used a rate of flow corresponding to the determined 3 and 4 hours doubling time. Two different plant strains were also used. Fig. 15 shows the course of the basic cultivation curves in the first fermentation vessel at a rate of flow equal to a doubling time of 3 hours. The results were similar to those obtained in the laboratory. In the second tank the results were also similar to those in the laboratory experiments, the alcohol content decreasing to below 0·1% and the yeast dry matter increasing to a steady state of 1·5%. This applied also for experiments with a rate of flow corresponding to a doubling time of 4 hours, exepting the fact that the equilibrium dry weight was already about 1·5% in the first vessel and the content of alcohol about 0·2—0·3%.

Table 5 shows the values for $Q^{N}_{CO_2}$ on glucose and maltose and for Q_{O_2} on maltose for yeast from the first fermentation vessel for both strains. Strain 1 was used for all fermentations in the laboratory. It can be seen that in these experiments not all relations found in laboratory cultivation could be confirm-

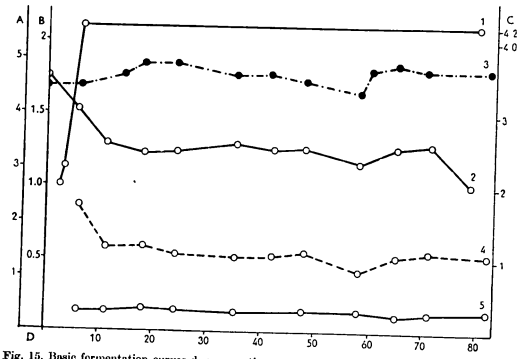

Fig. 15. Basic fermentation curves during continuous flow cultivation of baker's yeast on beet molasses wort containing 3% of invert sugar in a semi-pilot plant apparatus.
A — pH; B — % of dry matter, alcohol and invert sugar; C — flow of wort in l/hour; D — time in hours. Curves: 1 — flow of wort; 2 — % of dry matter; 3 — pH; 4 — % of alcohol; 5 — % of invert sugar.

ed. The $Q^{N}_{CO_2}$ values in strain 1 on glucose did not show such a deep decrease with a rate of flow corresponding to a doubling time of 4 hours and a drop of from 290 to 220, this being the fall found with a rate of flow corresponding to a doubling time of 3 hours in the laboratory. This was even more evident in the $Q^{N}_{CO_2}$ values on maltose. These decreased in experiments a higher rate of flow. The decrease in affinity to maltose could not, of course, be explained by the extent of multiplication. It was evident, however, (table 5) that even here in all cases a considerable ability to increase activity during maltose fermentation was preserved. Thus yeast appeared more active here than in laboratory experiments from the point of view of glucose and maltose fermentation. This can be explained by the fact that the preparation of the inoculum for laboratory experiments and for those with larger volumes differed fundamental-

Table 5

Q_{O_2} values on glucose and maltose and Q_{O_2} on maltose in yeast from the 1st fermentation vessel

Flow rate equivalent to doubling time	Hour of fermentation	Maltose $Q^{N}_{CO_2}$ 1	2	3	Maltose Q_{O_2} 1	2	3	Glucose $Q^{N}_{CO_2}$ 1	2
		Strain 1, one stage fermentation							
3	5	73 3	176·5	218·7	83·2	80·0	75·2	255·0	249·0
	15	117·8	202·0	210·0	104·0	77·7	61·0	256·5	249 0
	35	131·0	203·0	198·8	110·5	67·7	65·8	273·0	273·5
	65	97·7	202·0	224·0	109·5	67·0	58·0	314·0	318·5
	95	65·2	150·0	193·5	103·5	77·7	63·3	271·0	290·2
4·0	5	130·0	222·0	234·0	106·5	96·3	90·0	291·0	273·0
	17	151·5	181·5	163·5	113·5	74·7	60·8	215·—	223·—
	46	129·5	196·5	182·0	110·5	98·7	64·3	221·0	232·0
	72	140·0	170·5	173·5	88·7	72·7	56·7	—	—
	96	135·5	178·0	172·0	114·0	75·8	58·7	221·0	229 0
		Strain 2, two stage fermentation							
4·0	5	148·0	199·0	226·0	114·0	97·3	71·1	294·0	280 0
	15	142·5	231·0	222·0	139·5	105·0	87·9	315·0	293·0
	38	146·0	215 0	216·0	137 0	105·0	80 5	255·0	268 0
	62	135 0	222·0	228·0	143·0	107·5	90 0	285 0	298·0
	85	122·0	225·0	219·0	110·5	89 0	66·3	278 0	289 0

1, 2, 3 — values for 1st, 2nd and 3rd hour of manometric determination. The endogenous values are not subtracted.

ly. For laboratory experiments the inoculum was prepared by transferring a culture grown on 10° Bg malt wort to molasses wort fortified and containing 3% of invert sugar. Cultivation was carried out in a shaker. Pitching yeast for larger volume experiments was prepared on more concentrated wort of 14° Bg, a mixture of 14° Bg molasses wort and 14° Bg malt wort. This wort was inoculated with a corresponding volume of culture grown on 14° Bg malt wort and cultivated under slight aeration. These preparatory conditions probably affected the development of enzymatic systems responsible for glucose and maltose fermentation. Plant strain 2 showed even higher activity for glucose and maltose fermentation which it maintained for the whole time of the incubation period. The strain is not only somewhat different morphologically from strain 1, but is also cultivated differently under laboratory conditions and renewed from plant fermentation. The values for Q_{O_2} on maltose were about 110 for strain 1, i. e. about the same as in laboratory fermentation. They were somewhat higher in strain 2.

The values of the above mentioned tests for yeast in the second vessel showed a similar decrease in comparison to those of the first vessel, as was observed under laboratory conditions. $Q^{N}_{CO_2}$ on glucose fell to 190—200, on maltose to

about 50 with a small capacity under these conditions to increase the activity to 70 in the second and 80 in the third hour of the manometric determination. The values for Q_{O_2} remain at about 80—90.

The capacity of the apparatus used was planned so as to obtain an amount of yeast enabling pressing and thus quality tests (macrotests). We also studied baking strength and keeping properties of pressed yeast.

Table 6

Values for $Q^N_{CO_2}$ on glucose and maltose, Q_{O_2} on maltose and baking strength in pressed yeast from continuous flow cultivation

Flow rate equivalent to doubling time	Hour of fermentation	Baking strength min.	Maltose						Glucose	
			$Q^N_{CO_2}$			Q_{O_2}			$Q^N_{CO_2}$	
			1	2	3	1	2	3	1	2
		Strain 1, one stage fermentation								
3·—	15	138*	32·5	32·5	45·7	109·0	100·5	69·8	208·0	251·5
	35	145*	121·0	196·0	191·5	112·5	80·7	71·1	278·8	200·0
	63	135*	145·3	241·0	265·0	131·0	96·2	84·3	251·0	287·0
	95	140*	51·7	127·5	171·0	88·0	75·4	61·2	256·5	274·5
4,—	18	140	130·0	163·5	157·5	111·0	81·5	69·0	256·0	237·0
	43	130	122·5	141·5	156·0	99·5	76·4	61·3	217·0	197·5
	73	140	122·5	142·0	144·5	94·7	72·3	62 2	227·0	192·0
	95	125	74·7	137·0	147·5	93·7	67·4	48·3	183·0	185 5
		Strain 2, two stage fermentation								
4 —	18	145	61·9	83·3	102·5	89·5	69·0	51·6	226·0	240·0
	40	140	30 2	34·2	43·0	66·2	66·2	78·2	201·0	226·0
	66	145	38·9	45·3	70·5	68·3	53·2	48·5	196·5	205 0
	95	130	65·0	95·3	164·5	137·0	75·0	—	—	—

Determination made in pressed yeast kept for 24 hours at about 5 °C. 1, 2, 3 values for the 1st, 2nd and 3rd hour of manometric determination. The endogenous values have not been subtracted.
* Baking strength determined without addition of salt.

Table 6 shows the values for $Q^N_{CO_2}$ on maltose and glucose, for Q_{O_2} on maltose and the baking stregnth of pressed yeast. The values given in the first two experiments with strain 1 are from yeast separated and pressed from the 1st fermentation vessel. It was kept at 5 °C for 24 hours. Pressed yeast with both rates of flow maintains a considerable fermentation rate on glucose and maltose in comparison with yeast taken directly from the fermentation vessel. Yeast from the 15th hour of fermentation was an exception. The oxygen consumption on maltose was also high. The values for $Q^N_{CO_2}$ and Q_{O_2} on maltose decreased considerably and also the ability to increase the fermentation rate on maltose varied a lot in pressed yeast from the 2nd fermentation vessel. This was in agreement with the above results with yeast from the 2nd vessel.

The baking strength in the case of pressed yeast from flow cultivation is 125—145 minutes of dough test. The baking strength in the case of pressed yeast from a rate of flow corresponding to a doubling time of three hours was determined without the use of kitchen salt as it was found that this yeast was more osmosensitive than in other experiments. In other experiments the differences in baking strength were small with or without salt. The changes in baking strength did not correspond to the considerable variability of the $Q^N_{CO_2}$ values on maltose and to the difference in the ability, under these conditions, to increase the activity to this substrate. Thus baking strength was 140 min. in pressed yeast from the 15th hour of cultivation while the $Q^N_{CO_2}$ on maltose is 32·5. There was hardly any ability to increase the rate of maltose fermentation in this yeast. Yeast from the 95th hour also had a baking strength of 140 min. with a $Q^N_{CO_2}$ of 51·7 and a considerably increased rate of fermentation. Yeast from the 35th hour had a baking strength of 145 min. and a $Q^N_{CO_2}$ of 121 and also increased its affinity to maltose under these conditions. The same applied for pressed yeast from the second vessel. Here also yeast with a small maltose fermentation rate and a small ability to increase its activity to this substrate had the same baking strength as yeast with high $Q^N_{CO_2}$ values in the previous experiments. In all these cases the intensity of the aerobic breakdown of maltose, however, was higher than the intensity of its anaerobic fermentation. These relations are especially apparent on the graphical representation of the course of CO_2 evolution or oxygen consumption by this yeast. As an example we show the CO_2 evolution and oxygen consumption on maltose in pressed

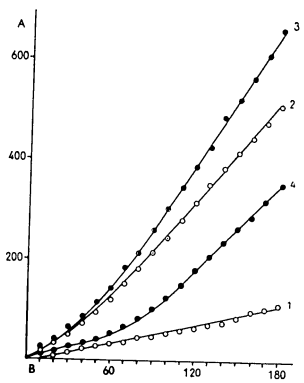

Fig. 16. Fermentation of maltose by pressed yeast from continuous flow cultivation at a flow rate equal to a doubling time of 3 hours.
A — µl CO_2/hour/mg dry matter; B — time in minutes. Curves: 1 — yeast from the 15th hour of cultivation; 2 — yeast from the 35th hour of cultivation; 3 — yeast from the 63rd hour of cultivation; 4 — yeast from the 95th hour of cultivation.

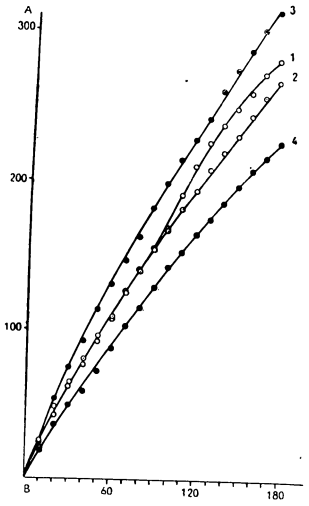

Fig. 17. Oxygen consumption of pressed yeast from continuous flow cultivation at a flow rate to a doubling time of 3 hours.

A — µl O_2/hour/mg. dry matter; B — time in minutes. Curves: 1 — yeast from the 15th hour of cultivation; 2 — yeast from the 35th hour of cultivation; 3 — yeast from the 63rd hour of cultivation; 4 — yeast from the 95th hour of cultivation.

yeast with a rate of flow corresponding to a doubling time of 3 hours. Fig. 16 shows the course of CO_2 evolution on maltose. Curve 1 is for yeast from the 15th cultivation hour and a baking strength of 138 minutes, curve 2 from the 35th hour of cultivation and a baking strength of 145 minutes, curve 3 from the 63rd hour and a baking strength of 135 minutes and curve 4 from the 95th hour with a baking strength of 140 minutes. The course of the curves indicates that the maltose fermentation increased gradually as the affinity to the substrate increased. The course of oxygen consumption, on the other hand, as shown in Fig. 17 was different. In all cases there was a high oxygen consumption and the difference between CO_2 evolution and oxygen consumption (Fig. 16, curve 1 and Fig. 17 curve 1) was especially evident in presed yeast from the 15th cultivation hour. Fig. 18 complete the picture of the fermentation ability in pressed yeast. It shows the CO_2 evolution during manometric determination of glucose fermentation. As can be seen from the curves the CO_2 evolution from glucose was rapid in all cases and on the whole varied only slightly.

In view of the disagreement between the baking strength and aerobic and anaerobic maltose fermentation in pressed yeast obtained during continuous flow cultivation we studied the same relationships in yeast from various stages of commercial production of baker's yeast by the feed method. The

results obtained are given in table 7. The baking strength in the 1st, 2nd and 3rd generation of pitching yeast are practically the same — 130 — 135. The fermentation rate of glucose is fundamentally high, but quite different from that of maltose. The fermentation of maltose is slight in the 1st generation and somewhat higher in the third and there is no ability to increase the fermentation rate. The 2nd generation has the highest fer-

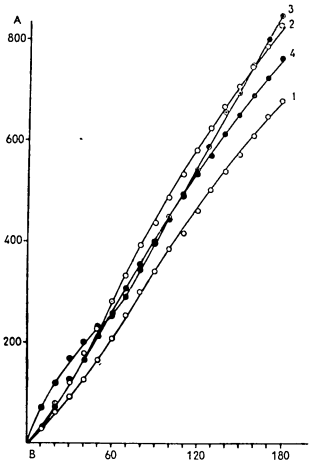

Fig. 18. Fermentation of glucose by pressed yeast from continuous flow cultivation at a flow rate equal to a doubling time of 3 hours.

A — µl CO_2/hour/mg. dry matter; B — time in minutes. Curves: 1 — yeast from the 15th hour of cultivation; 2 — from the 35th hour; 3 — from the 63rd hour; 4 — from the 95th hour of cultivation.

Table 7

Values for $Q^N_{CO_2}$ on maltose and glucose and for Q_{O_2} on maltose, baking strength of yeast from different stages of yeast production

Yeast	Baking strength min	Maltose						Glucose	
		$Q^N_{CO_2}$			Q_{O_2}			$Q^N_{CO_2}$	
		1	2	3	1	2	3	1	2
1st generation	135	9·2	4·1	6 1	48·0	57·0	73·0	238·0	221 0
2nd generation	130	57·1	79·3	125 0	59·5	57·9	55·5	282·0	240 0
3rd generation	130	31·7	27·5	27·5	41 0	41·0	37·3	203 0	190 0
Final stage									
5th hour of feed	—	45 0	76·8	123·0	78·8	86·0	67·6	288·0	253 0
10th hour of feed ...	—	18·5	14·2	13 1	37·5	44·7	45·0	174·5	144·0
12th hour of feed ...	—	17·6	15·1	14·1	32·0	32·0	40·8	148·0	124·5
Final stage	160	3 5	3·5	12·8	36·0	29 3	31·2	165·0	144·0

1, 2, 3 — values for 1st, 2nd and 3rd hour of manometric determination. The endogenous values are not subtracted.

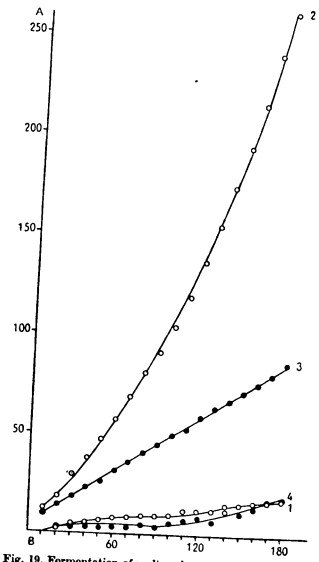

Fig. 19. Fermentation of maltose by yeast from different stages of yeast production by the feed method.
A — µl CO_2/hour/mg dry matter; B — time in minutes. Curves: 1 — yeast from the 1st generation; 2 — from the 2nd generation; 3 — from the 3rd generation; 4 — saleable yeast.

mentation rate of maltose and also has a considerably increased ability to ferment maltose as can be seen from Fig. 19 (curve 2) which shows the course of CO_2 evolution during anaerobic fermentation of maltose. In final saleable yeast, conditions are similar, as can be seen from curve 4, Fig. 19 and from table 7. The intensity of the aerobic breakdown of the maltose is in all cases greater than the rate of CO_2 evolution under anaerobic conditions (cf. table 7 and Fig. 20). This difference is especially considerable in the 1st generation of pitching yeast. It is evident from the $Q^N_{CO_2}$ values on glucose and maltose in yeast from different fermentation times during the production of saleable yeast that the fermentation ability develops in the 5th hour of flow. At that time there is also a considerable ability to increase activity during the anaerobic breakdown of maltose. In the 10th and 11th hours the fermentation rate of the maltose is small and the above ability has been lost.

It can be seen from a number of the above results that the fermentation of maltose by yeast and the ability to increase the fermentation rate both vary considerably. In a number of cases we found no relationship between the fermentation rate of maltose and the baking strength (within a range of 125—145 min.) Even in those cases, however, in which yeast with a good baking strength did not have the ability to ferment maltose more rapidly or

increase its activity under those conditions to this substrate, the intensity of the anaerobic breakdown of the maltose was great and was linear on the whole. We believe, therefore, that strictly anaerobic fermentation of maltose is not a reliable test related to the rising of flour. Under normal conditions of

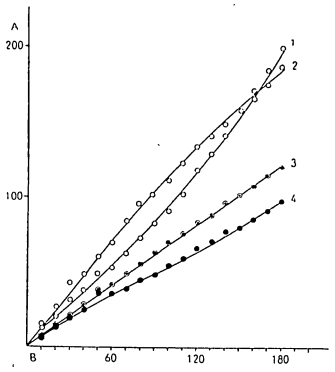

Fig. 20. Oxygen consumption on maltose by yeast from different stages of yeast production by the feed method.
A — µl CO_2/hour/mg dry matter; B — time in minutes. Curves: 1 — yeast from the 1st generation; 2 — yeast from the 2nd generation; 3 — yeast from the 3rd generation; 4 — saleable yeast.

determination of the fermentation rate of maltose by yeast the latter ferments maltose in an artifial substrate during shaking in a closed cultivation vessel from which air has not been expelled by an inert gas (White 1954). This difference in method may explain the difference between our results and those in literature. These conditions will, however, have to be verified. It must further be taken into consideration that yeast in dough ferments maltose only after the fermentation of saccharose and levosine. The fermentation of these substrate can favourably affect increased activity of the yeast for maltose fermentation as was shown by Blair (1954) in quantitative fermentation of maltose in the presence of a small amount of glucose. In addition flour contains substances stimulating the rate of adaptation of yeast. Thus the fermentation of maltose in flour may proceed more rapidly than in synthetic solutions. The

rate of such stimulated adaptation depends on the overall physiological state of the yeast, which was good in the mentioned cases. It is important, however, from the practical point of view that baking strengths of 125—145 minutes were obtained in pressed yeast from continuous flow cultivation. This indicates good baking properties.

Another sign of quality requested in good baker's yeast are the keeping properties. This was studied in pressed yeast from continuous flow cultures using two tests: the liquification of yeast in a Petri dish at 35 °C and the rate of autolysis. The keeping property as tested by liquification was 49 to 120 hours, mostly 75—80 hours. It is thus lower than in usually required for good yeast (120—144 hours). No conclusions can as yet be drawn from the results concerning the effect of cultivation length or rate of flow on the keeping property. The keeping property changed even in the same experiment but the lowest time mentioned was found only in yeast at the beginning of the experiment. The rate of autolysis was about the same as in the laboratory experiments. Here too the yeast had a higher autolytic rate at the beginning of cultivation 70 or 80% of broken down proteins — which settled down at various levels during the cultivation process. At a rate of flow corresponding to a doubling time of 4 hours they remained at 70% and for 3 hours and in strain 2 at 50 to 60%. At the moment we have no relationship between the keeping property and rate of autolysis. It is apparent, however, from the rate of autolysis that the keeping properties thus studied remain at a certain level during the experiment and do not change considerably. The relatively lower keeping properties found with the liquification test in a Petri dish can be explained mainly by the increased protein content in yeast of 50—52%. On the hole, however, the results indicate that a keeping property of 120 hours can be achieved if sufficient attention is paid to this problem.

Yields (grams of yeast dry matter from 100 g of saccharose in the wort) differed according to the rate of flow of the wort and according to whether a one-stage or two-stage cultivation process was used. In one stage cultivation and a rate of flow corresponding to a doubling time of four hours the yields were about 50% and at a rate of flow for a doubling time of 3 hours about 40%. In two stage cultivation and a rate of flow corresponding to a doubling time of 3 hours the yield was 51% and about 53% with a rate of flow corresponding to a doubling time of 4 hours.

MORPHOLOGY OF YEAST FROM CONTINUOUS FLOW CULTIVATION

In conclusion I should like to describe shortly a phenomenon observed in all our experiments. Morphological changes were observed in yeast cultivated by the continuous flow method. The inoculum for our laboratory experiments

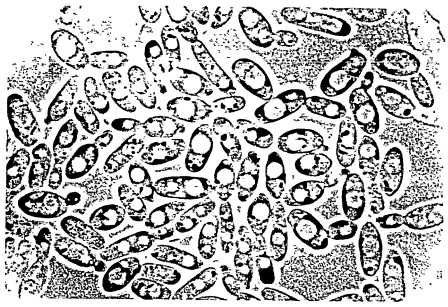

Fig. 22 Yeast from the 120th hour of continuous flow cultivation. Enlargement 1 : 1500 (photo J. Fuda).

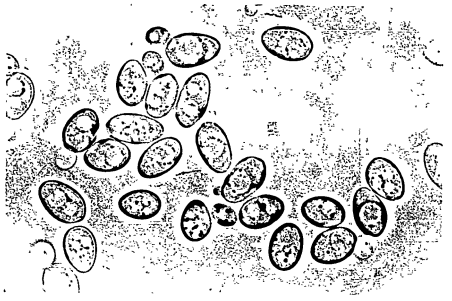

Fig. 21 Yeast used for inoculation of continuous flow fermentation. Enlargement 1 : 1500 (photo J. Fuda).

was prepared under shaking on molasses wort containing 3% of invert sugar. Under these conditions the fermenting cells of the inoculum were 7·38 × 5·04 μ in size and the proportion of their length to width was 1·46. Fig. 21 shows a microphotograph of yeast used for inoculation in our experiments. It is evident that these are normally sized yeast cells. Although it is known that in batch cultivation both the size and volume of yeast changes, the proportion of length to width is only slightly affected, usually changing to lower value. Also during feed this proportion changes only slightly from an average of 1·46 to 1·57.

Under our conditions during continuous flow cultivation of yeast, however, there are considerable changes in the shape of the yeast cells. During the feed phase of cultivation the size of the yeast cells is 6·86 × 4·38 μ (i. e. a proportion of 1·57) and this changes fairly rapidly during the flow phase to 8·34 × 4·06 μ, a proportion of 2·05. Fig. 22 is a microphotograph of yeast from the 120th hour of continuous flow cultivation at a rate equal to a doubling time of 3 hours. It can be seen that there are considerable morphological changes.

These changes in our experiments occurred at all flow rates with excessive or insufficient amounts of biotin, in experiments in which the wort was fortified with further vitamins and trace elements and in the second yeast strain used in semi-pilot experiments. We do not yet understand this phenomenon and are studying it further.

CONCLUSIONS

We have not found a more complete report in accessible literature concerning the basis of the process of continuous flow cultivation of yeast. It is true that this problem has already been worked out technologically, but the published reports do not pay attention to a detailed analysis of this process. This is perhaps due to the fact that at that time the theory of continuous processes had not yet been elaborated. A number of methods of flow cultivation of baker's yeast and their modifications have been patented. From these it is evident that they are based on empirical data and it is difficult to decide which process is the best. Perhaps that is also the reason why continuous flow cultivation of baker's yeast has not been applied to a large extent as yet. The results of these earlier reports, however indicated that fundamentally this process is realizable. In our work we studied, as far as possible all problems related to continuous flow cultivation of yeast, especially their physiology. In our opinion it is the physiology of yeast that indicates its quality as required for practical purposes. This is an atractive question as the process of the feed method of yeast production itself has not been worked out sufficiently.

Our results showed that beet molasses must be fortified with a certain amount of biotin during continuous flow cultivation of baker's yeast. In our conditions 1 μg of biotin per g. yeast grown proved the most suitable. The

significance of biotin for yeast growth is well known, yet we may assume that the requirement for biotin may be influenced by the strain of the yeast used. When biotin was added maximum yields were obtained in semi-pilot experiments equal to 51—52% as calculated per added invert sugar. This is the yield attainable by the feed method on good beet molasses. We did not attain the maximum yield reported for beet molasses fortified with biotin by White (1954). Also the results of the dough test for yeast from our continuous flow cultivation (125—145 minutes) and the stability of these values throughout 120 hours of cultivation indicate that continuous flow cultivation can be applied successfully. The problem of keeping properties, however, will have to be studied further and the results obtained indicate that this problem can be solved successfully. From the practical point of view the problem of an oxygen supply is also important during the continuous flow production of yeast. The results from the laboratory experiments of Maxon and Johnson (1953) are not sufficient. We are studying this problem further in our laboratory.

It follows from a comparison of the results of physiological changes in yeast from batch cultivation and those from continuous flow cultivation that the latter, at a more rapid flow rate, are in a physiological state corresponding to the end of the first part of the growth curve in batch cultivation. It is important practically and theoretically that at a flow rate conrresponding to the maximum yield of the apparatus the physiological state of the yeast is quite stable throughout the experiment. This was confirmed by Málek (1955) and was reported by Herbert (1956). It is characteristic of yeast from continuous flow cultivation at a more rapid flow rate that it can increase the rate of maltose fermentation. The ability to breakdown maltose anaerobically, however, is very variable, although aerobically this is not the case. This is especially evident from different samples of pressed yeast in which we found that strictly anaerobic fermentation of maltose is not a reliable test with regard to the dough test.

A further interesting fact follows from the above results: yeast of a defined physiological state which breaks down maltose aerobically loses the ability to do so anaerobically. At the same time the aerobic and anaerobic intensive breakdown of glucose is preserved. Thus the anaerobic breakdown of maltose in its aerliest stages must be completely different from the aerobic breakdown of the same sugar. It is the subject of further research whether this difference is due to physico-chemical conditions occurring during fermentation or whether it is due to different enzymatic equipment of the cell.

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STAT

THE EFFECT OF ENVIRONMENTAL FACTORS ON THE GROWTH RATE OF BAKER'S YEAST IN AERATED CONTINUOUS FLOW CULTURE

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A deeper study of industrial cultures in all their stages of development is interesting both from the theoretical as well as from the practical point of view and offers the possibility of controlling the life processes of microorganisms used in the fermentation industry. According to the teaching of Mičurin certain changes of mass transfer arise in a living organism under the influence of environment. Marked changes occur in yeast cells in a twelve-hour feed cycle under plant conditions with adequate aeration, where every hour the amount of yeast in the nutrient medium increases, while the nutrient content continuously decreases and finally vanishes.

Simultaneously metabolic wastes gradually accumulate in the medium as well as water soluble substances such as molasses non-sugars; this leads to a rise in the density of the medium. It is considered that during the twelve-hour aerated feed-culture with yeast of respiratory type a certain development cycle occurs — from the progressive growth of the young generation from the seed culture in the first four hours of cultivation to the violent multiplication of the yeast cells in the second period from the fourth to the eighth hour. In the last period it appears that the yeast cells end their developmental cycle and they pass over in the tenth hour to a state of maturity and quiescence. In cultivating yeast with continuous aeration of the medium and of feeding of nutrients, whose amount is increased at hourly intervals, the accumulation of the yeast mass occurs at different velocities in various phases of the twelve-hour process. This circumstance led to more thorough study of the physiological state of the yeast cells and of the influence on their fermentation activity of such environmental factors as the concentration of sugar in the medium and of the degree of aeration of the medium for various amounts of yeast in the fermentation tank. The goal of this work was to find suitable conditions for the long period flow culture of baker's yeast.

The experimental research work of I. P. Astachova concerning the consumption of oxygen in the accumulation of mass of *Saccharomyces* yeast during its flow culture on molasses preceded the work in the field of the biology of yeast. It was found by direct experiments that one gram of compressed yeast under these conditions consumes 80–100 mg of oxygen per hour at the maximum rate of growth. It is thus necessary to supply the growing yeast with assimilable oxygen in an amount corresponding to the amount of yeast found in the tank.

Clarification of our notions regarding the chemical mechanism of processes of sugar assimilation in relation to the increase of yeast mass during aeration of the medium as well as the comsumption of sugar in alcoholic fermentation helps us to find the optimum conditions for long period cultivation of yeast by the flow method. The mechanism of the accumulation of yeast mass has not previously been adequately studied. It has, however, been experimentally shown that the composition of the medium — the presence of metabolitic products and of growth substances — is of importance in relating the consumption of sugar to the growth and multiplication of yeast in media containing the necessary amount of oxygen. The growth substances are constituents of enzymes and coenzymes participating in the assimilation processes and in the accumulation of protoplasmic substances which occur by a non-fermentative process. Under these conditions accumulation of cell protoplasm occurs in deficient media in the initial stages of sugar decomposition, in the hexosemonophosphate state and in the conversion of the media sugar in the Krebs cycle with the formation of tricarboxylic acids.

Since the rate of growth of yeast in relation to the measured consumption of sugar and the increment of a given amount of yeast is very important for correct feeding of the growing yeast mass, our work was therefore directed to investigating this relationship. The purpose of our research was to compare the rate of the overall increase of the yeast mass in dependence on the consumption of sugar on the increment of yeast during each hour of growth under aerobic conditions. The overall amount of yeast, its concentration in grams per litre of medium, is certainly of importance for the consumption of sugar in relation to the metabolic reactions of yeast at various ages. The experimental work was therefore carried out in the laboratory experiments in fermentation equipment ensuring the cultivation of 30—35 g of yeast per litre of medium. Other concentrations of yeast up to 55 to 65 g/litre of medium were also tested under pilot plant conditions.

For the purpose of studying the effect of the nutrition regime of the yeast *Saccharomyces cerevisiae VII — T* on the rate of growth and multiplication we decided to carry out the cultivation with continuous outflow of part of the nutrient medium carrying yeast cells from the fermentation vessels. This could act favourably on the physiology of the yeast remaining in the fermenter. The volume decrement in the fermentation vessels was compensated by

feeding the appropriate amount of nutrient medium and water so that the amount of medium in the vessels remained constant.

The experiments in cultivating yeast were carried out in special fermentation vessels of a total volume of ten litres containing seven and a half litres of molasses medium which was aerated. The conditions for the cultivation of the yeast — the temperature and pH of the medium, the amount of inoculum and its quality, and the composition of the nutrient medium fed to the apparatus — were constant.

The following determinations were carried out in the course of the experiments:

1. Determination of the weight of compressed yeast with 75% water in samples taken every hour. The rate of growth of the yeast was calculated in percent of the weight of the yeast mass in the fermentation vessel at the beginning of every hour or per gram of yeast (specific rate).

2. Determination of the size of the cells by measuring their diameter. The percentage of large cells with a diameter of 10 to 12 μ, average with diameter 7 to 9 μ, small with diameter 4 to 6 μ, was determined by counting 100 yeast cells.

3. The rate of budding of the cells was determined in a microculture and the ratio of adult colonies was determined after a lapse of 16 to 18 hours, where the large ones contained more than 10 cells, the average ones consisted of 6 to 8, and the small ones contained 2 to 4 cells. Samples for inoculation in the microculture were removed every hour.

It was observed that large cells form large colonies of yeast containing 10 to 15 cells in the microculture. Cells of average size gave rise to colonies with 5 to 9 cells, while the small cells have colonies with 2 to 4. These were clearly young daughter cells which were still in the growth stage. Very young cells also occurred; these were still not capable of rapid growth and multiplication and did not bud after 16 to 18 hours.

4. The amount of sugar fed to the apparatus during one hour was determined and compared with the increment of yeast mass during this period. From this we found the ratio of the weight of sugar to that of the newly grown yeast.

5. In the first series of experiments the yeast was cultivated during a twelve hour period by the normal method used in the baker's yeast industry (Table I).

Comparison of the rate of growth of yeast with the morphology of the yeast cells and the consumption of sugar during various periods shows that the gradual deceleration of the rate of growth of yeast at the ninth hour corresponds to a decrease of the relative consumption of sugar at this period to 430 mg/g of yeast cells grown per hour. As a result the volume of 100 yeast cells changes and actually decreases from 11,400 μ^3 to 8,000 μ^3. The amount of small cells rapidly increases to 65%. The specific rate of growth of the yeast

Table 1

Change of the rate of growth of yeast with periodic feeding

Time hour	Molasses %	Amount of yeast		Increment %	Consumption of sugar in g		volume of 100 cells μ^3
		Overall g	Increment g/hr		g/hr	per g of yeast	
0	8	50	—	10	9·2	—	
1	—	55	7	12	—	—	
2	6	62	9	15	6·9	0·77	11300
3	7	71	11	17	8·05	0·73	
4	9	82	15	18	10·35	0·70	11400
5	10	97	20	20	11·5	0·57	—
6	11	117	28	24	12·6	0·45	10100
7	12	145	32	22	13·8	0·43	—
8	13	177	32	18	14·5	0·45	8300
9	14	209	29	14	16·5	0·57	—
10	10	258	14	6	11·5	0·8	8000
11	—	252	8	3	—	—	
12	—	260	—	—	—	—	

Total amount of yeast obtained: 260 grams.
Net yeast yield (inoculum substracted): 210 grams.
Yield of yeast: 80% based on molasses containing 46% of sugar.

mass decreases during the eleventh hour to 1·06 as a result of the inadequate supply of sugar and its effect on the increment of the yeast mass in the preceding hours of the cultivation.

The amount of newly grown yeast and the volume of the yeast cells gradually decreases towards the end of the twelfth hour of cultivation.

In the second series of experiments we extended the period of cultivation of yeast to fifty hours. The nutrition regime of the yeast was the same as in the first series of experiments in the first ten hours of the accumulation of yeast mass. In the succeeding hours (12 to fifty) the hourly feed of molasses was always the same, that is 31·5 g or 14·5 g of sugar per hour for 7·5 litres of medium.

The medium gradually flowed out of the apparatus at the rate of 14% of the total volume of medium (1 litre) per hour. At the same time new nutrient medium and water were fed into the apparatus, thus compensating the volume decrement.

A study of the morphology of the yeast cells and of the rate of their growth showed that under these conditions where the period of cultivation of the yeast is increased with an outflow of 14% of the volume of the apparatus per hour, the amount of small cells incapable of rapid growth gradually increases; during the twelfth hour small colonies with daughter cells predominate in the microculture. After 24 hours the volume of a hundred cells sharply decreases to 7,500 μ^3. Simultaneously the yeast concentration falls from 30 to 32 grams during twelve hours to 27 grams per litre.

With the same flow rate of medium the relative consumption of sugar with respect to the weight of the residual yeast increases and reaches 600 to 650 mg per gram of yeast increment. As a result of this the rate of growth of yeast

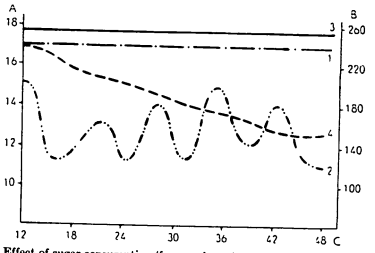

Fig. 1. Effect of sugar consumption (from molasses) on the rate of growth of yeast.
A — % increment of yeast; B — amount of yeast in grams; C — time in hours.
Increment of yeast per hour in percent: 1 — with adequate nutrition; 2 — with insufficient nutrition.
Amount of yeast in the equipment: 3 — with adequate nutrition; 4 — with insufficient nutrition.

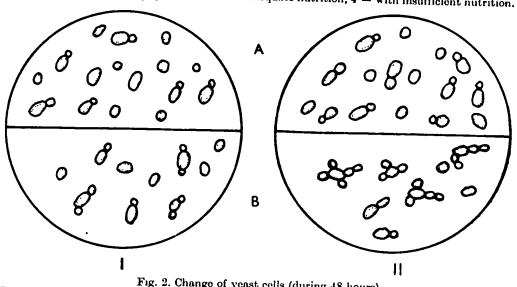

Fig. 2. Change of yeast cells (during 48 hours).
I — insufficiency of nutrients; II — normal amount of nutrients; A — microscopic examination
B — microculture.

gradually begins to increase towards the 24th hour, its total amount in the apparatus remaining constant for a certain period and then decreasing again towards the end of the second day (Table 2).

Table 2

Rate of growth of baker's yeast in flow culture with an insufficiency of nutrients

Time hours	Yeast Total in unit g	Increment of yeast		Sugar Consumption g/hr		Yeast quality	
		g/hr	% of total weight	Total	per g of yeast	volume of 100 cells in μ^3	dough test*) in min.
12	240	36	15	14·5	0·4	8500	13
14	236	37	13	14·5	0·45	—	—
16	231	25	11	14·5	0·55	8200	—
18	225	24	11	14·5	0·60	—	—
20	217	25	12	14·5	0·58	7500	13
22	209	24	12	14·5	0·6	/	—
24	205	24	12	14·5	0·6	7500	—
26	198	21	11	14·5	0·68		—
28	189	26	14	14·5	0·54	7300	16
30	185	24	13	14·5	0·6	/	—
32	181	23	13	14·5	0·63	7000	—
34	178	25	14	14·5	0·55	—	20
36	176	20	12	14·5	0·63	6500	
40	167	19	12	14·5	0·71	—	—
42	162	19	12	14·5	0·71	6500	28

Yeast obtained 732 + 110 = 842 g.
Yield of yeast 70·5 % based on molasses with 46 % of sugar.
*) Ostrovský's method.

The amount of yeast leaving the apparatus gradually drops: in one litre of medium flowing out this quantity there was first of all 32 grams, towards the end of the first day it did not exceed 27 grams, and after 36 hours of operation of the equipment, the amount of yeast fell to 22 grams.

The yeast increment during this period amounted to 19 to 20 grams per hour, i. e. at most 12% of the weight of the yeast in the unit does not compensate the outflow of yeast with the medium withdrawn. The total amount of yeast in the unit gradually falls under such a feeding regime and the rate of increment of yeast mass fluctuates (Fig. 1).

At the same time the morphology of the yeast changes rapidly. At the 42nd hour small cells predominate (Fig. 3), the amount of average cells is markedly lowered, and large cells are missing (Fig. 2—I.).

Samples of the yeast withdrawn after twelve and 48 hours required a different period of time for the dough test: the first 13 minutes and the second 28 minutes, which represents a more than two-fold decrease of the fermentation activity of the yeast.

Comparison of the volume of yeast cells with the consumption of sugar per hour with respect to the life processes of the yeast showed that the decrease of volume of the cells is caused by the inadequacy of the nutrition of the yeast; small yeast cells accumulate as is related in accordance with the changes of

their biochemical properties — with the loss of the rate of growth and the decrease of their fermentation activity.

The third series of experiments with long-period flow cultivation of yeast was related to changes of the rate of growth of the by raising the consumption

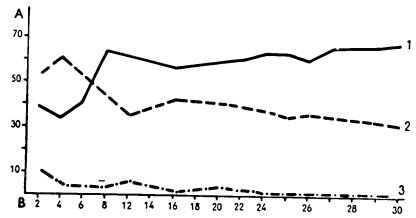

Fig. 3. Fluctuation in the % amounts of cells of various sizes with insufficient nutrition.
1 — small cells; 2 — medium cells; 3 — large cells; A — percent; B — time in hours.

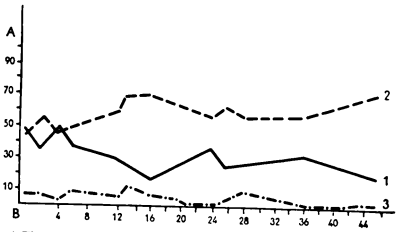

Fig. 4. Fluctuation in the % amounts of cells of various sizes with normal nutrition.
1 — small cells; 2 — medium cells; 3 — large cells; A — percent; B — time in hours.

of sugar per unit weight of yeast grown. A series of experiments was carried out on growing yeast with an increased amount of sugar up to 630—650 mg/g of yeast increment. At the same time the increment of yeast mass remained constant during the long cultivation and was characterised by a specific rate of growth of the yeast of 1·17/hr (Tabel 3).

In subsequent experiments with long period culture of yeast with correct feeding of the nutrient medium and of the calculated amount of sugar, the

Table 3
Rate of growth of baker's yeast in flow culture with a sufficiency of nutrient

Time hours	Yeast Total in unit	Increment of yeast		Sugar Consumption g/hr		Yeast quality	
		g/hr	% total weight	total	per g of yeast	volume of 100 cells in μ^3	dough test*) in min.
10	253	43	17	27·0	0·63	9500	—
11	253	43	17	27·0	0·63	9500	13
12	253	43	17	27·0	0·63	9500	—
24	253	43	17	27·0	0·63	9300	12
48	253	43	17	27·0	0·63	9500	—
60	253	50	20	31	0·62	9300	13
66	285	57	20	35	0·63	9300	—
72	285	57	20	35	0·63	9300	13

Yeast obtained 3025 g.
Yield of yeast 75 % based on molasses with 46 % of sugar.
*) Ostrovský's method.

increment of biological mass remained constant for 60 hours. At the same time the rate of growth of yeast was quite high as it had to compensate the decrement of yeast withdrawn from the unit each hour. The total amount of yeast in the unit also remained constant (250 g or 33—35 g/l).

It is interesting to note that during the period of the third day an acceleration of the growth of 1·2 to 1·23 was ascertained, which increased the amount of yeast in the fermentation vessel of 270 to 290 g; this state was maintained for a long time under the conditions that the nutrient medium was added in good time, that a sugar consumption of 630—650 mg/g of increment of yeast mass per hour was provided for and that the outflow of medium was accelerated to 1,5 l/hr. The morphology of the yeast in samples after 24—36—48—56 hrs. did not differ significantly. Yeast cells of medium size predominated (Fig. 2 — II). The small yeast cells had a constant concentration of about 38% of the total amount of yeast (Fig. 4).

Baker's yeast can multiply for a long period under conditions of aerated flow culture on molasses media by maintaining the correct feeding of nutrients calculated both for the increment of yeast as well as for the metabolic reactions of the growing yeast occurring in the fermentation vessel.

The total increment of the UP—T yeast strain amounted to 17% of the weight of yeast per hour where under long period cultivation cells of medium size (7—8 μ in diameter) prevailed for three days. These cells have a constant rate of growth and multiplication. The fermentation activity of the yeast did not change and the dough test on a sample of yeast obtained at the end of the third day was quite unchanged.

Good results were obtained in checking this procedure of yeast culture in pilot plant and plant scale units.

SOME ASPECTS OF CONTINUOUS CULTURE OF FOOD YEAST

Z. Fencl and M. Burger

In the period before the Second World War the first experiments were made in an endeavour to change the process of growth of microorganisms from the closed-system type (i. e. the type in which they grow according to the classic logarithmic curve) to the open-system type in which it is theoretically possible to extent a certain part of the logarithmic curve ad infinitum.

Among the first papers those of Utěnkov, from about 1922, Lebeděv (1916) and, independently, of Málek (from 1935) should be mentioned. Both Utěnkov and Málek were of the iopinion that by removing metabolic products and dying-off cells through the afflux of new substrate microorganisms could be prevented from passing from the logarithmic phase to the stationary one, continuous cell division thus being ensured. For instance, Málek (1943) proved with *Escherichia coli* that this microorganism retains the average rate of multiplication for a whole month without any observable degeneration. In his paper he pointed out also that together with the theoretical importance of, for example, the possibility of studying microbial physiology under dynamic conditions this method could have also practical importance, particularly in technical microbiology.

Lebeděv developed his method independently and systematically especially in the field of practical application (1936). In his work he studied conditions leading to the change of single-stage alcoholic fermentation into continuous flow fermentation. In his book he described laboratory and industrial equipment necessary for continuous flow alcoholic fermentation.

Development regarding the production of living matter in the form of production of food yeast was accelerated by the Second World War when a lack of foods and vitamins raised productional requirements, especially for *T. utilis*. For economic reasons the single-stage fermentation process was gradually replaced in production by the semi-continuous or continuous one. In England a type of continuous production of food yeast on the laboratory and industrial

scale was then developed (Anon., 1944). Sugar cane molasses served as raw material and the production itself was brought nearer its source. During the war it was Germany where production of food yeast on various raw materials was developed, particularly on cellulose production waste. The original single--stage flow production developed mainly by Fink was changed to the continuous type as a logical consequence of technical progress. There also a new method of introducing oxygen into the medium was materialized, using aeration and mechanical stirring. The new method of fermentation on this basis was applied by Waldhof & Co. and is now applied in a number of countries. The fermentation process was changed from the single-stage type to the continuous type mostly on a purely empirical basis without any detailed study of the physiological changes of the microorganisms and without any experiments investigating the possibility of mathematical and kinetic generalization of relationships existing in the continuous method of culture of microorganisms.

This task was taken up first by Monod (1950) and by several other workers such as Maxon, Johnson (1953), Maxon (1955) and others. A general report concerning these problems was presented by Gaden (1956). Mathematical calculations were based on the assumption of a steady state in the fermentor.

As follows from the literature given at the end of this paper, the steady state is limited only by the generation time of the microorganisms, provided that the entering medium is of full value as far as the microorganisms are concerned and that the other conditions of fermentation are optimal. The full value of the medium can be achieved by adding deficient nutrients. A sufficient amount of oxygen, which is one of the conditions of aerobic culture, can be provided by means of suitable transportable equipment (aerating and stirring). For practical application of continuous culture in the production of living matter, e. g. of *Torula*, it is, however, necessary for economic reasons to consider the source of carbon as the invariable factor together with the amount of accessible oxygen brought into the medium by means of efficient aeration usually connected with mechanical stirring. These two factors become then the limiting ones for the generation time of the yeast and thus also for the production of living matter. From the well-known relationships discussed by the above-named authors it is possible to deduce the conditions of maximum production fermentation. As has been shown experimentally the available substrate is then utilized successfully and the capacity of the production equipment exploited to its maximum. It is commonly known that during the batch process the yeast is not capable of utilizing the available sugar immediately and turning it into living matter, but rather that it glycolyzes it partly with the production of alcohol or its derivatives. As has been shown by Maxon and Johnson (1953) volatilization of these products during aeration leads to losses in this type of living matter production. The possibility of limiting the amount of substrate during continuous flow diminishes the losses caused by the formation

and evaporation of alcohol because the fermentation is arranged in such a way that the sugar added in the vessel is immediately assimilated so that its concentration is very low, usually not exceeding 0,1%. Stickland (1956) who, in studying the Pasteur effect, concerned himself also with the affect of sugar in medium on the production of alcohol, proved that the production of alcohol increases from 0,04 to 0,40 with a sugar concentration increased from 0,2 to 5%, while the amount of assimilated sugar remains approximately constant. In the first case 1·80 of sugar was assimilated in all, in the second case 2·20, i. e. only 22% more, whereas the production of alcohol was increased by 100%. It follows that under the given conditions of flow the formation of alcohol and thus also its losses due to evaporation are minimal. In this way the comparatively high yields of continuous culture as compared with those of batch culture can be explained. In our experiments (Málek et al. 1958) concerning the growth of *Torula* on molasses we achieved quite commonly yields of 64 to 65% of dry weight of yeast when related to the used amount of sugar. Agarwall (1949) almost reached these yields in his studies concerning the maximal yield of *Torula* on molasses using a batch culture in Erlenmeyer flasks in a vibrator without aeration of the medium by air and thus preventing losses of alcohol due to evaporation. This type of culture is, however, impossible on a larger scale. Also the duration of the culture process was too long (about 8 to 16 hours). In our case the content of the fermentor was renewed usually in the course of 3·5 to 4 hours. This rapidity constitutes one of the reasons why the continuous flow method is applied to an ever increasing extent in practice. The capacity of the equipment is exploited much more than in a single-stage (batch) culture. Maxon and Johnson (1953) maintain that in the fermentor of the Waldhof type 9·1 gr. of dry weight of *Torula* per litre can be obtained per hour. 13·2—15·5 gr. of dry weight per litre per hour is given as maximum. Such high economy of the capacity of the equipment can be achieved only by means of continuous culture when it is possible to limit the substrate concentration to obtain a permanently minimal level of sugar. The above-named authors applied a medium containing initially 10% of glucose and nevertheless its concentration in the fermentor medium did not exceed 0·1 gr. The fermentation process requires a high rate of passage of oxygen. In the given experiment, for instance, the rate was 310 millimoles O_2/hr per litre of substrate.

In one of our older papers (Leopold, Fencl 1955) concerning semi-continuous yeast growth on waste liquor from citric acid production we obtained 2·5% of dry weight of *Torula* in a "well-fermented" medium at a rate of 250 ml/litre per hour. We used organic acids for substrate-gluconic, citric and others — together with remainders of sugars (about 1·5%). The yield per litre of volume was 6·25 gr. of dry yeast per hour. We did not use the modern type of fermentor as fermentation vessel, but the so called Fink's cylinder with no stirring mechanism. The feed of substrate was not continuous but took place at one

hour intervals. Although this type of culture cannot eliminate the immediate increase of substrate concentration, which, as has been shown above, causes necessarily certain losses, our yields of dry weight per 100 ml of substrate were on the average 0·5% higher as compared with batch culture. The high concentration of raw material is of advantage for the separation of yeast in separators as it means also higher economy in exploiting their capacity. The attained high yields and high economy of the apparatus are interesting also because all the above — mentioned data of other authors refer to fermentation processes where hexose had the function of a carbon source. When other waste products, such as, for instance, sulphite liquors are used the yields and economy of the fermentation space are usually lower; mostly 45% of the total amount of sugar added.

So far we have studied mostly the type of fermentation process where a single organic substance (glucose) served as the source of carbon and where it is consequently relatively simple to reach a steady state even in one flask only.

It is well-known that a steady state is very difficult to establish in a single fermentor if the aim of the fermentation process is not just the production of microorganisms, but the formation of some other product, such as, for example, alcohol, antibiotics or microorganisms with certain qualities (e. g. baker's yeast), or if there are several sources of carbon assimilated at different rates. For such cases it is more convenient to use a battery of fermentors. In our case where the living matter production is concerned it is quite clear from theoretical considerations that one fermentor is sufficient for optimal production with the exception of cases when there are several sources of carbon present in the medium. Maxon (1955) points out that the generation period depends on the rate of assimilation of the substrate and on the optimal conditions of exploitation of the fermentation space. He maintains that if, for example, two sources of carbon are present in the medium, one of which is assimilated faster than the other, it is more convenient to profit from the different generation periods and to achieve a higher production of living matter in the same volume but in two fermentation flasks.

It is also known that sugars are assimilated by the yeast cell gradually, preference being given to glucose. Then, if in a single fermentation tank complete utilization of more utilizable sugars is desired, the rate of exchange of the medium must be adapted to the generation corresponding to the assimilation of those sugars the utilization of which is slower. This means practically that such a steady state must be established in the fermentation tank in which the level of preferentially assimilated sugars, e. g. glucose, is practically nil, and one of the slower assimilated sugars, e. g. galactose, determines the rate of flow. If we connect two flasks together in the first one mostly assimilation of glucose takes place. The rate of flow in this flask is proportional to the generation time of the yeast for glucose. In the second flask the other sugar

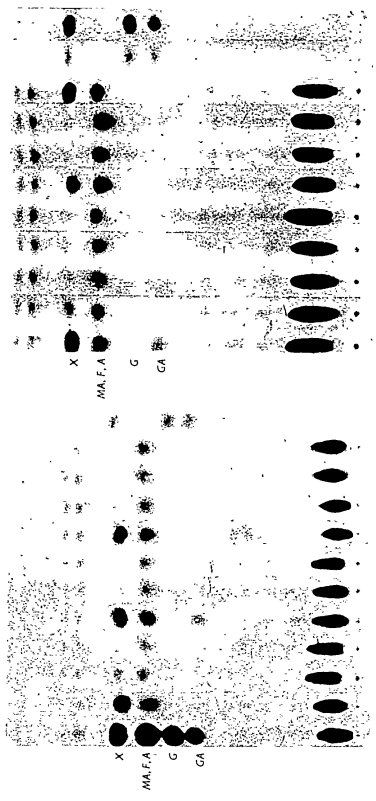

Fig. 1a

Fig. 1b

Assimilation of sugars by *Torulopsis utilis*, strain 4.

x: hours of culture; F_1, F_2, F_3: first, second and third fermenting vessel; J: collecting tank; ST: standard solution of galactose, glucose, mannose and xylose; X — xylose, A — arabinose, G — glucose, GA — galactose, S sample of sulphite liquor, MA — mannose, F — fructose,

is assimilated, e. g. galactose, and if the steady state is not to be disturbed the rate of flow in this flask must be proportional to the generation time of the yeast for galactose. As the generation time for glucose is with most yeasts shorter than that for galactose, it is possible to assimilate more substrate per unit of time with the same initial amount of yeast, i. e. that in the first fermentation flask the rate of multiplication of yeast and the living matter production is higher than in the second one and thus the capacity of fermentors is increased by the difference in increments between the first and the second flask, because if we split the fermentation process into two mutually independent parts the increment will be limited in each flask by the generation time corresponding to the rate of multiplication on galactose.

As far as we know these theoretical considerations have not yet been proved experimentally. It is possible to raise the objection that in assuming gradual assimilation of sugars they disregard the fact that with minimal concentrations of sugars, such as exist in the course of fermentation, the laws of polyauxia need not be necessarily valid. Thus Burger (1958) for instance, who studied the mechanism of polyauxia and who maintains that this phenomenon is caused by a mutual competition of the sugar at the yeast cell surface, used concentrations that were of a higher order of magnitude than those existing under the conditions of continuous flow culture. On the other hand, however, our experiments (Málek et al. 1957) with continuous yeast culture on sulphite liquors using *Torula* prove that the present sugars are assimilated gradually and thus our result can be taken as an indirect proof of the above-mentioned theory.

In order to elucidate this problem we should like to mention one of our experiments. Non-diluted sulphite liquor was heated to approximately 90 °C and then 1% $(NH_4)_2SO_4$, 0·05% cryst. $MgSO_4$, and 0·02% KCl were added. The liquid was decanted while warm, the pH of the clear medium adjusted to 5·5 and then sterilized for 45 minutes at an over-pressure of 1 atm. The pH dropped to 5·1 in the course of sterilization. The sulphite liquor was not treated any more so that fural and SO_2 remained in it. Phosphate in the form of KH_2PO_4 was added during the fermentation process in such an amount that its concentration with respect to the volume of the added substrate was 0·2%. After this treatment the sulphite liquor contained 3·2% of reducing substance, of which 2·6% were sugars, 0·016% fural, 0·015% free SO_2, 0·057% SO_2 bound by aldehydes, and 0·2% volatile acids estimated as acetic acid. As follows from the figure representing chromatographic analyses of sulphite liquors (Fig. 1 — S) the sugars present are a mixture of xylose, mannose, fructose, arabinose, glucose and galactose. We used the fermentation apparatus constructed by J. Ričica (1958). Its description is given in another report in this symposium. The rate of flow of oxygen was 120 millimoles per litre of substrate per hour. The substrate volume in the first flask was one litre. In the experiment three flasks

were connected but the fermentation process itself took place only in the first two while the third one served for control purposes. The rate of flow was 360 ml/hr. In 1 ml of "fermented" substrate there was 12·7 mg. of dry weight of yeast in the waste liquid from the second fermentation flask. The yield was maximally 49·2% of yeast dry weight related to the total sugar in the mash. Fig. 1 shows the chromatographic separation of sugars present in samples of substrate in the course of fermentation taken at different hours during the fermentation process. It follows from the figure that in the first flask glucose is always fully assimilated (F 1) and a marked decrease of those sugars can be observed there which overlap during chromatography and form a single spot containing mannose, fructose and arabinose. In some cases (F 1 at 9 hrs., 19 hrs. and 39 hrs.) the waste liquid from the first fermentor contains also galactose. Beside this sugar and the above mentioned remainders of sugars (mannose, fructose and arabinose), from which fructose is certainly preferentially assimilated while arabinose remains intact during the whole fermentation process, as shown by analytical tests, the waste from the first tank contains also xylose. Xylose and galactose are then completely assimilated in the second or third tank. The presence of these sugars in the waste from the first tank shows that even in the case of minimal concentrations of sugars in solution glucose is assimilated preferentially together apparently with fructose, and only then do the other sugars follow. This means that the generation time and the doubling time dependent on it are measured according to which sugars are assimilated in the first tank and which in the second one. Under these conditions a system of two interconnected flasks is able to ferment a higher amount of substrate per unit of time then two independently working fermentors, while the yield of dry matter per unit of substrate is identical. It is obvious that for determination of steady state conditions it is necessary to consider also the change in the dry weight of the yeast in the first and second tank and to choose their volumes in relation to the amount of sugar present. The aim of our study, however, did not lie in observing these changes and generation times and therefore our results can serve only as an indirect proof of the considered theory.

For a basic study it is not convenient to use sulphite liquors because together with sugars also volatile organic acids can be assimilated, the assimilation of which does not take place in polyauxia with respect to the sugars — acetic acid is assimilated together with sugar (Fencl 1958). For this reason the utilization of organic acids affects the generation time as an unknown factor and these problems can be solved only on an artificial substrate.

These conclusions can be modified to a certain extent by the adaptation of microorganisms in the course of continuous flow culture to various sugars whereby the generation time is affected in such a way that the generation times for both sugars are more alike. However, (Málek et al. 1957) we did not

obtain in the course of flow culture of T. utilis on sulphite waters any strain differing in its capacity to assimilate sugars present in the medium. Nor did we find any quantitative changes in adaptive systems catalyzing the assimilation of sugars. None of the microorganisms that we used was adapted to the assimilation of arabinose and in no case was this sugar utilized. The amount of arabinose remained practically the same in the third fermentation flask as in the second one, even in cases when it was present as the only sugar. A sugar very hard to assimilate by yeast (Lechner 1940), arabinose can be assimilated to a certain extent by adapted yeasts. It is, however, more significant that when a strain of *Torula* is used which is not adapted to galactose the concentration of this sugar in the medium does not change during the fermentation process.

This low ability of microorganisms to adapt themselves to a new substrate during their flow culture is probably to a certain extent connected with the above mentioned polyauxia which makes it impossible for an adaptive enzymatic system to be formed in such a short time in the last fermentation flask. Otherwise it would be impossible to explain the very fast formation of adaptive enzymes necessary for overcoming poisonous components of the substrate. In our experiments, for instance. *T. utilis* adapted itself to fural to such an extent that, after being placed on a substrate containing pre-hydrolyzates of deciduous tree wood with a much higher content of fural, it grew at once normally although before its passage through flow culture it did not grow at all on this substrate (Málek et al. 1957). Verbina (1955) also reached the conclusion that adaptation proceeds much faster in continuous culture; she studied the adaptation of yeast to some antiseptics such as sodium pentachlorophenolate by comparing the rates of adaptation in batch and flow cultures. She needed 76 days, i. e. 76 transfers of yeast at 24 hours intervals to a medium with a gradually increasing concentration of pentachlorophenolate in order to adapt the yeast to a concentration of 0·001% of antiseptic starting with 0·00025%. At a relatively slow rate of flow, when the content of the flask renews itself once in 24 hours, she reached the same effect in 17 days. In the first case she was compelled to inoculate the yeast anew every 4 to 25 days when transferring it on to an unchanged concentration of antiseptic if damage of the yeast was to be prevented. In the continuous flow experiment she increased the concentration of the antiseptic every 48 hours by 0·0001%. The acceleration of adaptation can be explained here by the fact that the yeast was in a physiologically active form.

The existing experience with continuous flow culture shows that this method has not only a number of advantages for industrial fermentation, but maked it also possible to study the physiology of microorganisms from new points of view. So far however, the majority of papers studying food yeast have been concerned almost exclusively either with empirical observation or mathematical

interpretations of flow kinetics or with the conditions of oxygen regime. Very little has been done with regard to the problem of the physiological requirements of yeasts in flow culture and to the state of the cell itself. It is obvious that intensified multiplication taking place under constant conditions must reveal all deficiencies of the medium much more easily than a batch culture. Thus, for instance, according to our experience it is necessary to supplement the medium with malt extract or corn-steep when *Torula* is grown continuously on molasses. An analogous, rather frequent occurrence is the formation of pseudomycelium in the fermentor. We have encountered it ourselves (Leopold, Fencl 1955) and it is described also by other authors (Fink 1948, Fink, Gailer 1954). Our work has shown that the formation of pseudomycelium is not caused only by a lack of oxygen in the upper part to the foaming substrate, but also by a change in the physiological state of the cell due to the effect of nutrients (Leopold, Fencl, Palivec 1954). These small deficiencies in microbial nutrition which do not manifest themselves in batch culture become fully apparent only during the intensified formation of living matter in flow culture when, through repeated multiplication, yeast loses supplies of some essential compounds if it is impossible to renew them from the medium. The study of physiological problems should be among the principal tasks in the field of continuous flow fermentations.

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SOME EXAMPLES OF CONTINUOUS CULTURE

K. R. Butlin

During the past 18 years, microbiologists at the Chemical Research Laboratory of the Department of Scientific and Industrial Research at Teddington, England, have engaged in various activities involving some form of continuous culture of microorganisms, either specifically for the production of chemicals or for producing masses of cell material. This paper deals with three of these activities and concentrates information which has hitherto been scattered in various journals not easily available. None of them involves continuous culture in the strict esoteric sense in which it is regarded by other contributors to this Symposium. Two of the subjects illustrate the practical application of "continuous" techniques, one with pure, one with mixed cultures; the other concerns an unusual continuous microbial process occurring in nature. The items, which will be considered in chronological order, are as follows:

1. The war-time production of food yeast at Teddington on laboratory and pilot plant scales, which was later developed on an industrial scale in Jamaica. This aerobic process involved a preliminary "incremental" period of growth with continuous addition of medium until the fermenter was full, followed by a continuous period at constant volume; pure culture techniques were employed, though they could not be fully maintained in the pilot plant.

2. The "continuous" production of elementary sulphur in Cyrenaican lakes, a natural process depending mainly on the combined action of two types of strictly anaerobic microorganisms: sulphate-reducing bacteria (*Desulphovibrio*) and photosynthetic sulphide-oxidizing bacteria (*Chlorobium* and *Chromatium*). The process involves an ecological community of the greatest interest, set in a primitive environment, which may well have been a feature of early geological times.

3. The production of hydrogen sulphide by anaerobic fermentation of raw sewage sludge enriched with calcium sulphate, using mixed cultures, unsterile conditions and semi-continuous techniques. This process has potencial industrial importance for the production of elementary sulphur.

STAT

PRODUCTION OF FOOD YEAST

Investigations on the production of food yeast, using *Torulopsis utilis*, were carried out at Teddington during the war years (1940—45) mainly with the object of providing a valuable protein rich in B vitamins for human consumption at a time when these were scarce. But even at that time long-term objectives were kept in mind. Perhaps the majority of people live on diets deficient in vitamin B, especially those whose staple foodstuffs are maize, polished rice and other carbohydrate foods, e. g. Africa, China, India, Latin America. This widespread deficiency could be corrected, at least partially, by the production of food yeast from many waste products or from surplus agricultural products and so lead to a great increase in health and efficiency. In particular, local surpluses of sugar and molasses in many tropical and subtropical areas could be utilized for yeast production with great benefit for the people of those areas. The main reason for past failures to exploit this process successfully was not insufficient need or demand, but failure to produce the yeast at a sufficiently low cost, i. e. efficiently. It is in this respect that an efficient continuous process would be most likely to help, and it is for this reason that our earlier and rather crude attempts to develop continuous production of food yeast are described here. The very considerable progress made during the past few years in both theoretical and technical knowledge of continuous culture may lead to a solution of this important problem. Allied to it is that of the production of fat by yeasts and other microorganisms, to which Kleinzeller (1944, 1948) has made such a notable contribution. The author recalls with pleasure the fact that Dr Kleinzeller carried out some of his early work with *Torulopsis lipofera* at the Teddington laboratories, using the apparatus and plant described below.

Detailed descriptions of the equipment and methods of operation employed at Teddington and at Jamaica have been published (Anon., 1944; Thaysen, 1957). The subject has been reviewed by Dunn (1952) and White (1954).

LABORATORY AND PILOT PLANT INVESTIGATIONS AT TEDDINGTON

Apparatus and equipment

Laboratory

The fermenter consisted of a pyrex glass cylinder 12 cm in diameter and 80 cm high, with a total volume of 10 litres giving a 7 litre working capacity. The cylinder was mounted on an aluminium base, fitted with an outlet tube for sampling and emptying and an air inlet. Compressed air (25 litres/minute)

was passed through a sterile cotton wool filter and then through three ceramic blocks, mounted on the base, into the fermenting liquid, producing minute bubbles about 10 microns in diameter. The cylinder was closed at the top by an aluminium disc with inlets for adding molasses solution and nutrient salts, and holes for a thermometer and an aluminium cooling coil. In later models, all aluminium was replaced by stainless steel. The apparatus was sterilized internally by passing steam through the porous blocks for an hour.

Pilot plant

The fermenter was a cylindrical vessel (diameter 85 cm, height 3·75 metres) constructed of aluminium, of a total volume of 400 gallons (1800 litres) and a 300 gallon working capacity. Air was blown through 8 ceramic candles (30 cm × 5 cm diameter) at the rate of about 1000 l/min. The temperature (30 °C) was regulated by water flowing through aluminium coils. The vessel was sterilised by covering the candles with water, injecting live steam and boiling for an hour. These were vessels for boiling and settling the molasses solutions.

Description of process

Organism

Torulopsis utilis was normally used. A special strain, *T. utilis var. major*, approximately double the size of the ordinary strain, was developed by being grown in the presence of camphor (Thaysen, Morris, 1947), but it was unstable and reverted to its normal size during fermentation, though Thaysen claims (1957, p. 174) that the larger strain remained permanent for at least 7 years (this may have been a later isolate). *T. utilis* was chosen because it was known to grow easily and was rich in vitamin B. Perhaps other organisms, e. g. *Endomyces vernalis*, *Candida arborea*, *Oidium lactis*, may prove to be better for certain desirable specific properties.

Raw materials

The chief raw material used was cane molasses, since it was decided quite early on that any large-scale production should take place in a sugar-producing area such as the West Indies: The choice of cane molasses was fortunate, since it is rich in biotin, which is needed for maximum growth (White and Munns, 1950). Many other materials were tried, some in the pilot plant, with considerable though varying degrees of success, e. g. potatoes, bracken, straw, waste bananas.

General procedure

The two essencial conditions for the maximum production of yeast are intense aeration and low sugar concentrations. If these are not observed, alcohol is produced at the expense of yeast. There can be considerable variations in the method of maintaining these conditions. The method adopted at Teddington was based on the industrial process for producing baker's yeast.

Incremental growth phase

The fermentation vessel was halffilled with molasses solution containing about 0·5% of sugar to which phosphate (as a filtered solution of arsenic-free triple superphosphate) and ammonia were added to bring the concentration of phosphorus (as P) to 0,006% and that of nitrogen (as N) to 0·024%. The pH was adjusted to 4·5, the wort being inoculated with sufficient *T. utilis* to give a population of 100—200 × 10^6 cells/ml and aerated vigorously for 1—2 hours. The yeast was then assumed to be in an active growth phase and "incremental" addition of stronger wort containing 10% sugar was started. It was known from preliminary experiments that the weight of yeast cell material could, in favourable conditions, be increased hourly by a factor of 1·4 and that a 60% conversion of sugar to protein could be achieved. If, therefore, it was expected that 10 g of new yeast cells would be produced during the first hour of incremental growth, 16·7 g sugar was supplied. During the second hour, 16·7 × × 1·4 = 23·38 g sugar was added and so on throughout the "incremental period". Addition of nitrogen and of phosphate were increased hourly by the same factor 1·4, the inicial requirements being calculated by analysis of the yeast cells. In practice, the molasses solution was run in continuously, the rate being increased hourly, but the other nutrients were added in batches hourly. Hourly determinations of "formol nitrogen" and of pH were made. The pH was kept at about 4·2—4·5 and was normally controlled by an addition of sodium hydroxide; but if more nitrogen was required, as indicated by the formol figure, ammonia was used. The fermentation was carried out at 30 °C and continued until the limit of capacity of the fermenter was reached, usually in 8—9 hours; the yeast was then harvested or the "continuous" phase was started (see below). The final population reached at the end of the "incremental" period was usually about 2000—2200 million yeast cells/ml; beyond this point the percentage conversion of sugar to protein decreased. The final wort contained 2·0—2·3% (dry weight) of yeast and the inicial inoculum had multiplied about 20 times.

"Continuous" growth phase

Every hour a quarter of the total value of fermenting wort was run off and replaced by an equal volume of molasses containing 4·5% of sugar, together with appropriate amounts of nitrogen and phosphorus. In the circumstances prevailing at the time of the experiments — shortage of labour, lack of facilities for preparing sufficient wort etc. — it was impossible to keep the continuous process going for more than 1—2 days. It was still vigorous and comparatively free from infection at the end of this period, and later experience on an industrial scale in Jamaica showed that continuous runs of seven days were normally possible.

Harvesting and drying

In the pilot plant experiments, the final liquor was centrifuged, the cream being washed with clean cold water and centrifuged again. The resulting yeast suspension was immediately passed over rollers heated by steam, from which it emerged as a light-yellow powder containing about 5% of moisture.

In the light of modern fermentation techniques and recent developments in continuous culture, the methods described above appear to be both complicated (the "incremental" phase) and crude and "rule of thumb". They had the merit, however, of working smoothly, of being easy to control, and of giving comparatively high yields of yeast; but this was probably because yeast is easy tu manipulate and cane molasses an excellent medium for growth.

COMMERCIAL PRODUCTION OF FOOD YEAST IN JAMAICA

The results of the pilot plant experiments at Teddington were sufficiently encouraging to enable the British Government to finance the erection of a commercial food yeast plant in Jamaica, British West Indies. The West Indies Sugar Company of London acted as technical and commercial agents for the undertaking. The plant and equipment have been fully described elsewhere (Thaysen, 1957; p. 186; Floro et al., 1948) and only a short summary will be given here.

The fermentation process was based on that used in the pilot plant at Teddington. The fermentation plant consisted of ten stainless steel vessels, each with a total volume of 3000 gallons and a fermenting capacity of 2000 gallons (9000 litres), fitted with stainless steel water coils for temperature control. Aeration was conducted through „Porosent" phosphobronze candles capable of delivering 350 c. ft. (10 000 litres) of air per minute. Molasses solutions and chemical nutrients were supplied from overhead constant-head tanks through calibrated nozzles which permitted any mixture of molasses and other nutrients

to be supplied at any desired constant rate during both the "incremental" and continuous growth periods.

The fermentation was carried out at 30 °C and at pH values between 3·9 and 4·4. After building up the yeast population by the "incremental" method as described above, the continuous process was started, 25 per cent of the fermenting liquor being replaced each hour. It was possible to maintain this continuous process for periods as long as one week. Very little trouble was experienced with infections, and the principle factor limiting the duration of the fermentation process was clogging up of the candles, which led to inefficient aeration. The final product, after centrifuging, washing and drying was a "light cream-coloured powder, having a pleasant nutty flavour and containing 45—50% of protein, 2% of phosphoric acid and the whole range of B-vitamins in balanced proportions (aneurin, 20 micrograms per gram; riboflavin, 60; nicotinic acid, 400). (Floro et al, 1948.)"

While in some respects the process showed promise, it was not found possible to produce the yeast at sufficiently low cost; the yields were not as high as those obtained with the pilot plant and the costs of aeration, power and steam were considerably greater than had been estimated. For these and other reasons it was decided to cease production. It should be remembered however, that the methods employed were comparatively rudimentary. Considerable progress has since been made in fermentation technology, efficiency of aeration and knowledge of continuous culture. If these were applied to the production of yeast, it is quite probable that the process could be made economically successful. What is certain is that there would be almost an unlimited demand for this product if it were produced cheaply enough.

CONTINUOUS PRODUCTION OF SULPHUR IN CYRENAICAN LAKES

In May 1950 the author had the opportunity of examining the production of elementary sulphur in several Cyrenaican lakes. These lay in a remote area, a stretch of desert and salt marshes south-west of El Agheila, which is on the Gulf of Sirte, 200 miles south-west of Benghazi. Four lakes were examined: one, though smelling strongly of hydrogen sulphide, produced virtually no sulphur; from the other three about 200—300 tons of sulphur were recovered annually by local Arabs using hand dredges made of hemp, though the total amount formed would be larger. The lakes were small; the largest, which was circular, being about 100 metres in diameter.

The appearance of the sulphur-producing lakes was most striking. The main body of water, in brilliant sunshine reflected a vivid milky blue, though a bottle sample was virtually colourless with a slight haze. Bordering the blue was an

uneven band of red gelatinous material, stretching in some places several yards from the banks in shallow water. Bulbous formations could be seen in this red material and a few red masses were floating in the water There was a strong smell of hydrogen sulphide. A deposit of finely divided sulphur, 15—25 cm in depth covered the bottom of the lakes, and large lumps of calcium sulphate were also found there. The temperatures of the water in the lakes varied within the range of 30—34 °C and in each case the water was overflowing into the surrounding desert, showing that the lakes were continuously fed by warm springs. The fourth lake, producing no sulphur, contained none of the red material.

A detailed account of our investigations, both in Cyrenaica and subsequently at the Chemical Research Laboratory, Teddington has appeared elsewhere (Butlin, 1954) and can be summarized as follows. The water was saturated with calcium sulphate and contained various other mineral salts, but only traces of organic materials. Numerous sulphate-reducing bacteria were present. The red gelatinous material bordering the lakes consisted of masses of coloured photosynthetic bacteria, mainly *Chromatium* (red) and *Chlorobium* (green). All these organisms are obligate anaerobes. Blue-green algae, fish (*Cyprinodon*) and a few aerobic bacteria were also present. Our observations, supported by many experiments carried out later with mixtures of pure cultures of the organism concerned, led to the conclusion that most of the sulphur was produced by a combination of two microbial reaction: 1. sulphate-reducing bacteria converted sulphate to sulphide which 2. the coloured photosynthetic bacteria oxidized to sulphur. At first it was not at all clear how the sulphate-reducing bacteria obtained energy for the reduction of sulphate, since the organic content of the water was very low. Experiments with mixtures of pure cultures of sulphate-reducing bacteria and of *Chlorobium* and *Chromatium* in suitable culture media with no carbon source other than $NaHCO_3$ showed that *Chlorobium* or *Chromatium*, which can satisfy their own carbon requirements by photosynthesis from CO_2, are also capable of providing suitable organic materials for the sulphate reducers. Thus, for the production of sulphur in the lakes, only inorganic compounds were necessary, with sunlight as the primary source of energy. It is possible, of course, that the continuous supply of organic matter (however small) from the springs, as well as any hydrogen evolved by microbial action from the bottom of the lakes, contributed to the final result. Also, atmospheric oxidation of sulphide was undoubtedly responsible for some of the sulphur, but only for a very small amount, as shown by the small quantities of sulphur produced in the fourth lake mentioned above.

The point of interest to this Symposium is that, in a remote desert area, sulphur is being produced continuously by microbial reactions. In its simplest form we can consider it as proceeding according to the following sequence:

$$\text{Sulphate} \xrightarrow{\textit{Desulphovibrio}} \text{sulphide} \xrightarrow[\textit{Chromatium}]{\textit{Chlorobium}} \text{sulphur}$$

in near autotrophic conditions, using sunlight as the primary source of energy. The process is not, of course, strictly continuous. It is complicated by darkness, climatic conditions, adventitious addition of organic matter (foliage, animal and bird excrement etc). Also, Chromatium is able to carry the oxidation of sulphide, through sulphur, to sulphate, and thus part of the process becomes continuous in another, cyclical, sense.

We have been able to reproduce these reactions in the laboratory using mixtures of pure cultures of sulphate-reducing and photosynthetic sulphide-oxidizing bacteria in batch processes. Investigations using modern techniques of continuous culture would be not only be most interesting, but might well be profitable.

MICROBIAL PRODUCTION OF SULPHIDE FROM SEWAGE SLUDGE

The United Kingdom is largely dependent on imports for its sulphur supplies, both of elementary sulphur and pyrites. Should these fail, the economy of the country would be seriously affected. This problem caused some concern during the war (1939—45), but the situation became much more serious in 1950, when imports of elementary sulphur were curtailed. Many schemes to lessen our dependence on imports were then discussed, and one of them was the possibility of using bacteria to produce sulphur from indigenous materials. It was mainly for this reason that the natural production of comparatively large quantities of sulphur in the Cyrenaican lakes, described in the preceeding section, was investigated in the hope that it would give useful information for industrial application. It was quite obvious, however, that the mechanism of sulphur formation in the lakes, depending as it did for its energy on sunlight, could not be applied in the United Kingdom, where cloud and fog are more common than sunshine; but the mass action of sulphate reducers in the lakes encouraged the hope that these organisms might be used industrially. They had been studied at the Chemical Research Laboratory at Teddington for nearly 20 years, mostly from the point of view of preventing them from growing: they cause serious losses by corroding buried metal pipes and can be a great nuisance in causing noxious smells (Butlin, 1949). In past years, however, considerable progress had been made in growing them rapidly and in quantity and with high activity in producing hydrogen sulphide. If hydrogen sulphide could be produced economically, its oxidation to sulphur (or to sulphuric acid) could safely be left to the chemical engineers, who had devised highly efficient industrial processes for it. The microbial problem therefore

resolved itself into evolving an economic process for producing hydrogen sulphide from sulphate by means of sulphate-reducing bacteria.

Everything had to be cheap — raw materials, plant, running costs — for sulphur is itself a cheap raw material. Moreover, the raw materials had to be available regularly and in sufficient quantities to make tens of thousands of tons a year, for it was of little use to make less. Two main materials were required: sulphate, and an organic reducing agent to serve as a source of energy for the reduction and as a growth medium for the bacteria. Supplies of sulphate presented little difficulty. Not only is England largely built on calcium sulphate (gypsum or anhydrite), but large quantities are available as industrial wastes. The search for suitable organic reducing materials was much less simple. Some potentially useful sources were only available in relatively small quantities or their supply was unreliable or erratic e. g. waste effluents from the fermentation industries. In this connection it is most interesting to find that conditions in Czechoslovakia are different from those in Great Britain, for Grégr (1957) describes an efficient process for the production of hydrogen sulphide from the waste waters of yeast plants; these waste waters were chosen because of their relatively high control of sulphate and organic matter. After an exhaustive survey of all the waste materials available in Great Britain, the conclusion was reached that the only material suitable biologically, likely to be economic and available in large enough quantities was raw sewage sludge and calcium sulphate.

LABORATORY EXPERIMENTS

The first results were not encouraging. Experiments with sterilized sludge enriched with sulphate and inoculated anaerobically at 30 °C with pure cultures of sulphate reducing bacteria yielded only traces of sulphide. We then tried unsterilized sludge and crude cultures of sulphate reducers obtained from sewage. This produced some sulphide but it took over six month to yield 1% of sulphide (as S) calculated on the weight of sludge used. However, by repeated subculture, cultures of greatly increased activity were developed; the rate of production of sulphide was eventually increased to 1% in five days. All these results were obtained in batch experiments, but it soon became apparent that it would be more advantegeous to work out a semi-continuous method similar to that practised in some large sewage works for the production of methane. In this process, a certain proportion (usually $\frac{1}{10}$ or $\frac{1}{15}$) of the fermenting sludge is removed daily and an equal quantity of raw sludge is added. Eventually a laboratory process was developed on these lines but adapted to the production of sulphide (Butlin et al., 1956). The fermenting vessel held 1 litre of sludge enriched with 5% of $CaSO_4 . 2 H_2O$, and each day a proportion was removed

and replaced by an equal volume of raw sludge (with 5% of $CaSO_4 . 2 H_2O$). The sulphide produced was swept out, as hydrogen sulphide, by a continuous stream of a suitable gas, either methane or nitrogen containing about 30% of CO_2. The pH remained at $7 \cdot 1 \pm 0 \cdot 1$ without adjustment. The final gas mixture (usually 5—10% H_2S; 60—65% CH_4, 30% CO_2) was collected over acidified saturated brine in inverted graduated separating funnels. The yields of H_2S depended both on the rate at which the sweeping gas was blown through the sludge and on the proportion of sludge removed and replaced each day. If a quicker turnover of sludge — desirable economically — was required a more rapid rate of sweeping was necessary to give yields equal to those obtained with a slower turnover of sludge. For example (and very approximately), to obtain similar yields for turnover periods of 10 and 20 days, it was necessary to sweep the fermenting sludge with 12 times its volume of gas per day for the day period, compared with 8 times for the 20 day turnover period.

In general, these experiments indicated that a yield of H_2S of at least 1%, which sometimes rose to 1·8%, depending on the "quality" of the sludge, could be expected, i. e. 100 tons of wet sludge, containing 5% of solids, should yield at least one ton of sulphur. Moreover, the process worked smoothly, was not sensitive to normal variations in conditions and the fermentation process revived quickly if interrupted by mechanical difficulties. The semi-continuous process could be kept up almost indefinitely: one such fermentation has been operated "continuously" for every 3 years and is still as active as ever. No sterilization of apparatus or materials was needed. No attempt was made in these laboratory experiments to make the fermentation fully continuous, for the simple reason that sewage sludge will not move easily and continuously through the comparatively narrow tubes used.

PILOT PLANT TRIALS

Because of the encouraging results obtained in the Teddington laboratory, it was decided to develop the process on a larger scale. The London County Council authorities kindly agreed to carry out experiments at the Northern Outfall Sewage Disposal Works at Beckton, East London. An experimental fermentation plant was modified for this purpose. The present plant consists of a fermentation vessel of a 50 gallon (225 litre) capacity, fitted with heating coils, a stirrer, a gas diffuser, storage tanks and a gas holder. The mode of operation is essentially the same as with the laboratory experiments. The fermentation of the raw sewage sludge enriched with calcium sulphate is carried out at 30 °C, the process is semi — continuous (with turnover periods of 10 or 20 days, as required) and H_2S is swept out by passing normal "sludge gas" (70% CH_4 + 30% CO_2) through the gas diffuser.

The tests carried out on the pilot plant during the past year have substantially confirmed the results obtained in the Teddington laboratory experiments. Yields vary somewhat with the nature of the sludge, but normally lie between 1·0 and 1·5% (i. e. 100 tons of sludge should yield 1—1·5 tons of sulphur) in the case of a 10-day turnover period. At this stage no attempt is made to recover elemental sulphur from the final gas mixture (5—10% H_2S, 60—65% CH_4, 20—30% CO_2). Neither has the process been made fully continuous. But no great difficulty is anticipated on either of these points, which will be easier to introduce during the next stage of development of the process.

The pilot plant experiments also confirmed what had been suspected in laboratory experiments, viz. that the sulphide fermentation process caused the final sludge to settle better than it did after the methane method of fermentation. While this has nothing to do with the actual microbial production of sulphur, it could well have a decisive influence on its economics, for after fermentation the sludge (whether for methane or sulphide) must either be dried or transported somewhere; and the less water there is the better. This adventitious effect of the sulphide process would save sewage authorities very considerable sums of money.

SEMISCALE TRIALS

Arrangements are now being made for trials on a semi-scale plant, using a 100,000 gallon (450,000 litre) fermenter. Sulphur will be recovered by one of the well known processes, e. g. the Girbitol process, for which a plant can be bought "off the shelf"; it is very efficient and needs little control.

Here we may perhaps be allowed to indulge in some speculation, which the author is careful not to confuse with reality. A very large sewage disposal works handling 6000 tons of raw sludge daily (as at Beckton) could, if it were decided to use all of it for making sulphur, produce 60 tons a day or over 20,000 tons a year. If it were possible to use all the sludge in the United Kingdom, one million tons could be made annually in this way. This is manifestly impossible since most of the sludge is produced at various centres in amounts too small for economic processing, but an annual total of 100,000—150,000 tons from the larger centres of population is not out of the question. These figures, admittedly speculative, give some idea of what could be done if the need arose.

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USE OF CONTINUOUS CULTURE METHOD FOR ALCOHOLIC FERMENTATION OF MASHES OF SOFT WOOD HYDROLYSATE

K. P. Andreev

The classical contributions of S. V. Lebedev (1936) have opened great perspectives in the industry for the continuous fermentation method. This method has become the main method of alcohol fermentation of sugar containing substrates from raw materials of no nutritional value (wood hydrolysates, agricultural waste, sulphite liquor; Šarkov 1950, Kaljužnyj 1955, Kaljužnyj, Andreev 1956). Finally, continuous fermentation is being used in production where molasses and starch-containing raw materials are employed (Gladkij 1949, Berenstein, Skoblo, Guljajev 1953, Jarovenko 1948).

Both the multiplication capacity of yeast and its fermentation are simultaneously established during the continuous fermentation process in the fermentor. At the same time the growing yeast is continuously removed from the fermentor with the spent mash. The continuous fermentation is mainly characterized by the constant conditions of existence of the yeast. The whole procedure of the alcohol fermentation takes practically place in one tank, i. e. the first, where approx. 85—90% of the fermentable sugar is used up. Therefore the yeast remains a considerable time in the substrate which contains great quantities of alcohol and only little sugar. Under these conditions the rate of yeast multiplication is greatly reduced.

According to the observations of M. J. Kaljužnyj (1948), in plants producing alcohol from hydrolysates and sulphite liquor the amount of sugar fermented per hour by 1 kg of yeast is relatively constant for each type of yeast.

It was found by E. E. Drubljanec in a hydrolysate plant that, for example, 4—7 kg of sugar are fermented by 1 kg of *Saccharomyces* yeast, and 7—11 kg of sugar by one kg of *Schizosaccharomyces*. Therefore the regulation of the fermentation rate, i. e. the regulation of the flow of the fermenting mash through the fermentor, requires a constant and definite concentration of yeast in the fermenting mash. In the hydrolysate industry this regulation is achieved

by the backflow to the first tank of the yeast separated from the spent mash. The loss of yeast, due to ageing and destruction, but also due to sedimentation caused by a loss of fermenting activity, is compensated by the continuous multiplication of yeast. Therefore the assay of the rate of multiplication during the continuous fermentation is of great theoretical and practical interest.

The rate of multiplication can be expressed by a mathematic constant as in any physico-chemical or biochemical process. The rate constant of multiplication "K" indicates the amount of yeast (in kg), formed in one hour from one kg of yeast.

The method of continuous fermentation is based on the fact that in the course of the process an equilibrium is established when the concentration of substrate and of the products of metabolism are maintained at a constant level by a continuous inflow of fresh substrate and removal of the same volume of fermented or partially fermented substrate.

The continuous culture makes it possible to estimate the rate constant of multiplication under continual and easily repeatable conditions. Furthermore, the economic coefficient is estimated, i. e. the accumulation of dry weight of yeast — αg/g and of alcohol — βg/g, expressed per unit of the sugar utilized by fermentation. The continuous culture method makes it possible to eliminate the effect of the lag phase on the result. From the point of view of practice the continuous culture method enables substantiation of the continuous fermentation method as used in industry and the devising of new ways of its improvement.

AN APPARATUS FOR CONTINUOUS CULTURE

The apparatus consists of an elevated container 1 containing a sterile store of substrate (Fig. 1), a dosage appliance (2), (3), (4) and (5), and a fermentation vessel placed in a water thermostat II.

The fermentation vessel of 350—450 ml capacity is provided with a stirrer which is inserted through a stopper with a sulphuric acid seal. The stirrer is operated by a warren motor at 60 r. p. m. A constant volume of the liquid in the fermentation vessel is maintained by an overflow tube which opens into the wall of the vessel. Considerable difficulties were encountered with the exact dosage of substrate when the continuous method was put into operation in this apparatus. The regulating taps, capillary fittings and other appliances designed to regulate the outflow of liquid by changes of the flow were entirely unsuitable due to their rapid clogging. In addition, it was rather difficult to obtain a preset rate by regulating the rate of flow of liquid according to the number of drops in a given period of time. Therefore a special dosage appliance was constructed enabling a dropping inflow of sterile liquids containing sediments.

A store of sterile substrate is placed in a container (1), closed with a rubber stopper and sealed with Mendělejev cement. Two tubes pass through the stopper: the first enables only preliminary dilution of the substrate in the container at the start; the other tube reaches to the bottom and regulates the filling of dosage appliance (2). The container functions according to the principle of Mariotte bottles. As soon as dosage appliance (2) is lowered below the orifice of the tube, the liquid from container (1) starts to flow and at the same time air enters through a cotton filter into the container. The dosage appliance having been filled with liquid to the given level, the further flow of liquid from container (1) into the dosage appliance is stopped.

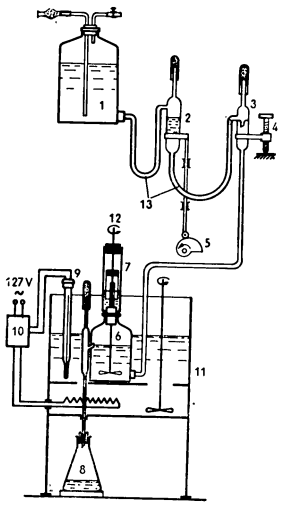

Fig. 1. Diagram of the apparatus for continuous culture.

1 — Elevated container (Mariotte-container); 2 — Dosage appliance; 3 — Dropper; 4 — Regulating screw; 5 — Cam (Archimedes spiral); 6 — Fermentation vessel; 7 — Sulphuric acid seal; 8 — Container for mash; 9 — Contact thermometer; 10 — Electronic relais; 11 — Water thermostat; 12 — Stirrer, 60 r. p. m., 13 — Flexible tubing.

On lifting the dosage appliance no backflow of liquid into container (1) occurs, the liquid having been replaced by the air which entered the container on filling the dosage appliance; instead, the liquid flows to dropper (3), whose drainage tube is placed somewhat higher than the orifice of the regulating tube. The rate of flow of liquid is governed by the volume of the dosage appliance and the time necessary for its lifting.

The lifting and lowering of the dosage appliance is operated by a spiral cam of a warren motor provided with a reduction gear. The cam revolves 3·3 times per hour. With each revolution of the cam the dosage appliance is lowered for three minutes, filled with liquid and then lifted and emptied within 15 minutes. Since no thin tubing is used in the system the dosage appliance can deliver even suspensions. At an inflow rate of 30—40 ml of liquid per hr. the exact dosage differs at the most by 2% from the set amount.

For the removal of samples for analysis and samples for the estimation of yeast concentration a weighed centrifuge tube, inserted in a cooling mixture, is used instead of container (8). The removed sample is quickly centrifuged and thus the yeast separated from the liquid. The liquid is analysed for sugar and alcohol contents, the yeast sediment is washed, centrifuged once more, the water decanted and the sediment dried at 56—60 °C in vacuo to constant weight.

According to the analytical results and the rate of flow of the fermenting liquid the rate constant of yeast multiplication, the sugar required for the alcohol formation and the amount of yeast formed per fermented unit of sugar are estimated, i. e. the economic coefficient is calculated.

The rate constant of multiplication K, the economic coefficient and the yield of alcohol are calculated after the establishment of constant characteristics and after the minimum 6—10 volumes of substrate have passed the fermentation vessel.

FUNDAMENTALS OF CONTINUOUS CULTURES

Let us derive the balance equation for the estimation of the rate constant of yeast multiplication. We designate the volume of liquid in the fermentation vessel V, V = constant; x is the average concentration of yeast cells in the liquid of the fermentation vessel. Let us now assume that in an infinitesimal time period dt a volume dy of fresh mash is introduced into a fermentation vessel and the same amount of fermented mash glows out. Then the original amount of yeast cells Vx is reduced by $x\,dy$ due to the outflow of fermentated mash. At the same time, due to multiplication of the yeast at the rate K, the amount $(Vx' - x'\,dy')\,K\,dt$ of fresh cells are formed. At the end of the dt period the concentration of yeast cells in the fermenting vessel has changed by dx. Now the equation of the balance of yeast cells can be written:

$$Vx - x\,dy + (Vx - x\,dy)\,K\,dt = V(x + dx) \qquad (1)$$

On integration of this equation within the limits of $0 - y$, of 0 to t and of x_0 to x, and their solution for K, we obtain:

$$K = \left(\frac{y}{v} + \ln\frac{x}{x_0}\right)\frac{1}{t} \qquad (2)$$

If the process is stable and the yeast cell concentration constant, i. e. $x = x_0$, then

$$K = \frac{y}{vt} \qquad (3)$$

i. e. in this case the rate constant of yeast multiplication equals the volume of the liquid passing through the fermentation vessel. However, the rate constant of yeast multiplication depends on the yeast concentration. Therefore equation (3) is valid only for a given yeast concentration, which is formed in a given time period, and is related to the flow rate of the substrate and its content of fermented sugar.

According to the data of V. A. Utenkova-Rancan (1954) and L. I. Jasinskij (1953), which were confirmed by our experiments, the economic coefficient α in the case of complete utilization of sugar is more or less constant for each type of yeast. It is, possible, therefore, to calculate the optimal flow rate, which is dependent on the economic coefficient α and on the sugar content in the substrate.

The stabilisation of the process is conditioned by $x = \text{const.}$, i. e. the yeast concentration must be constant. Therefore in the infinitesimal time period dt the amount of newly formed yeast is $\alpha c\, dy$, where c denoted the concentration of fermentable sugar. The balance equation is now:

$$\alpha c\, dy = (Vx - x\, dy)\, K\, dt \tag{4}$$

After integration within the same limits we obtain:

$$K = \frac{y\alpha c}{vtx} \tag{5}$$

Comparing equations (3) and (5) we find that the stability of the process can be maintained only when

$$\frac{\alpha c}{x} = 1\,, \quad \text{or} \quad x = \alpha c \tag{6}$$

Equation (6) shows that during the stabilisation of the continuous fermentation process the yeast concentration is proportional to the sugar concentration and to the economic coefficient.

The calculated relationships make it possible to calculate the necessary flow rate of the fermented substrate through the apparatus in the course of the continuous fermentation process. When expressing the flow rate of fermented substrate in volume units per hr., i. e. $V = t = 1$ according to equation (5), we obtain:

$$y = \frac{Kx}{\alpha c} \tag{7}$$

Now during the stabilisation of the process $\frac{x}{\alpha c} = 1$, and $y = K$.

Thus we come to the conclusion, already shown by Monod (1950), that the continuous culture is in equilibrium (in the absence of inhibiting factors) when the flow rate of substrate equals the rate constant of multiplication of yeast.

This conclusion is of practical importance. Knowing the value of the rate constant of yeast multiplication, in a given substrate, the economic coefficient and the content of fermentable sugar c, the maximal possible yeast concentration x and the duration of the total cycle of the fermenting vessel can be predicted:

$$x = \alpha c\,; \quad T = \frac{1}{K} \tag{8}$$

Many experiments on the fermentation of industrial hydrolysate mashes with adapted *Saccharomyces*, carried out in the flow apparatus, have made it possible to establish the average values of α and K under conditions similar to those of the hydrolysate industry:

$\alpha = 0{\cdot}2$, expressed in pressed yeast with 75% of water content
$\alpha = 0{\cdot}05$ expressed in dry weight
$K = 0{\cdot}06$

If the hydrolysate mash contains 2% of fermentable sugar, the possible yeast concentration is:

$$\alpha c = \frac{0{\cdot}2 \times 2 \times 1000}{10} = 4 \text{ g/l}$$

The rate of fermentation (cycle of the fermentation vessel):

$$T = \frac{1}{K} = \frac{1}{0{\cdot}06} = 16{\cdot}67 \text{ hr}$$

CONTINUOUS CULTURE AT INCREASED YEAST CONCENTRATIONS

It should be pointed out that the above considerations of the behaviour of continuous yeast culture apply in the case of intensive stirring of the fermented substrate in the fermentation vessel, when the yeast concentration in the outflow liquid equals that of the fermentation vessel. However, if a portion of the yeast for some reasons or other remains in the vessel, the conditions of equilibrium are changed.

Let us denote the yeast concentration in the system by x_1 at the beginning of the time period t, and x_2 at the end of this period. We assume that during this short period the volume y passes through the vessel. The concentration of yeast in the outflowing mash is x_3. The yeast balance for the interval t is expressed by the following equation:

$$Vx_1 = y\alpha c = yx_3 = Vx_2 \tag{9}$$

where V represents the volume of the fermenting liquid in the vessel (in accordance with the conditions for the continuous culture, V const), and c has the same meaning as above.

This equation can be transformed as follows:

$$x_2 = x_1 + \frac{y}{v}(\alpha c - x_3) \tag{10}$$

On examining equation (10) it is found that $x_2 - x_3$, i. e. the culture is in equilibrium, when $\frac{y}{v}(\alpha c - x_2) = 0$, or when $\alpha c = x_2$.

When $\alpha c > x_3$, then $x_2 > x_1$. In this case the yeast concentration in the fermentation vessel increases. In order not to affect the equilibrium of the development of the culture, the rate of flow must be increased according to equation (7). This enables continuous culture to be carried out with rates of flow which considerably exceed the rate of multiplication of the yeast. This observation was made by I. Málek (1956) in experiments with continuous cultures of bacteria, where the culture partially sedimented on the walls of the vessel. In some of these experiments the substrate was exchanged within 40—120 sec., although under given experimental conditions the culture could duplicate its population only within 20—30 min.

It follows from equation (10) that even if the yeast concentration in the outflowing liquid x_3 is lower than the multiplication of the yeast αc, the concentration of yeast and also the rate of flow y in the fermentation vessel x steadily increases. However, in practice no unlimited increase of the yeast concentration and rate of flow can be observed. On increasing the yeast concentration to a definite limit x_{limit} the dying off of the yeast increases although a complete amount of sugar and other nutrients are available. The rate constant of yeast multiplication decreases ($K_{\text{limit}} = 0{\cdot}025—0{\cdot}035$), thus reproducing the general rate of yeast fermentation. The dying off of the yeast is connected with the dissimilation process. The yield of alcohol, expressed per unit of the fermented sugar, decreases due to the formation of subsidiary products. An increase in the flow rate becomes uneconomical. The maximum rate of flow is established automatically:

$$y_{\text{limit}} = \frac{K_{\text{limit}} \times x_{\text{limit}}}{\alpha c} \tag{11}$$

It appears rather difficult to establish the magnitude of the limiting values x_{limit}, and it may even be impossible because of the many factors affecting them. First, the amount of yeast leaving the fermentation vessel, x_3, does not remain constant. Thus for example, when working with a yeast forming a fine sediment, the outflowing amount increases with an increase of the yeast concentration in the vessel until the equation $x_3 = \alpha c$ is no longer valid and the continuous culture achieves equilibrium. On the other hand when working with a flaky yeast the outflow decreases with an increased yeast concentration in the vessel, but the yeast begins to sediment in the vessel and becomes inactive. In this case continuous culture tends inevitably towards equilibrium.

Secondly, it appears that the flow rate is also limited by the reaction of alcohol formation.

The experimental results of M. J. Kaljužnyj (1955) and also our experience with the continuous fermentation process using yeast backflow in hydrolysate plants show that the maximal concentration of yeast during the continuous fermentation of hydrolysate amounts to 35—40 g/l weight of pressed yeast 75% of water. The flow rate corresponds to a complete cycle of the fermenting vessel in 3—3·5 hr., instead of 16·67 hr., when the fermentation process is carried out at a rate corresponding to the natural multiplication of the yeast, i. e. when $x = \alpha c$.

This result confirms the possibility of continuous rapid fermentation of hydrolysate mash with multiple utilization of the yeast at high concentration of yeast in the fermenting substrate.

This continuous fermentation process with the use of separators for the backflow of yeast from the mash is now being employed in all hydrolysate plants in the USSR (Šarkov 1950). The average concentration of yeast (75% water) is maintained within the range of 17 to 25 g/l and the cycle of the fermentation vessel at 6—8 hr. These conditions are optimal and provide a maximal yield of alcohol from the fermentated sugar.

LABORATORY APPARATUS WITH YEAST BACKFLOW FOR CONTINUOUS CULTURE

In 1953 M. J. Kaljužnyj succeeded in separating from yeast from the Archangelsk hydrolysate plant a flaky branching yeast corresponding to true *Saccharomyces*, designated Av-I. Later he separated a yeast of similar qualities from the Leningrad hydrolysate plant, called Lv-4.

The branching yeast differs from that forming a fine sediment in that it absorbs little or none of the colouring matter of the hydrolysate wort; furthermore, due to its greater sedimentation rate it is more easily separated from the dispersed lignin which contaminates the yeast when separating the mash (Andreev 1954).

The discovery of the Av-1 and Lv-1 types of yeast made it possible to establish a continuous culture with a higher yeast concentration in the fermenter.

The laboratory plant for continuous fermentation with a yeast backflow (Fig. 2) consists of two fermentation vessels connected in series (1, 2) placed

in a water thermostat, (3). The constant experimental temperature is regulated by a toluol-mercury-thermoregulator with an electronic relay (4) and an electroheater (5).

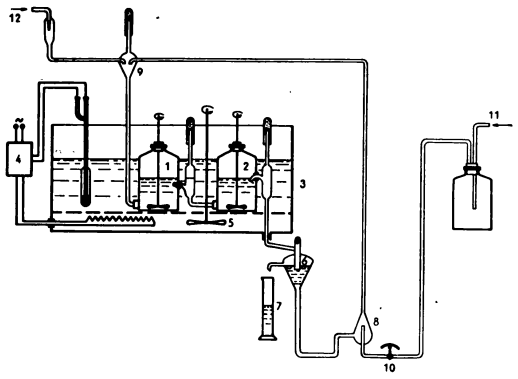

Fig. 2. Diagram of apparatus for continuous culture with yeast backflow.
1 — and 2 — Fermentation vessels; 3 — Water thermostat; 4 — Thermoregulator; 5 — Stirrer; 6 — Sedimenting tank; 7 — Container for mash; 8 — Airlift; 9 — Mixer for yeast and wort; 10 — Stopcock for regulation of air supply; 11 — Air or CO_2 inlet; 12 — Wort inlet.

For the separation of the yeast from the mash a conical separator (6) is used. The fermented mash is collected in a measuring cylinder (7) and the concentrate independently led from the apex of the cone through a capillary tube into the airlift (8). Through the airlift the yeast concentrate is led through a capillary tube into the mixer (9) where with the aid of the above described dosage appliance fresh mash is also introduced. The amount of the backflowing yeast concentrate is regulated by a change of the air or CO_2 supply into the airlift; this is achieved by a stop-cock (10) and the lifting of the airlift.

As fermenting vessels tubular vessels of 1·5 l volume are used. Openings in the vessel walls serve for the overflow of the mash from one vessel into the other and for the outflow of fermented mash into the separator, the openings being closed by rubber stoppers through which overflow tubes are led. Both

vessels are provided with stirrers operating at 60 revolutions per minute. This mixture ensures an even suspension of yeast in the fermenting substrate. If the stirrers are out of motion for only a short time the yeast rapidly sediments. The rate of complete sedimentation of the mash in the first fermentation vessel is 0·033 mm/sec. The working volume of the fermentor is, vessel 1 : 720 ml, vessel 2 : 700 ml, the separator: 180 ml, e. g. a total of 1600 ml.

The air supply for the transport of the yeast concentrate is effected by a water pump and a water manostat, the supply of CO_2 out of an cylinder being effected by the same manostat.

In this plant we fermented factory produced wort prepared from soft wood hydrolysate, which contained 2·55% of fermentable sugar and to which the usual amount of phosphorus and ammonium salts was added. The experiment lasted 15 to 21 days.

The flow rate during the experiment was kept constant and corresponded to the complete renewal of the liquid in the apparatus within 8 hrs. The volume of the yeast concentrate which was led back from the separator to the mixer was 200—400 ml/hr. The yeast concentrate in the apparatus during the experiment increased steadily from 13 g/l to 35 g/l.

The maximal yield of alcohol was achieved at a yeast concentration of 24·5—29·1 g/l and represented 56·7 l per 100 kg of fermented sugar. When the yeast concentrate in the apparatus reached 30 g/l, the yeast started to sediment, irrespective of the stirring. The yield of alcohol per 100 kg of fermented sugar was then reduced to 52·2 l.

In all experiments the concentration of the fermentable sugar in the first vessel was less than 0·3 — 0·25%. The spent mash obtained from the separator did not contain more than 0·02% of fermentable sugar and approximately 1—1·5 g/l of yeast.

Laboratory experiments confirm the possibility of continuous rapid fermentation of hydrolysate wort with a partial backflow of yeast, using a continuously working mash separator. This conclusion was confirmed in experiments at an experimental pilot plant (Andreev, Bulondr 1955), where approximately 3 m^3 hydrolysate mash was fermented per hour, and fermentation process lasting 7·5 hrs.

CONCLUSIONS

1. The rate constant of yeast multiplication is an important criterium of the viability and efficiency of yeast culture under given concrete conditions of the substrate used.

2. The continuous culture enables estimation of the rate constant of yeast multiplication under constant, easily reproducible conditions. In addition,

the economic coefficient is estimated, i. e. the accumulation of dry weight of yeast — αg/g and alcohol — βg/g per unit of sugar utilized during fermentation.

3. Continuous culture makes it practically possible to substantiate the continuous fermentation process used in industry and to device its further improvement.

4. Continuous culture is in equilibrium when the flow rate per hour through the working volume of the fermentation vessel equals the rate constant of yeast multiplication, i. e. when $y = K$. However, this rule is valid only when the yeast concentration in the outflowing mash equals the average yeast concentration in the fermentation vessel.

5. If the yeast formed during the fermentation process is for some reason or other partially withheld in the fermentation vessel and its concentration in the outflowing mash is lower than its natural multiplication, i. e. $x_2 < \alpha c$, then the flow rate may be considerably higher (2·5 to 4·5 fold) than the multiplication rate of the yeast.

6. If the yeast is partially withheld in the fermentation vessel or reintroduced from the fermented mash, the yeast concentration in the fermentation vessel and the flow rate increase only up to certain limited value.

After this maximal yeast concentration in the fermentation vessel has been reached, the rate constant of yeast multiplication decreases to such an extent that a further increase of the flow rate is impossible.

7. During the fermentation of hydrolysate wort from soft wood the limiting values of yeast concentration and rate constant of yeast multiplication were estimated in a series of observations:

$$x \cong 35—40 \text{ g/l}\,; \quad K \cong 0{\cdot}025—0{\cdot}035\,.$$

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THE VIABILITY AND FERMENTATIVE ACTIVITY OF YEAST DURING THE CONTINUOUS FERMENTATION OF WOOD HYDROLYSATE

M. J. Kaljužnyj

Wood hydrolysates prepared in various ways do not represent a complete medium for the vital activities of microorganisms. Of substances suitable for the biosynthesis of the cell mass of microorganisms they contain approximately 3·0% of carbohydrates and some parts per million of phosphorus and nitrogen. On inoculation with small amounts of yeast the development of the yeast is inhibited, not only due to lack of nutrients and vitamins, but mainly due to the presence of toxic substances (Šarkov, 1950). Many observations (Zubkova et. al. 1936; Kaljužnyj et. al. 1955; Leonard and Hajny, 1945) showed that with a small inoculum up to 30×10^6 yeast cells per ml of hydrolysate, or about 3·0 g/l, may accumulate in the mash, corresponding to about 0·1 of the sugar weight of the substrate. With this amount of cells the fermentation process proceeds slowly and the alcohol yield is low. Therefore it was found difficult to ferment hydrolysates with a natural yeast population.

Thus the development of a continuous fermentation method became necessary. During the fermentation by this method conditions are established where the population of a stationary culture increases several fold, accompanied by a decrease of viability of the old yeast cells and an increase of young cells. In addition, the number of cells increases with each renewal of the substrate, and thus their weight increases in relation to the initial sugar content of the wort from 1 : 10 at the first renewal to 1 : 1, or even more in the case of continuous utilization. Periodicity is excluded by the character of the procedure and the sugar is dynamically utilized. Reintroduction of the whole yeast mass must be considered, however, as artificial oversaturation of the substrate with cells which represent a rather heterogenous population from the point of view of their age, especially during continuous work. The majority of these cells consists of resting forms which have finished their development, only 12—15% budding. Therefore the fermentation process takes place with an

old and weakened culture. In order to increase the activity of such a culture it is necessary to define more precisely the nutritive regime.

It is known from literature (Kvasnikov and Boguslovskaja, 1956; Lebedev, 1936; Häglund, 1914) that the fermenting activity of yeast reaches a high level only when proliferation also takes place during the fermentation process and the decreasing activity of old cells is compensated by the high activity of young ones. If this proliferation does not take place, the fermentative activity declines. Under conditions of flow of nutrient substrate microorganisms proliferate and produce more intensively (Aristovskaja, 1955). Monod (1950) and Málek (1956) point out that a culture under flowing conditions is characterized by self-regulation and that its concentration can be maintained at an arbitrary level which, of course, cannot exceed that conditioned by the supply of nutrients.

Self-regulation of the number of cells in a continuous fermentation with a backflow of yeast is confirmed by experience in plants where this method has found application. However, there exists a basic difference between the self-regulation of a continuous culture with an outflow of cells with the substrate and the method where cells are reintroduced for fermentation. In the first case self-regulation takes place with a young population consisting of a great number of proliferating cells. This state may possibly serve as a criterium for evaluation of the maximal activity of the culture.

Other conditions of self-regulation occur with the backflow method for an old population, where the cells consist mainly of resisting forms. Here the self-regulation does not characterize a maximal accumulation of cells under given conditions (this maximum has been considerably exceeded), but characterizes the viability of a concentrated culture under conditions of a lack of nutrients. Therefore self-regulation does not characterize the activity of the culture, but indicates only the number of cells viable in the case of minimal utilization of the nutrients. In order to evaluate the activity of such a culture a quantitative analysis of the metabolism and formation of products was necessary. In order to use the continuous culture method with backflow of yeast it was thus necessary to establish in the first place the number of viable cells in a wood hydrolysate wort under conditions of varying cycles of backflow, and secondly, the amount of sugar which ensures a high productivity of yeast. At the same time the conditions of removal of weakened yeast and the dynamics of the alcohol fermentation in wood hydrolysate wort were examined.

EXPERIMENTAL METHODS

The experiments were carried out with different cultures of yeast and varying cell numbers in 400 ml flasks. A wood hydrolysate of pH 4·8—5·2,

containing 2·8% of fermentable sugar, was used. The wort was then supplemented with superphosphate and ammonium sulphate (both 0·3 g/litre). Fermentation was carried out at varying numbers of times of renewal of the substrate per day; the state of the culture after a single renewal corresponded thus to the stationary conditions of the experiment, while a four to sixfold renewal corresponded to the conditions of a continuous culture. At the end of each fermentation cycle the yeast was centrifuged, the spent mash decanted and the yeast again mixed with the substrate. After uniform distribution of the yeast a sample of the suspension was removed for counting of the cells in a counting chamber and for estimation of the increase and dying off of cells during the fermentation process. The number of dead cells was estimated according to their dying with colouring substances of the hydrolysate (Nizovkin and Ochrimenko, 1947).

Experiments directed towards study of the activity of yeast and of the dynamics of the fermentation process were carried out under industrial conditions. The amount of reducing sugars (RS) in the wort was estimated using the ebulliostatic method. The amount of alcohol in the mash was estimated by the flotation method of VNIIGS.

VIABILITY OF DIFFERENT YEAST CULTURES IN THE CASE OF SINGLE RENEWAL OF THE MEDIUM PER DAY

It has already been pointed out that during the fermentation cycle up to 30×10^6 cells accumulate per ml, or about 3 g per litre. This population number at the time of removal of the cells with the substrate as well as the stability of their composition limit the rate of fermentation. In order to accelerate the process, it was of importance to use repeatedly the yeast separated from the spent mash, this yeast representing a great store of enzymes. For this reason it was necessary to evaluate the increase of cells during the fermentation process under conditions where the amount of yeast starter was 3—6 times greater than the number of the natural population.

The yeast culture A_3, a rapidly proliferating yeast, and strain R XII, all cultivated on beer wort, were tested. In order to evaulate their viability in wood hydrolysate wort an inoculum of 10 g of yeast per litre was used, the substrate being renewed once a day. The concentration of reducing sugar in the wort was 2·85%, and that of fermentable sugar 2·11%; thus 1 g of yeast fermented 2·11 g of sugar per day. It follows from analyses (Table 1) that with each culture dead cells appeared on the second day. On the fourth day the number of living cells decreased on an average to 10×10^6/ml. In the following period an average of 100×10^6 cells per ml survived and this level was maintained. The culture A_3, which was isolated from the yeast at a hydrolysate plant, proved to be of higher activity. This experiment showed that

under stationary conditions with reintroduction of yeast and renewal of substrate the number of the culture population considerably increases,

Table 1

	Orig. number of cells 10^6/ml	Time of sampling (hr)								Mean of experiment
		24	48	96	120	144	168	192	216	
		Culture A_3								
Living cells 10^6/ml	120·0	124	111·3	110·1	107·6	110·8	121·5	106·5	129·6	
Dead cells 10^6/ml	0	0	8·7	9·9	21·4	18·7	25·5	53·2	71·6	
Sugar fermented, %		2·07	—	2·11	2·10	2·11	2·07	2·09	2·08	
Yield of alcohol per 100 kg RS/litres		40·0	—	38·6	38·6	41·0	38·6	37·0	37·7	38·8
		Rapidly proliferating yeast culture								
Living cells 10^6/ml	87·0	87·0	91·1	78·0	97·8	87·0	102·5	96·4	95·3	
Dead cells, 10^6/ml	0	0	6·4	14·0	22·2	16·0	20·5	43·1	51·9	
Sugar fermented, %		2·02	2·07	2·11	1·93	2·02	2·03	2·08	2·10	
Yield of alcohol per 100 kg RS/litres		38·1	37·7	38·6	37·2	37·2	37·7	37·7	37·2	37·7
		Strain XII								
Living cells, 10^6/ml	96·0	96·5	85·8	87·0	108·3	120·0	100·2	102·3	105·7	
Dead cells, 10^6/ml	0	0	4·9	6·5	15·4	29·0	48·8	52·4	86·8	
Sugar fermented, %		2·02	2·11	2·11	2·07	2·05	2·09	2·10	2·04	
Yield of alcohol per 100 kg RS/litres		40·0	—	38·6	37·2	37·7	37·7	38·1	36·0	37·9
Average number of living cells from three cultures	101·0	102·4	96·0	91·7	104·5	105·9	108·0	101·7	110·2	

In another series of experiments the culture A_3 was compared with culture L_{23}, which was sub-cultivated for many generations in wood hydrolysate wort. 20 g of yeast were used as inoculum, which corresponded to 2·44 g of sugar/day × g. It was calculated that 1 ml contained 132×10^6 cells of culture A_3 and 150×10^6 cells of L_{23}. From these figures it follows that the viability and increase of cells depend on the type of culture and mainly on the presence of nutrients.

VIABILITY AND PRODUCTIVITY OF YEAST UNDER VARYING CONDITIONS OF INOCULATION

In a second experimental series the effect of the renewal number of substrate on the viability of the culture A_3 accumulating in wood hydrolysate wort was investigated.

The fermentation was carried out with varying inocula; the substrate was renewed 1—5 times a day; thus the daily quantity of sugar per g of yeast varied. Furthermore, in an additional flask, inoculated with 50 g yeast per litre, the wort content of phosphorus and nitrogen was increased by the addition of 2 g $(NH_4)_2HPO_4$ per litre. For the first three concentrations the duration of the experiment was 10 days and 7 days for the concentration of 50 g per litre. The initial count of cells, their increase, dying off and alcohol productivity are shown in Table 2 as average values for the duration of the experiments.

Table 2

Fermentable sugar in mash %	Number of renewals of media per day	Yeast inoculum g/l	Yeast inoculum 10^6/ml	Daily level of sugar g/g of yeast	Increase (+) or decrease (−) of living cells 10^6/ml	Yield of alcohol 1 per 100 kg sugar	Yield of alcohol % of the theoretical yield
2·8	1	10	123	2·8	+ 8·3	53·8	83·8
2·8	1	20	227	1·4	+ 2·2	52·8	82·0
2·8	3	20	240	4·2	+ 6·9	54·0	84·0
2·8	1	40	460	0·7	−10	51·8	80·5
2·8	3	40	466	2·1	− 3·8	52·1	81·0
2·4	1	50	750	0·48	−35·0	45·7	71·0
2·4	5	50	754	2·4	0·00	50·0	77·8
2·4 + $(NH_4)_2HPO_4$	5	50	754	2·4	+ 2·2	49·3	76·4

It follows from these data that in the case of an inoculum of 10 and 20 g per litre the yeast not only survives, but that proliferation of the cells takes place corresponding to the quantity of sugar available. Thus at a level of 4·2 g of sugar and a threefold renewal of the wort the yeast weight increases from the original 20 g to 27 g per litre. The alcohol yield produced by the yeast is closely related to the proliferation of the cells. Lower values of the alcohol yield and the state of the yeast were obtained with inocula of 40 and 50 g per litre. In these variants a threefold renewal of substrate per day did not lead to proliferation of the cells. With a sugar content of 2·1 g and less a considerable decrease of the cell number in 1 ml substrate can be observed as compared with the itinial number; at a level of 2·4 g of sugar, both in the usual wort and in that with an addition of $(NH_4)_2HPO_4$, only the surviving of inoculated, resting cells is found. Thus an increased nutrient level of phosphorus and nitrogen does not produce an increase of yeast.

In these experiments a clear example of self-regulation of the amount of yeast in the case of insufficient nutrition was observed. On the strength of these experimental results it is concluded that a yeast concentration of 20 g per litre is well suited for continuous fermentation of wood hydrolysate wort with yeast backflow.

FERMENTATION COEFFICIENT AND PRODUCTIVITY OF THE CULTURE

Experiments in which a small amount of sugar was fermented by a great inoculum showed that the process is carried out by resting cells, without proliferation, even if the substrate is repeatedly renewed. However, the fermentation of sugar with small inocula leads to cell increase and also to an increased alcohol yield. This increase of cells is but small and does not exceed one half of the cell number of the natural population. It may be asked whether it is possible to obtain in a concentrated culture an increase of cells greater than the cells number of the natural population, and how much sugar will be required to form one g of yeast under these conditions.

In order to solve this problem it was necessary to estimate the fermentation activity of a young culture in wood hydrolysate wort.

The relationship between the fermentative activity and the yeast concentration, found earlier (Slator, 1908), enables the coefficient of the rate of fermentation to be calculated. However, for the yeast which is required for the fermentation of wood hydrolysate wort, it was sought to estimate the fermentation coefficient experimentally under industrial conditions.

Yeast A_3, separated in the plant, was mixed with wood hydrolysate wort in such proportion that its final concentration was between 2·3 to 10 g per litre. The duration of the fermentation process was defined as the time required from the mixing of the wort with the yeast to the moments when the reducing sugar in the wort ceased to fall off, the R. S. value being controlled each hour. The results of these experiments are shown in Table 3. The last column shows

Table 3

Volume of fermenting mixture m^3	Fermentable sugar kg	Living yeast D kg	Living yeast a g/l	Duration of fermentation "t" (hr)	Product $a \times t$	Fermentation coefficient for 1 kg of yeast per hr $K = \frac{m}{D \times t}$
49	557	115	2·35	16·0	37·6	0·303
48	553	139	2·90	13·0	37·7	0·306
48	535	149	3·10	12·0	37·2	0·300
47	450	153	3·26	11·0	35·9	0·270
47	451	192	4·10	8·0	32·8	0·296
20	210	137	6·85	5·0	34·2	0·306
19	206	153	8·05	4·5	34·2	0·300
20	220	188	9·4	4·0	37·6	0·294
17	187	175	10·3	3·5	36·0	0·305
					Average	0·300 kg

the average rate of fermentation of sugar by one kg of yeast per hour according to the formula $K = \frac{m}{D \times t}$, where K = fermentation coefficient, m = = the amount of fermentable sugar, D = living yeast in kg, and t = duration of fermentation.

These experiments show that 1 kg of yeast ferments on the average 0·3 kg of sugar per hour, i. e. 7·2 kg in 24 hrs.

From these experiments it follows that the fermentation coefficient represents the ratio of sugar weight to yeast weight and duration of active fermentation, when the cells are not starving and their alcohol producing capacity thus not being impaired. It might be assumed that with such a level of carbohydrate nutrition conditions would be established for an increased multiplication of cells even in a concentrated culture.

In order to confirm this assumption the hydrolysate was fermented with inocula of 10·20 and 40 g per litre at a sixfold renewal of substrate during 24 hrs. The amount of fermentable sugar per one g of yeast was between 1·26 to 10·12 g, and in one variant of this experiment it corresponded to the fermentation coefficient, i. e. amounted to 7·59 g per hr. The average results of these experiments for a period of 8 days (Table 4) confirmed that on increasing the daily amount of sugar up to 10·12 g with an inoculum of 10 g per litre, the additional yeast multiplication above the original amount represented on the average $37{\cdot}2 \times 10^6$ cells/ml, i. e. $7{\cdot}2 \times 10^6$, more than the cell number of the natural population.

The total number of living cells in 1 ml increases in 8 days from 120×10^6 to 375×10^6. In the initial phases the yeast was not capable of fermenting the given amount of sugar in time and the separated mash contained a high amount of nonfermented sugar.

Table 4

Yeast inoculum	10			20				40	
Number of renewals of media per day	1	2	4	1	2	4	6	4	6
Sugar per g of yeast	2·53	5·06	10·12	1·26	2·53	5·06	7·59	2·53	3·79
Increase of cells 10^6/ml	8·0	20·0	37·2	0·0	27·5	42·2	56·2	6·0	9·0
Yield of alcohol from fermented sugar, % of theor.	75·6	77·6	76·8	77·2	77·7	77·5	82·2	76·6	77·4
Nitrogen in yeast, % of dry weight	6·83	7·27	7·60	6·40	6·81	6·96	6·65	6·71	6·8

Of particular interest is the fermentation process with an inoculum of 20 g per litre and a sugar amount of 7·59 g, corresponding to the activity coefficient of the yeast. The cell increase per ml here was $56{\cdot}2 \times 10^6$, thus exceeding twice the cell number of the natural population. In this experimental variant the cell population corresponds to a continuous rather than stationary culture. In addition to increased cell multiplication an increased alcohol yield is found. Therefore it may be concluded that the capacity for alcohol formation is closely related to the state of vegetation of the cells. The fermentation coefficient for an inoculum of 20 g per litre can thus be considered as the main factor guaranteeing a sufficient increase of cells and their high productivity.

REMOVAL OF WEAKENED YEAST DURING CONTINUOUS FERMENTATION

It has been shown above that the use of yeast under conditions of insufficient nutrition leads within a short time to self-regulation of the number of cells in the population. The viability in this case is not determined by the concentration of cells, as assumed by Bayle, but by the conditions of the medium, by the level of nutrition. The last experimental series convinces us that the level of self-regulation should not serve as criterium for the maintenance of a high activiy of the culture, as believed in industrial practice, but instead the level of carbohydrate nutrition, ensuring a preponderance of cell multiplication over their dying off, should be used as criterium. As shown by the experiment, already on the fifth day a doubling of the cell number can be observed as compared with the initial count, due to active multiplication of the cells. It is clear that a double amount of cells requires an increase of the medium inflow. However, under industrial conditions, the amount of sugar supplied for fermentation cannot exceed a certain limit. Therefore the only way of maintaining a high metabolic activity of the cells is removal of the weakened cells with normal nutrition of the yeast mass.

Considering the different activities of suspended and sedimented cells an attempt was made to carry out the removal of yeast from the sedimented mass. A suspension of yeast in hydrolysate wort or fermented mash was placed into a cylider of 9 litres capacity, provided at its lower end with a funnel and a stopcock of 25 × 15 mm boring, and left standing for one hour. Then by a rapid turn of the stopcock one litre of mash was removed together with the sedimented yeast, which was then estimated by weighing after separation from the mash. The sedimentation value was calculated according to the formula

$$PS = \frac{(B - A) \times 100}{8 \times A \times T},$$

where PS = percentage of sedimentation, A and B the concentrations of yeast in g/l before sedimentation and in the sediment, 8 the volume of the mash in litres from which the yeast sedimented in time T.

Table 5

Dependence of sedimentation on the inoculum and concentration of sugar in the mash

Sugar in mash %	Yeast, g/l		Sedimentation per hour %
	in suspension	in sediment	
Mash without fermentable sugar	010·0	4·0	5·0
	022·5	13·0	7·2
	025·0	20·0	10·0
	035·0	35·0	30·0
	040·0	121·0	38·0
	045·0	181·0	50·0
2·86	20·0	5·0	3·1
0·71	20·0	10·0	6·2
1·30	25·0	6·8	3·4
1·70	25·0	27·0	13·5
1·60	30·0	6·2	2·6
0·61	30·0	85·0	35·3
2·86	40·0	11·0	3·0
0·65	40·0	17·6	49·0

Experiments showed (Table 5) that in a spent mash not containing fermentable sugar, the sedimentation of yeast was lineraly related to its concentration. Thus at 25 g/l, 10% or even as much as 15% of the yeast sediments per hour; at a concentration of 35—45 g/l, up to 50% of the yeast sediments per hour.

In a fermenting mash with a rapid evolution of carbon dioxide and at a sugar concentration higher than 1%, a suspension of 30 or 40 g/l remains suspended and there is only a small percentage of sedimentation. However, at a sugar concentration of less than 1% and a weak CO_2 formation, the suspension shows the same picture as the mash without sugar, i. e. up to 50% of yeast sediments. This shows that continuous fermentation of wood hydrolysates with high concentrations of yeast is not feasable due to the rapid sedimentation of the yeast. With an inoculum of 20 g/l the situation is greatly different. Here the main portion of yeast remains in suspension and only a small amount of sedimentation occurs, irrespective of whether the CO_2 evolution is high or low. Therefore the concentration of yeast should not exceed 20 g/l for continuous fermentation of wood hydrolysate with a sugar content of 3·0—3·5%, since with these yeast concentrations little sedimentation occurs even during stoppages of work.

Yeast from a hydrolysate plant, fermenting for a prolonged period at a concentration of 18—20 g/l with a fourfold renewal of medium per day was divided into two fractions — sedimenting and suspended, — and these were examined for their alcohol forming capacity. Experiments showed (Table 6) that sedimenting yeast ferments more slowly and gives a lower yield of alcohol than suspended yeast.

Table 6

No.	Yeast	RS in wort %	Alcohol content of mash			Duration of fermentation hr
			volume %	yield per 10 kg sugar	% of theor. yield	
1	Suspended	3·26	1·38	54·7	85·0	10
	Sedimented	3·26	1·28	50·4	78·2	18
2	Suspended	3·18	1·39	57·4	89·0	8
	Sedimented	3·18	1·33	54·9	85·0	20
3	Suspended	3·00	1·37	58·5	90·8	12
	Sedimented	3·00	1·31	56·0	86·9	18

Therefore a fourfold renewal of medium per day does not remove the inactivation of the sedimenting yeast. The removal of yeast sediment from the fermenting tanks represents thus an indispensable part of the regime of continuous fermentation with backflow of yeast.

THE DYNAMICS OF FERMENTATION OF WOOD HYDROLYSATE WORT

Carbon dioxide plays a fundamental role in the intensification of fermentation and maintenance of yeast in suspension by its mixing effect of the contents. In order that this factor be better utilised when working with high concentrations of yeast, the dynamics of fermentation of wood hydrolysate was studied in a plant where tanks of 100 m³ capacity were connected in series. Continuous fermentation was carried out with a backflow of separated yeast and at a flow rate of 25 m³ of mash per hour. The degree of sugar fermentation in the individual tanks is shown in Table 7.

Table 7

Plant consisting of	RS in wort %	Yeast g/l	Fermentation of sugar in % of initial tank No.				Fermented total %	Duration of fermentation hr.
			1	2	3	4		
2 tanks	2·96	20	76·8	3·7	—	—	80·5*)	$7^{hrs.}10^{min.}$
3 tanks	2·92	14·2	69·2	8·6	2·7	—	80·5	$9^{hrs.}30^{min.}$
4 tanks	2·97	10·2	61·8	16·1	2·0	1·9	80·0	$11^{hrs.}40^{min.}$

*) The residual sugars in the mash are pentoses.

The results show that under all variations of work the main portion of sugar is fermented in the first tank. In the remaining tanks at the most 0·2% sugar of the mash is fermented, this requiring, however, more time than the main fermentation.

This finding can be readily explained by the fact that the end of the fermentation proceeds under considerably changed conditions, since the CO_2 which brings about mixing of the mash and thus ensures better contact of the yeast with the sugar, evolves mainly in the first tank. It follows thus that the fermentation of wood hydrolysates by the continuous method should be carried out in a plant consisting of one or two tanks.

CONCLUSIONS

The conditions of fermentation of wood hydrolysates were examined, using the method of renewal of substrate, in order to establish the basis for continuous fermentation with yeast backflow. The following results were obtained:

1. During fermentation with repeated use of yeast a greater number of cells survive in 1 ml of renewed substrate than in substrate without renewal.
2. Under conditions of renewal of substrate the viability and increase of yeast cells are controlled by the amount of nutrients in the medium.
3. The carbohydrate nutrition is the decisive determinant of the accumulation of yeast and its productivity.
4. In wood hydrolysate one g of yeast (75% water content) ferments in the course of the fermentation period on an average 0·3 g of sugar per hour, or about 7·5 g per 24 hr.
5. At a daily level of 7·5 g of sugar per 1 g of yeast, the sugar level being achieved either by renewal or flow of medium, the state of the yeast corresponds more to continuous than stationary conditions and gives high yields of alcohol also with an inoculum of 20 g/l.
6. For the preservation of a high productivity of yeast continuous fermentation of wood hydrolysates should be carried out in a plant consisting of two tanks with removal of the sedimenting weakened yeast.

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FORMATION OF NEUTRAL SOLVENTS IN CONTINUOUS FERMENTATION BY MEANS OF *CLOSTRIDIUM ACETOBUTYLICUM*

JOSEF DYR, JIŘÍ PROTIVA and ROMAN PRAUS

This paper deals with the formation of neutral solvents in the continuous fermentation process by means of *Cl. acetobutylicum*. Continuous fermentation has been applied with advantage to some classical fermentation processes even on a pilot plant scale. Attempts have been made to use this continuous method also for other industrial fermentation processes but investigations carried out so far have been only on laboratory or semi-pilot plant scale. This applies also to the acetone — butanol fermentation process which is a comparatively young branch of the fermentation industry. Althought acetone-butanol batch fermentation has been successfully employed for decades in different countries, few reports have been published on the continuous cultivation of strains of *Cl. acetobutylicum* producing neutral solvents.

The report of Logotkin (loc. cit. by Málek, 1955) is one of the available papers on the continuous cultivation of *Cl. acetobutylicum* and deals with the evolution stages of *Clostridia* under various conditions of a continuously supplied fresh cultivating medium. However, no data on the course and yields of the fermentation are given. Another work by Nowrey and Finn (1956) describes the repeated propagation of a culture of *Cl. acetobutylicum*. On the contrary, a number of papers have been published which seem to reject the possibility of carrying out acetone-butanol fermentation by the continuous method.

Many investigators (Dyr, 1952; Kutzenok and Aschner, 1952; Hejnalová, 1955; Nowrey and Finn, 1956) showed that repeated transfers of the culture of *Cl. acetobutylicum* at the stage of acid formation resulted in a diminished ability to form neutral solvents in the following stages of the fermentation, in a longer acid forming period and after a certain number of transfers it brought about physiological degeneration of the culture.

These findings were elucidated in different ways. Some authors, admitting

the evolution cycle of *Cl. acetobutylicum*, suppose that the culture, which owing to repeated transfers at the acid formation stage did not end its evolution cycle with spore formation, impairs the process of its evolution and loses its ability to respond to the outer environmental conditions with the formation of neutral products. The above mentioned authors, Nowrey and Finn reported, that the butyric acid bacteria degenerated after a number of transfers owing to the spontaneous selection of mutants at a low pH, which was reached at the initial stage of fermentation. Thus acetone-butanol fermentation appeared to be unsuitable for the continuous method inevitably requiring prolonged propagation period of the culture without losing their physiological activities in any way.

In the present paper we report the results of some of our experiments with the continuous acetone-butanol fermentation technique on laboratory scale, carried out in 1956—1957.

METHODS

MICROORGANISMS AND CULTIVATION

Cl. acetobutylicum, strain Ca 3, isolated by Dyr in 1946, was used for our experiments. The strain was maintained by conventional microbiological practices in potatoe mash and transfererd once in two months, the culture being subjected to heat shocks. For experimental purposes fresh subcultures were always used.

Repeated vegetative transfers of the culture *Cl. acetobutylicum*, strain Ca 3, were performed in a potatoe mash containing approximately 4% w/v of starch. The culture was propagated after 12 or 24 hours by inoculating 500 ml of fresh potatoe mash with 10 ml of fermenting culture without the application of shocks. These subcultures were allowed to complete their fermentation for another 48 or 60 hours, respectively, and determinations of the acetone content and titratable acidity were made.

Continuous cultivation was carried out in a complex liquid medium consisting of tuber water and 4% w/v of glucose. The pH values of the medium prior to sterilisation was 6·5, the titratable acidity after sterilisation being 0·5 to 0·9 of 1 N NaOH.

CONTINUOUS FLOW SYSTEM

Continuous propagation and cultivation of *Cl. acetobutylicum* were carried out in cylindrical glass vessels fitted with side outlets. The contents of each vessel were continuously agitated by means of stirrers slowly rotating at 200 r.

p. m. Fresh medium was fed by means of a slightly modified Mariotte bottle based on the gravitational flow of the liquid under steady hydrostatic pressure, as described by Málek (1955) and further elaborated by Ričica (1956). This equipment is illustrated diagrammatically in Fig. 1.

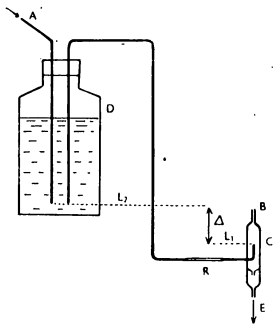

Fig. 1. Feeding equipment (schematic).
A a B — vent tubes; C — overflow tube; D — storage container; R — capillary resistance; L_1 — surface level of cultivating medium in the overflow tube; L_2 — theoretical level of cultivating medium in the storage container; Δ — value of hydrostatic pressure.

Throughout the experiment the storage container was connected with the outer atmosphere only with the vent pipe A reaching with its lower contracted end the bottom of the storage container. This arrangement made the feed independent from the level of the liquid surface inside the storage container, because the theoretic surface of the liquid was on the same level with the mouth of the lower end of the pipe A. The rate of the feed was controlled by adjusting the height of the surface levels (Δ) at the end of the overflow pipe C (L_1) and those of the storage container (L_2). The necessary fine control of the flow was attained by means of a capillary of a suitable diameter (R) incorporated into the flow system of the fresh medium. The feed system and the fermentation vessels were placed in a thermostatic chamber.

ANALYTICAL METHODS

Butyl alcohol and ethyl alcohol were determined by Johnson's method (1934), acetone according to Goodwin (1920) and glucose according to Shaffer-Hartmann (1921). The bacterial dry weight was determined by weighing, the titratable acidity by titrating 10 ml of the centrifuged cultivating medium with 0·1 N NaOH, using phenolphthalein as indicator.

EXPERIMENTAL

VEGETATIVE TRANSFER OF A CULTURE OF *CL. ACETOBUTYLICUM*

We started by checking earlier experiments by transferring vegetatively a culture of *Cl. acetobutylicum*, strain Ca 3. Forty transfers of the culture at 24 hours intervals were made. From the data in Tab. 1 (reproduced in concise form) it can be seen that even after forty transfers the culture did not tend to an increased formation of fatty acids, neither to a lower production of neutral solvents. In the course of repeated vegetative culture transfers only the appearance of the fermenting mash was changed. Approximately after the 30th transfer the fermenting liquid lost its characteristic appearance, the solid remainders of the mash did not rise to the surface and the foam formation was reduced. There is no doubt that together with the vegetative forms also a certain amount of cells with prospore and spores were transferred. They were formed owing to the heterogenuity of the cells in the culture already at the logarithmic phase of growth. This applied even in the case of transferring 24 hour old cultures. The experiment showed that repeated transfers of cultures without heat shocks and in the stage when most cells have not yet started sporulation did not necessarily cause lower physiological activity. By this the problem of the neutral solvent production by continuously propagated cultures of *Cl. acetobutylicum* was not entirely solved, since even cultures aged 24 hours, i. e. at the beginning of the second stage of fermentation, retained their ability to produce acetone, butanol and ethanol. It should be pointed out that the biochemical behaviour of the transferred cultures after forty transfers was studied only in the laboratory, not in the plant.

Table 1

Analysis of fermented liquors of vegetative passages of the culture of *Cl. acetobutylicum*, strain Ca 3

Passage	Acetone mg/ml	Titratable acidity	Passage	Acetone mg/ml	Titratable acidity
1.	3·86	3·2	9.	3·86	3·5
2.	1·64	3·3	10.	3·96	3·6
3.	4·83	3·2	15.	5·02	3·0
4.	4·15	3·0	20.	4·54	3·1
5.	3·86	3·3	25.	4·25	2·8
6.	3·48	3·1	30.	4·64	2·9
7.	3·28	3·2	35.	4·15	2·7
8.	2·70	3·1	40.	4·54	2·7

Cultures were transferred at 24 hours intervals.
Cultivating medium: potatoe mash containing 4 % w/v of starch.
Fermentation was completed in 72 hours.

Another series of experiments was made with cultures transferred after 12 hours into a fresh potatoe mash in a similar way as previously described. The yields of acetone and the values for the titratable acidity are given in Tab. 2.

Table 2

Analysis of fermented liquors of vegetative passages of the culture of *Cl. acetobutylicum*, strain Ca 3

Passage	Acetone mg/ml	Titratable acidity	Passage	Acetone mg/ml	Titratable acidity
1.	3·67	2·9	13.	4·25	3·0
2.	3·67	3·0	14.	3·67	2·9
3.	3·67	3·1	15.	3·38	2·9
4.	4·06	3·0	16.	4·06	3·0
5.	3·38	3·0	17.	4·30	3·0
6.	4·06	3·0	18.	4·06	3·0
7.	3·57	3·5	19.	4·74	3·0
8.	3·67	2·9	20.	3·72	3·0
9.	3·96	3·0	21.	3·81	3·2
10.	3·96	3·0	22.	3·52	3·2
11.	4·25	3·0	23.	3·91	2·9
12.	4·35	3·0	24.	3·43	3·0

Cultures were transferred at 12 hour intervals.
Cultivating medium: potatoe mash containing 4 % w/v of starch.
Fermentation was completed in 72 hours.

After the 24th transfer the experiment was stopped, the culture being in good physiological condition. It maintained its usual appearance until the 20th transfer when after further transfers reduced foaming was observed. In this experiment with transfers at 12 hour intervals the fresh media were inoculated with an inoculum in the logarithmic phase and thus the conditions resembled to a considerable extent those of the continuous flow system. Only a small number of cells which have acquired the ability to produce acetone was transferred together with the young propagating cells. This may be assumed owing to the observation that in batch fermentation the culture entered the second reducing stage in a short period of time, which implied great biochemical uniformity of the cells constituting the culture.

CONTINUOUS PROPAGATION OF *CL. ACETOBUTYLICUM*

With the results obtained in the previous experiments we started to cultivate *Cl. acetobutylicum*, strain Ca 3, under flow system conditions. Laboratory investigation of the continuous acetone-butanol fermentation required primarily a proper cultivation medium, so that simple and reliable feeding equipment might be used. For small operating volumes media of low viscosity, free of insoluble particles, proved to be the most suitable. A number of them was tested and the yields of acetone and fatty acids determined. They were made up of easily available materials, rich in growth factors and desired forms of assimilable nitrogen, such as corn-steep, malt germ extract, yeast autolysate and malt wort. Though most of the tested media showed desirable qualities in batch fermentation, all turned out to be unsuited for continuous cultivation. For all experiments described in this paper a liquid medium of tuber water and glucose was used.

Experiments attempting continuous propagation of the particular strain of *Cl. acetobutylicum* were started by following the effect of continuous propagation of a culture on its ability to form neutral products in the course of further batch cultivation. The experiment was carried out as follows: the culture was kept at the stage of intensive growth in the cultivation vessel into which fresh medium was fed at such a rate that the titratable acidity of the cultivation medium in the vessel or the cultivation liquid running out of the vessel was maintained at the same value. For this case, limiting values for titratable acidity, i. e. 2·5—3·0 ml 1n NaOH per 100 ml, were established. They were slightly lower than the maximum values obtained in the batch fermentation process. Preliminary experiments showed that 45—50 ml per hour of fresh medium had to be fed to maintain the fixed values for titratable acidity, provided that the capacity of the fermentation vessel was 250 ml. That indicated that theoretically once in 5 hours the contents of the vessel had to be changed.

Approximately 50 ml of the medium in the cultivation vessel were inoculated

Table 3

Analysis of fermenting liquor leaving the vessel (I) and after 72 hours of batch fermentation (II)

Hours	Glucose mg/ml	I					II				
		T. A.	B. mg/ml	A. mg/ml	E. mg/ml	B. C. mg/ml	Glucose mg/ml	T. A.	B. mg/ml	A. mg/ml	E. mg/ml
0	33·3	0·8	—	—	—	—	—	—	—	—	—
3	33·2	1·7	0·0	0 02	0·24	0·52	0·95	2·6	7·75	2·53	2·80
15	29 2	3·0	0 0	0 23	1·32	0·77	0·50	3·2	8·40	2·46	2·96
27	23·0	3 0	1·90	0 64	0·60	1·05	0·0	2·1	7·87	2·08	2·73
39	21·7	2·9	2·08	0·98	0·49	0·73	0 0	1·9	8·37	2·24	2·63
51	22·6	2·4	2·46	0·87	0 50	0·73	0·0	2·3	7·62	2·22	2·14
63	23·9	2·9	2·62	0·86	0·70	1·15	0·0	2·4	7 01	2·14	3·35
75	20·4	2·9	2·86	1·02	0·83	1·20	0 0	2·7	8·04	2·28	2·86
88	21·0	2·7	2·83	1·38	0·93	1·16	0 0	2·5	8·59	2·66	2·35
120	20·8	2·8	2·75	1·29	0·95	1·08	0·0	2·6	8·33	2·42	2·25

T. A. = titratable acidity; B. = butanol; A. = acetone; E. = ethanol; B. C. = bacterial cells
Cultivating medium: tuber water with 3·80 % w/v of glucose.
Flow rate: 80 ml/hr., dilution rate: 0·16 hr.$^{-1}$, hold-up time: 6·25 hrs.

with 5 ml of a 24 hour old culture of *Cl. acetobutylicum*, strain Ca 3, grown in potatoe mash. Three hours after inoculation the feed of the fresh medium was 20 ml/hr, after another five hours 50 ml/hr. During the experiment the appearance and the activity of the culture in the cultivation vessel were followed and 200 ml samples of the fermenting medium leaving the vessel were withdrawn and allowed to complete their fermentation in 72 hours. Then analyses were made, the results of 5 days continuous cultivation being given in Tab. 3.

At a theoretical total volume change of the vessel every 5 hours, the values of titratable acidity were 2·4—2·9. The bacterial dry weight had an initial rising tendency and after reaching the steady state it settled at the value of about 0·5 mg/ml of the medium. The adaptation of the culture to the new conditions was manifested also by the rapid utilization of the glucose and by the formation of neutral solvents, which had a rising tendency until the 60th hour of the experiment. Similarly, in the first 60 hours also the mobility of the *Clostridia* was increased. Morphologically the culture was uniform throughout the whole experiment and constituted by typical short cell forms. No spores were noticed. The yield of the experiment (with the exception of the first day) amounted to about 35% w/v. Rather unusual is the ratio of the solvents produced; the amount of butanol and ethanol was higher, whereas the acetone content was slightly lower in comparison with the batch fermentation process. (Tab. 4.)

Table 4

Comparison of yields and ratio of neutral solvents in batch and continuous cultivation of *Cl. acetobutylicum*

Cultivation	Yield of neutral solvents in % w/v	B. : A. : E.
Batch	32	4·5 : 2·7 : 2·8
Continuous	33	5·2 : 2·7 : 2·1

B. = butanol; A. = acetone; E. = ethanol.
Cultivating medium: tuber water containing 3·90% w/v of glucose, titratable acidity 0·7.
Batch fermentation was completed in 100 hours, the total hold-up time of four-vessel fermentation system being 30 hours.

From the average rate of glucose utilisation determined in the previous experiments and amounting to about 0·2% w/v per hour, it was assumed that 40% of glucose in the medium was utilized in 20 hours in the continuous fermentation process. Actually, however, more time was needed owing to the accumulation of metabolic end products in the medium and their inhibitive effects.

CONTINUOUS FERMENTATION

For the experiments with continuous fermentation by *Cl. acetobutylicum* three successively connected vessels fitted with slowly rotating stirrers of a total volume of 1500 ml were used. The flow system of the fermentation is illustrated in Fig. 2.

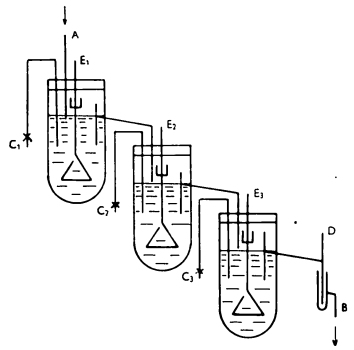

Fig. 2. Diagram of the three-vessel flow system.
A — inflow; B — effluent; C_1, C_2, C_3 — sampling points; D — vent tube; E_1, E_2, E_3 — mechanical stirrers.

According to the expected fermentation rate the flow rate was 50 ml/hr., so that the partial retention time was 10 hours and the total retention time 30 hours. In the first vessel only the growth stage and in the other two reducing stage should take place. This applied only if the cells in all vessels were approximately of the same age and if the medium passed through the whole series of vessels at a constant rate. However, the analytical cross-section of the continuous three-vessel-cultivation of *Cl. acetobutylicum* (Fig. 3) showed that even in the first cultivation stage there was a considerable percentage of aged cells, producing acetone as well as butanol and ethanol. The data for bacterial dry weight indicate that the propagation of the cells took place only in the first cultivation stage.

A considerably increased amount of fermentation products in the second vessel proved that predominantly reducing processes had set in. A slightly higher titratable acidity with small differences in the individual stages was in agreement with this observation. During the first four days of cultivation, the activity of the culture rose to such an extent that in the first cultivation

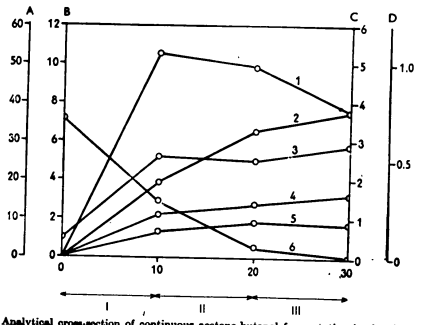

Fig. 3. Analytical cross-section of continuous acetone-butanol fermentation in the three-vessel flow system.

Cultivating medium: tuber water containing 3·6% w/v of glucose; titratable acidity 0·5. Flow rate: 50 ml/hr., dilution rate for the first vessel: 0·1 hr.$^{-1}$, total hold-up time: 30 hrs. Curves: 1 — dry weight of bacteria; 2 — butanol; 3 — titratable acidity; 4 — acetone; 5 — ethanol; 6 — glucose. Ordinate: hold-up time in hours: abscissa: A — glucose mg/ml; B — neutral solvents mg/ml; C — titratable acidity; D — dry weight of bacteria mg/ml. Abscissas marked by Roman figures sinclinate the retention times in individual vessels.

vessel more than half of the final amount of butanol was produced and as much as 60% of the glucose originally present was utilized. From this it follows that the throughput rate was lower than the retention time in the first fermentation vessel, corresponding to the logarithmic phase of growth. Considerably more butanol and acetone were produced, whereas the value for ethanol slightly varied. Increased butanol formation was evident, especially in the second vessel, during the 4th day of fermentation. At the same time all the glucose present was utilized. On the 5th day, spores appeared also in the first cultivation stage and in the third cultivation stage filamentous bacterial forms in all three vessels were found the following day. Simultaneously, the titratable acidity rose in all stages and the formation of the final products

and the rate of glucose utilization were identified, the glucose being utilized the 7th day only to 60%. It was supposed that the activity of the culture weakened owing to the increasing amount of inhibiting products, especially that of butanol, in the first cultivation stage. Thus the bacteria propagated under permanently unfavourable conditions. The dilution rate was, therefore,

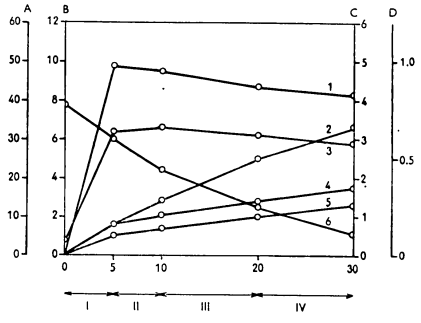

Fig. 4. Analytical cross-section of continuous acetone-butanol fermentation in the four-vessel flow system.

Cultivating medium: tuber water containing 3·9% w/v of glucose, titratable acidity 0·4. Flow-rate: 50 ml/hr., dilution rate for the first vessel: 0·2 hr.$^{-1}$, total hold-up time: 30 hrs. Curves: 1 — dry weight of bacteria; 2 — butanol; 3 — titratable acidity; 4 — acetone; 5 — ethanol; 6 — glucose. Ordinate: hold-up time in hours; abscissa: A — glucose mg/ml; B — neutral solvents mg/ml; C — titratable acidity; D — dry weight of bacteria mg/ml. Abscissas marked by Roman figures stand for retention times in individual vessels.

increased during the 7th day, so that the total volume of the fermenting system was changed once in 25 hours, which led to a partially improved state of the culture. The filamentous forms disappeared and the culture at the first propagation stage consisted of short motile cells. The yield rose during the 10th day to 34% w/v, the remaining content of reducing substances being 0·5% w/v. The average yield of the whole experiment, including the period of degeneration of the culture, was about 30% w/v of neutral solvents. The time required for utilization of the glucose present was by about 45% shorter compared with the time of the periodical method.

Attempting to avoid the difficulties in the continuous cultivation of *Cl. acetobutylicum* which may be caused by an intolerable amount of toxic metabolic

products as early as in the first cultivation stage, the cultivation system was divided into four stages, the first two of which had a capacity 250 ml, the other two 500 ml each. The time for the volume change in the first two stages was by 50% shorter than the time in the third and fourth stage. With this arrangement the exchange of the medium in the first vessel was doubled, so that it could be expected that no accumulation of toxic substances would occur at the stages with the greatest propagation rate of bacteria. The analytical cross-section of the experiment in the four-vessel-system is illustrated in Fig. 4.

With a retention time of 5 hours in the first cultivation stage half the time was required for doubling of the bacterial dry weight compared with the previous experiment. The bacterial dry weight in the course of a 12 days' experiment was the highest in the first cultivation stage. The enhanced flow was shown to favour the fermentation activity of the culture. On the 9th day of cultivation, the medium flowing out of the third vessel contained as little as 0·08% of reducing substances, indicating that the fermentation was sufficientlly completed in 20 hours. In spite of the initial activity the culture degenerated even in this case. The cells, short and extremely motile, changed into extended filamentous forms even in the first two cultivation vessels. These degenerated forms, reputedly having a low fermenting power, could not be removed even by the enhanced flow in the last three days of cultivation.

With regards to these unfavourable facts, the media used in the experiments were checked. They were subjected to batch fermentation to offer the possibility of comparing both methods of fermentation and active substances extracted from potatoes were estimated. Analyses of the total nitrogen in the potatoe tubers and in the extracted tuber water showed that only 29% of the total nitrogen was obtained in the preparation of the media, based on the amount of potatoes used. In this respect the employed medium was very poor in comparison with the natural fermentation mashes and it can be assumed that this was the cause of the degeneration processes in the continuous cultivation.

From the batch fermentation experiments data for the comparison of the various cultivation methods were obtained and changes in the final ratio of the neutral solvents produced by continuous fermentation were partially elucidated. The comparison of these experiments is shown in Tab. 4.

The batch fermentation in tuber water, supplemented with 4% w/v of glucose, proceeded comparatively slowly and it took 96—100 hours to utilize the glucose completely. The final ratio of solvents 4·5% w/v of butanol, 2·7% w/v of acetone and 2·8% w/v of ethanol was approximately identical to that of the continuous fermentation. The yields were 32% by weight in both for the continuous and batch fermentation processes, except for the time, which was three times as long in the case of batch fermentation.

The results obtained leave no doubt as to the possibility of employing the continuous method for acetone-butanol fermentation. The difficulties arising in the course of our experiments can be ascribed to insufficient differentiation of the growth- and neutral solvent production stage. In further experiments attention was called to the output of bacteria in the first vessel of the fermenting system. It can be assumed that the conditions securing the maximum output of bacteria at the initial stage of the fermenting system and shifting the productive stage to the final cultivation stage will have a decisive effect on the entire course of the continuous acetone-butanol fermentation process.

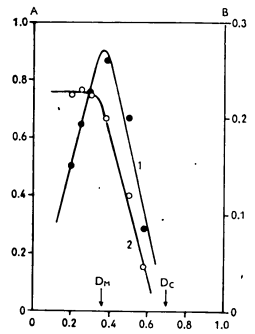

Fig. 5. Steady-state relatioships of bacterial concentration and output of bacteria to the increasing value of dilution rate in the continuous culture of *Cl. acetobutylicum*, strain Ca3.

Curves: 1 — output of bacteria; 2 — bacterial concentration. Ordinate: dilution rate D in $hr.^{-1}$; abscissa: A — bacterial concentration g/l of operating volume; B — output of bacteria g/hr./l of operating volume. D_M — maximum dilution rate; D_C — critical dilution rate.

Theoretical conditions for the growth of microorganisms in the continuous cultivation process, which are mentioned in the works of Monod (1950), Maxon (1955) and Herbert et al. (1956) indicate that the concentration of bacteria in the cultivating medium and the bacterial output, based on one volume unit of the medium in the vessel, are primarily affected by the volume ratio of the medium flowing through the fermentation vessel to the fermenting medium for a certain unit of time. Designating the volume of the fermenting medium as V and the volume of the feed as F, the ratio is F/V and is termed dilution rate D. Theoretically it may have the value from zero to infinity.

Actually, however, the dilution rate has a limited value. If the feed passes through the particular fermentation vessel containing a static microbial culture in the logarithmic phase of growth, the value of the dilution rate is above zero. If the dilution rate is increased and the steady state established in the fermentor, the bacterial concentration in the cultivation vessel changes with the increasing dilution rate only very little, whereas the bacterial output rises rapidly. If the dilution rate attains a certain value, termed the maximum dilution rate, the concentration of bacteria in the culture gradually falls and the bacterial output reaches its maximum. If the dilution rate is further increased

to the value termed "Critical dilution rate" both the concentration of bacteria in the fermentation vessel and the bacterial output falls rapidly and the cells are washed out of the fermentor.

A series of experiments was conducted with the varying dilution rates in the range of 0·20—0·60 and the uptake of glucose, concentration of bacteria,

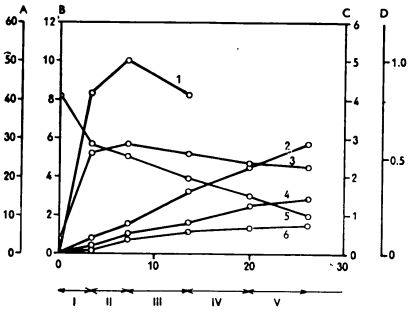

Fig. 6. Analytical cross-section of continuous acetone-butanol fermentation in the five-vessel flow system.

Cultivating medium: tuber water containing 4·1 % w/v of glucose, titratable acidity: 0·4. Flow-rate: 79·5 ml/hr.; dilution rate of the first vessel: 0·3 hr.$^{-1}$; total hold-up time: 26 hours. Curves: 1 — dry weight of bacteria; 2 — butanol; 3 — titratable acidity; 4 — acetone; 5 — glucose; 6 — ethanol. Ordinate: hold-up time in hours; abscissa: A — glucose mg/ml; B — neutral solvents mg/ml; C — titratable acidity; D — dry weight of bacteria mg/ml. Abscissas marked by Roman figures stand for the retention times in individual vessels.

bacterial output, titratable acidity and accumulation of neutral solvents in the medium leaving the first fermentation vessel were determined. The values obtained are shown in Fig. 5. The shape of the bacterial concentration curve and that for the yield of bacteria dependent on the increasing dilution rate were in full agreement with the theoretical assumptions.

It was shown that the optimum dilution rate for the production of the cellular matter of *Cl. acetobutylicum* under the conditions employed was approximately 0·3, which is slightly lower than the maximum dilution rate (D_M) the value of which is about 0·36. The critical dilution rate (D_c) derived by extrapolation is approximately 0·7.

Further experiments were conducted to follow the neutral solvent production

by continuous fermentation of a five-vessel cultivation system. The cultivating medium was fed in such a way that in the first vessel, where maximum propagation should have occured, the dilution rate approached the value of 0·3. Under those conditions the retention time of the first vessel was 3·3 hours, the time for the total volume change of the whole fermentation system being 26·0 hours. The experiment was stopped after 18 days of continuous fermentation. An analytical cross-section of the fermentation system on the 12th day of continuous fermentation is illustrated in Fig. 6.

DISCUSSION

These experiments showed that the culture of *Cl. acetobutylicum* kept its activity after repeated vegetative transfers in the laboratory stage. The results obtained with the culture of *Cl. acetobutylicum*, strain Ca 3, did not agree with some earlier published data. In the experiments of Hejnalová (1955) the degeneration of the culture set in after a few vegetative transfers. According to the statement of Nowrey and Finn (1956) the culture of *Cl. acetobutylicum* lost its characteristics only after four 24 hour transfers, which corresponded to about 23 generations. They are supposed to have confirmed the well known theory that butyric acid bacteria degenerate after a number of transfers owing to the spontaneous selection of mutants at low pH values in the first stage of fermentation. Kutzenok and Aschner (1952), experimenting with a selected strain of *Cl. butylicum*, observed anomalous behaviour of the culture already after the sixth or ninth passage without heat shocks. On further passages the cultures lost their activity till they died which occured after the eighteenth transfer at the latest. On the basis of available data and his own experience in acetone-butanol fermentation in the laboratory and plant, Dyr (1954) came to the conclusion that the culture of *Cl. acetobutylicum* must pass through all evolution stages and close its metabolis cycle if it is to be used in the plant. The culture not closing its life cycle after a number of generations, whether owing to unfavourable conditions of cultivation or repeated transfers of the culture in the logarithmic phase of growth (i. e. in the acid producing phase), does not accumulate certain enzymatic systems and is incapable of responding to the changed environmental conditions with neutral solvent production. In the experiments described in this paper employing a greatly active strain of *Cl. acetobutylicum*, we succeeded in keeping the culture in good condition even after forty vegetative 24 hour transfers and twenty four 12 hours passages. Roughly culculated, the culture produced 220 generations and 120 generations respectively. The number of transfers conducted was not limited, as shown by the unchanged activity of the culture at the end of the experiment.

Positive results were obtained also with the continuous propagation of *Cl. acetobutylicum* in one vessel only. Samples were taken from the continuous

fermentor in the course of a five days' experiment and allowed to complete their fermentation in the usual time with the average yield of solvents of 34%. The concentration of the bacterial dry weight in the cultivation vessel remained at the value of about 0·5 mg/ml, i. e., approximately 60% of the maximum amount obtained by batch fermentation. The doubling time of this experiment was about 3·5 hours, which was considerably longer than that of the logarithmic phase of growth in the batch fermentation process. The prolonged, so called "generation time" was not caused by the continuous cultivation itself, but was the result of the steady states attained in the culture, which were controlled by the dilution rate in the presence of excess nutrition sources (Monod 1950, Herbert et al. 1956). The dilution rate employed in these experiments for the *Cl. acetobutylicum* is far from being the maximum. Nowrey and Finn (1956), conducting continuous propagation of *Cl. acetobutylicum*, worked with a very low concentration of bacterial dry weight in the vessel. They obtained about 650 generations in a one vessel continuous propagation after a fortnight, which means that the "generation time" was a little longer than half an hour. The low yield of such a propagation is obvious.

In the three-vessel fermentation system, the propagation of microorganisms occured predominantly in the first stage at the dilution rate of $D = 0{\cdot}1$. When the steady state has been established, the time needed for doubling the dry weight (t_d) was about 7 hours. (t_d is a constant characteristic only for the continuous cultivation and can be compared with the "generation time" of a microbe in the logarithmic phase of growth of a static culture only at a maximum dilution rate. It is expressed by the following equation (Herbert et al 1956):

$$t_d = \frac{1}{D} \ln 2 = \frac{V}{F} \ln 2) \ .$$

The propagation phase even in the four-vessel system, where the first two vessels contained 250 ml each and where the dilution rate $D = 0{\cdot}2$ was used, took place practically only in the first cultivation stage at a doubling time of the bacterial dry weight of only 3·5 hours and a volume change time of about 5 hours.

Compared with the conditions for acetone-butanol batch fermentation in the same medium one of the advantages of the continuous fermentation was obvious; the time needed for the growth of the total bacterial dry weight in the continuous fermentation process was by 3/5 shorter when the steady state had been established than that of batch fermentation process lasting at least 12 hours. However, in spite of employing a doubled dilution rate complete separation of the propagation stage from the production stage could not be achieved. Owing to the accumulation of a considerable amount of fermentation end products the young proliferating cells were injured even in the first cultivation stage and lost their physiological activity and gradually degenerated. According to Čekan (1934), especially acetone and butanol were very toxic, butanol having a strong inhibitive effect on *Cl. acetobutylicum* at a concentration as low as 1% w/v. These difficulties with the continuous fermentation of *Cl. acetobutylicum*, strain Ca 3, were successfully avoided by increasing the dilution rate, which resulted in a lower concentration of toxic products in the medium and the regeneration of active cells. It is supposed that the separation of the propagation stage from the neutral solvent production stage could be attained either by adjusting the dilution rate in the vessels, where the propagation takes place or by inducing such carbohydrate conditions which would provoke competition for the substrate between the proliferating cells and the cells producing neutral solvents. Experiments with varying dilution rate showed that the best separation of the bacterial growth stage from the production stage was achieved at a dilution rate approaching the maximum, i. e., at the value of $D = 0{\cdot}3$. In continuous fermentation, conducted under these conditions in a five-vessel apparatus, the maximum bacterial growth occurred in the first vessel. In the first two vessels acids were formed and the neutral solvent production was most intensive in the third and further vessels. The number of generations after eighteen days was 190.

No morphological adaptation to the altered way of cultivation was observed in any of our experiments. The presence of filamentous forms and prolonged cells was always accompanied by diminished fermentation activity of the culture and obviously by a degeneration process. Similar morphological changes are often observed in degenerative batch fermentations. The formation of spores was observed only in the final stages of favourable continuous fermentation processes.

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