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# ENERGETIC AND KINETIC ASPECTS OF INDUSTRIAL FERMENTATIONS[1/]

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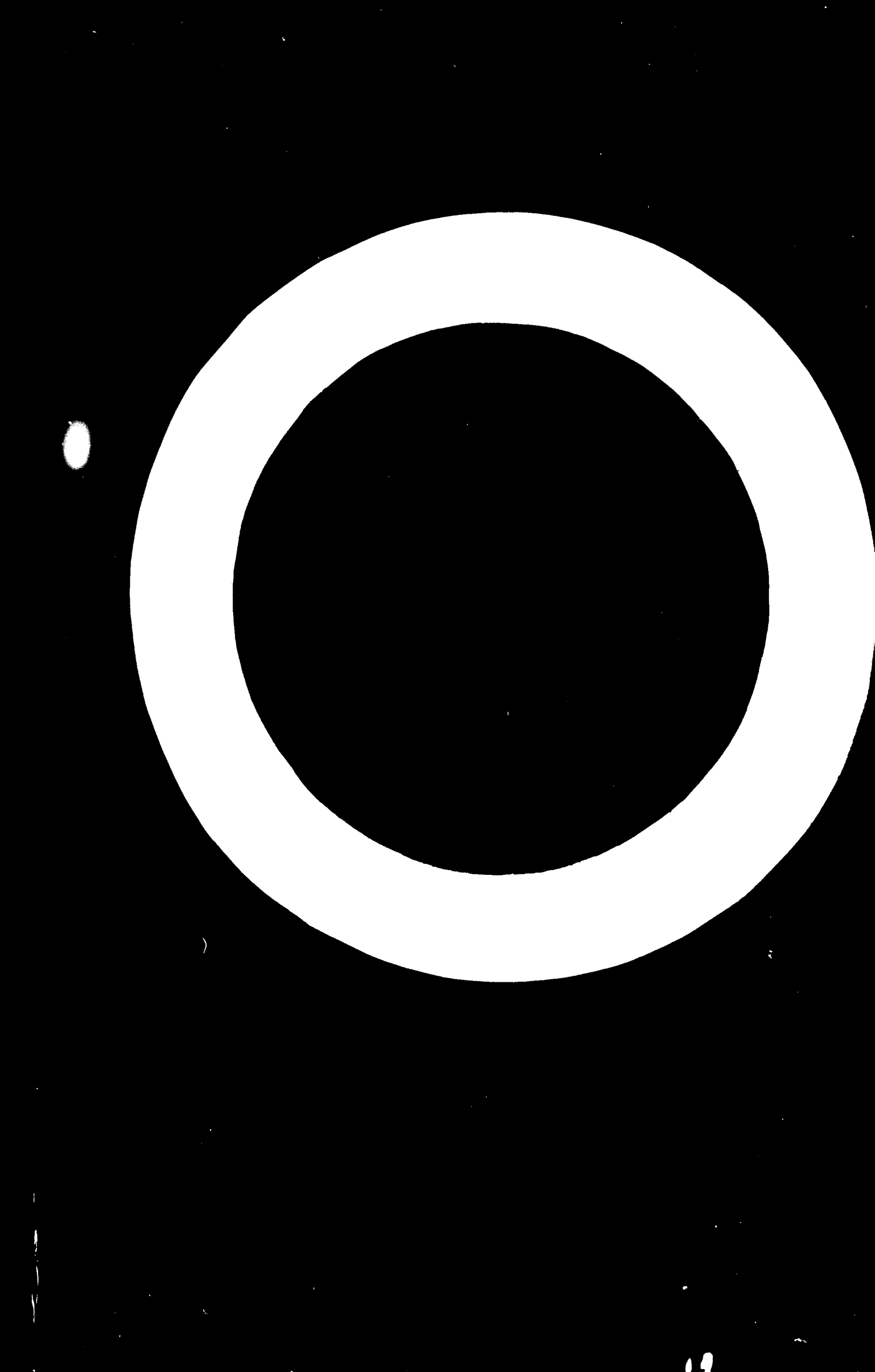

## 1. Definition of microbial kinetics

Kinetics in the chemical-physical sense deals with the science of rate of reactions, i. e. the determination of the rules and laws of these reactions, the study of the factors influencing their various parameters and characteristics, and the exploration of the mechanism (on a molecular level) of these changes of state. In biology this field of science can certainly be explored also, particularly in microbiology, where one is in the happy position of being able to deal with an enormous number of organisms, the relationship to chemical kinetics is becoming particularly obvious in view of the relatively high degree of regularity according to which microbial cells are able to reproduce or otherwise manifest their activities, and in view of the possibility of defining the environment of microbial cultures to a very high degree.

## 2. General kinetic aspects

It has been known for a long time that unicellular organisms exhibit a very characteristic way of reproduction, i. e. that each cell is in a position of dividing once within a given, constant & (usually short) time. Thus a geometric progression is obtained which in the chemical sense would be called an autocatalytic reaction, and defined according to the differential equation $\frac{dx}{dt} = kx$, where k indicates the rate of multiplication, or the relative rate of growth ($k = \frac{dx}{xdt}$). Many of the remarkable phenomena observed with micro-organisms are due to this property, but it is not the intention here to deal with these; instead it is proposed to consider, explore and discuss a few aspects of importance in the current trends of microbiological research which are tightly connected to some of these basic characteristics of microbial growth and reproduction.

It has been widely thought for a long time that the property of exponential reproduction is typical for unicellular organisms whereas multicellular micro-organisms, such as filamentous fungi would not reproduce according to this law. In this respect it will have to be admitted that the early observations dealing with microscopic observations on the reproduction of filamentous fungi had been forgotten in this modern age (where so many microbiologists have undergone a metamorphosis into biochemists). These authors found that there is a linear relationship between the logarithm of newly formed mycelial tips and time. Since growth, i. e. newly formed protoplasm, is limited to mycelial tips, it can therefore be expected that the culture as a whole will grow exponentially.

If now in reprospect we want to analyse why the exponential phase in cultures of filamentous fungi has been widely considered to be non-existent, it will at once be clear that this phenomenon was closely tied to the techniques of cultivation and methods of estimating cellular material. It is obvious from Fig. 1, that exponential growth of a culture of a filamentous fungus, i. e. Aspergillus oryzae, can be obtained over a reasonably wide range of mycelium content, i. e. from 0.9 to 160 mg/100 ml. The conditions for obtaining exponential growth of this fungus are that there is no limitation of access of oxygen or other nutrients to the mycelium, that there is no accumulation of toxic substances, and that the fungus is cultivated on a sufficiently large scale to allow the taking of large samples for the estimation of mycelium content at early stages of growth.

Fungal growth is often represented as an arithmetically linear plot of mycelium dry weight versus time. It should be noted that even if exponential growth occurs, as shown in the previous diagram, a considerable portion of the growth curve can, within the limits of the error of the experiment, be represented as a linear relationship. In Fig. 1 growth is plotted in two different ways.

it is obvious that the arithmetically linear phase corresponds to the late exponential phase and parts of the phase of decreasing rate of multiplication.

Other forms of representation have at times been used such as the representation of $\sqrt{x} = f(t)$, or $\sqrt[3]{x} = f(t)$. The latter has been particularly widely used, and it has been claimed that this is the function according to which the major phase, in fact the phase of uninhibited reproduction, could be represented. However, despite the possible wide range of application it is not advisable to consider a linear relation between $\sqrt[3]{x}$ and t as a phase of unlimited growth as is immediately obvious from the following comparison of differential equations.

| | | | |
|---|---|---|---|
| "linear growth phase" | : | $x = k' \cdot t$, | $\frac{dx}{xdt} = k'x^{-1}$ |
| "square growth phase" | : | $\sqrt{x} = k'' \cdot t$, | $\frac{dx}{xdt} = k'' \cdot x^{-1/2}$ |
| "cubical growth phase" | : | $\sqrt[3]{x} = k''' \cdot t$, | $\frac{dx}{xdt} = k''' \cdot x^{-1/3}$ |
| "exponential gr. ph. " | : | $\log x = kt$, | $\frac{dx}{xdt} = k$ |

Only in the exponential phase is the relative growth rate (rate of multiplication) constant, in all other cases this parameter is dependent either upon x or upon a function of x.

For many studies, e. g. for investigations on relevant factors influencing growth properties of microbial cultures, it will be possible to use either method of representation, since the values of the corresponding parameter will serve as a convenient yardstick to express changes in the growth characteristics. One will, however, have to be aware of the fact that in each case of the above equations a different phase of the growth curve comes under scrutiny.

The above considerations on the representation of the major phases of growth become of particular importance for assessment what is called lag phase. With bacterial cultures the latter has been defined as the time elapsing until exponential growth starts (in an idealized way). This definition is sensible, because it is only in this way that growth is considered from the point of view of the culture itself, rather than

from a subjective outside observer. Thus the good sense of the definition is illustrated in the following example (Fig. 2 ) where two cultures are started off with widely differing inoculum sizes.

Plotting the data arithmetically, and defining the lag phase as the time elapsing until this linear phase starts, would entail that inoculum size has really influenced the duration of the lag phase. This artifact is simply due to the fact that with a ten thousand fold increase in cell material (as assumed in the above example) the absolutely small increase of growth in the initial phases of development cannot be made out on an arithmetic plot, and the later occurring linear increase of growth is assumed to be the major phase of growth. In fact the same argument applies in principle also for a representation of $\sqrt[2]{x} = f(t)$ or $\sqrt[3]{x} = f(t)$ although to a lesser degree than in the case of a plot of $x = f(t)$. In each of these cases a more or less greater portion of the early exponential phase is considered as lag phase. Surely from the point of view of the culture itself, i. e. considering the small number of cells with which the small-inoculum culture had to start off, this is not fair.

Working with fungal cultures one usually represents growth in an arithmetic plot because it is very difficult to measure increases of growth in the same absolute range as in bacterial cultures, and only relatively advanced phases of growth are represented. It is tempting to define then also the lag phase as the time elapsing until linear growth takes place, which, according to the above considerations, should not be done, since the non-measurable phase may represent an exponential increase in cell matter, which was surely not the intention in the definition of the lag phase.

3. Factors influencing the various phases of growth curves.

Microbiologists have endeavoured to study relevant factors influencing the various parameters of growth curves, and this is surely very important too for industrial microbiologists. This approach, if

logically developed, sometimes referred to as empirical, has much to recommend it.

In the present discussion we propose to restrict ourselves to a few factors, i. e. the influence of the concentration of major nutrients and of trace minerals, as external factors and the influence of inoculum size as internal factor.

It has been known for a considerable time that the concentration of the source of carbon and energy in heterotrophic bacteria can be varied over wide ranges without affecting the rate of multiplication. Considering such a ubiquitous organism as Escherichia coli and many other bacteria and yeasts one invariably finds that glucose, in its function as source of carbon and energy has to be reduced to the order of a few mg/l in order to diminish rate of multiplication significantly. The practical result of this property is that bacterial cultures, once in the exponential phase, continue to grow with undiminished rate of multiplication until the source of carbon is exhausted to more than 99,9 %, when an initial concentration of sugar of as little as 1 % has been used. Therefore the usual thing with bacterial cultures in conventional laboratory media is that the growth curve changes its course very abruptly when it approaches the maximum yield of cell material as dictated by the carbon source concentration.

One should not, however, generalize this phenomenon too widely since a) there are reports in the literature that this phase of decreasing rate of multiplication can be quite extended in bacterial cultures, b) if the initial concentration of sugar is chosen very high, as is usually the case in industrial fermentations, it is generally to be expected that long before the sugar is exhausted there is accumulation of inhibitory substances in some form or other. The latter state of affairs exists frequently in industrial cultures.

Returning once again to the filamentous fungi one can observe that the so-called arithmetically linear phase is usually more pronounced here than in bacterial cultures, implying that the intermediate phase of decreasing rate of multiplication is also longer than in bacterial cultures.

## 4. Aspects of continuous cultivation.

It will be interesting to consider the implications of the aforementioned property in the continuous (steady-state) culture of microorganisms. The principle of steady-state culture with a homogeneous stirred fermenter is that the rate of dilution is equal to the rate of multiplication (i.e. the relative growth rate) of the organism. Thus, if a culture is operated batchwise and dilution of the culture with fresh substrate is started at a given time ($T_1$) during exponential growth at a rate such as to equal this (constant) rate of multiplication, a steady state (indicated by a cell content of $x_1$) is obtained as shown in Fig. 3.

It is obvious that a steady state can be obtained at any stage of the exponential phase with the same dilution rate. The equilibrium content of microbial cells is determined only by the stage of reaction (i.e. growth) at which the dilution was started. If, using the same dilution rate, continuous operation is started when the exponential phase has been passed, i.e. the phase of decreasing rate of multiplication or the maximum stationary phase, the culture will settle to an equilibrium cell concentration as indicated by the position $x_2$ at the end of the exponential phase. Higher dilution rates will of course result in a wash-out of the culture, at whatever point of the growth curve dilution with fresh substrate had been started.

Using lower dilution rates, however, at for example a given stage of growth in the exponential phase, it is easily understood that despite the continuous dilution the culture will go on growing exponentially until it reaches a point where the rate of multiplication equals the rate of dilution of position $x_3$ (or log $x_3$). It is at once clear that in steady-state culture using small dilution rates one will always work in a phase of decreasing rate of multiplication and the equilibrium concentration of cells is now directly dictated by the dilution rate. The lower the dilution rate the closer we reach the maximum yield of cells in the equilibrium concentration.

In as far as the magnitude of the equilibrium concentration in bacterial or yeast cultures with low substrate concentration is concerned (where the rate of multiplication decreases only when the concentration of the carbon source, as limiting factor, has reached very low levels)

it follows that this equilibrium concentration of cells is always close to the maximum yield of cell matter, as can be seen from the following calculation, where concentration of carbohydrate in the diluent is taken as 10 g/l and a noticeable effect on rate of multiplication assumed to occur at 10 mg/l:

Yield of cells in maximum = 10 y g/l and Yield of cells at the highest self-regulating dilution rate = (10 -0.01) y g/l

= 9,99 y g/l

This is the reason for a single stage continuous culture of bacteria being always technically and economically superior to a multistage continuous culture, since there is nothing to be gained by using a series of vessels.

How is the situation now in fungal cultures, (or in bacterial and yeast cultures with high substrate concentration), where the phase of decreasing rate of multiplication is usually very extended.

Our own strains of Aspergillus oryzae, A. niger and Penicillium chrysogenum at best showed an exponential phase lasting up to about 1,50 g/l of mycelium dry weight (MDW), which under ideal conditions could be approximated as having originated from about 3,0 g/l of carbohydrate. After the stage of exponential growth the maximum yield in batch cultures is reached approximately according to the equation

$$\frac{t}{\log x} = a \cdot t + b$$

where b is largely independent but a markedly dependent upon the concentration of carbohydrate used.

The practical conclusion is that after about 3.0 g/l of carbohydrate have been used in mould cultures in a medium containing originally 10 g/l, the highest possible dilution rate resulting in self-regulation of the steady state will leave us now with a residual carbohydrate concentration of about 7 g/l, whereas in bacterial cultures this was 0,01 g/l. In order to obtain almost complete utilization of the sugar in the case of fungal cultures, it is necessary therefore to operate at a dilution rate very much lower than the observed rate of multiplication in the exponential phase should a single stage operation be chosen. This state of affairs is graphically shown in Fig. 4.

Since in a single-stage operation the equilibrium concentration is dictated by the lowest reaction rate, it is now clear that in filamentous fungi there is something to be gained by a multitude of culture vessels operated in series. The first vessel can be operated at nearly the highest dilution rate possible, dictated by the highest rate of multiplication ($d_1$), followed by the next steps, $d_2$, $d_3$, ... dn, representing gradually smaller dilution rates until the desired degree of carbohydrate utilization has been reached. The overall dilution rate D would be the average of all the individual rates, $d_1$, $d_2$, ... dn. If an infinite number of such fermenters is used the overall dilution rate D can graphically be represented as the line joining the intersection points a and b. The value of D will always be higher than that of dn, thus showing that in the case of filamentous fungi a multitude of vessels in series, or indeed a plug-flow reactor, is superior to a single stage stirred fermenter.

5. Effects of trace elements and inoculum size.

Returning again to the batch culture we would like to consider the other extreme of nutritious factors: trace elements, i. e. salts of heavy metals, required sometimes only in the order of a few µg/l in order to exert a pronounced effect on the development of the cultures. It is clear that in the vast majority of cases there will be sufficient trace elements as impurities, even in very high grade commercially available chemicals, to allow development of most microbial cultures. The implications are that errors in assessing specific effects of trace elements can readily occur. In fact workers in this field have long known that chemicals and water have to be most carefully purified, that only high quality glass culture-vessels can be used, and that the inoculum has to be prepared in special ways, should any side effects be avoided.

In our studies on influence of trace elements we considered inoculum size to be an important intervening factor. However, it became soon clear to us that we were dealing here with a much more complicated phenomenon than we originally anticipated. Consider for example the diagrams in Fig. 5, where the development of cultures of Aspergillus oryzae is represented (arithmetically) in a fully synthetic medium prepared in the

same batch and inoculated with varying concentrations of washed mycelium(1). The fact that a smaller rate of growth and a lower maximum yield of mycelium is obtained if the inoculum size is reduced from 0.73 to 0.0073 mg/100 ml might suggest that some transfer of nutrients, such as trace elements has been responsible to allow better growth of the large-inoculum culture in this trace element-poor medium to which small amounts of ferric salts have been added as only micro element. However, if now inoculum size is further reduced to 0.000073 mg/100 ml a curious effect can be observed. There is full reversal of the previously described effect, since now rate of growth in the linear phase as well as maximum yield of mycelium are considerably higher than with the large-inoculum culture. Furthermore, the start of the linear phase takes place very much later with the smallest inoculum than with any of the larger ones. Last, but not least, it is to be noted also that the same effects can be observed if the concentration of sugar in the substrate is reduced from 30 g/l to 10 g/l.

It will be admitted that the above phenomena can hardly be explained on the basis of transfer of trace elements or other nutrients with the inoculum, particularly if it is being re-called that the above phenomenon has repeatedly been observed in our strain of Aspergillus oryzae with mycelium as well as with conidia in both trace element-poor and trace element-supplemented substrates. Furthermore, addition of $10^6$ conidia or pasteurized conidia to a medium inoculated with $10^3$ conidia per 100 ml of medium, did not influence the growth habit at all. In spite of all these observations it has to be noted that the trace element composition has a strong influence on the magnitude and on the qualitative properties of inoculum size effects.

Not only are there effects of individual trace elements but also synergistic and antagonistic effects. One system which has caught our attention particularly and which is of practical significance consists of a specially purified substrate with maltose as source of carbon and energy, ammonium sulphate and some other inorganic salts as its basal composition. In this medium Aspergillus oryzae, inoculated with a million conidia per 100 ml develops only poorly; with a thousand conidia, however,

there is rapid growth in the linear phase and very high maximum yields are obtained. In the absence of copper sulphate both the large-inoculum and the small-inoculum culture develop equally poorly. This extraordinary effect is not restricted to Aspergillus oryzae; we observed it with Penicillium chrysogenum, in a more restricted fashion, and there is a necessity that besides copper sulphate, traces of aluminium sulphate are added(2). With Penicillium chrysogenum too, the nutritious value of the conidial inoculum under our conditions of testing, could be neglected.

There can be no doubt that in our systems the described interdependence of inoculum size and trace element supply is due to other factors than the transfer of nutrients. This reasoning will also receive further support from the following observations: 1) The stage of culture development at which the trace elements are added is of importance, insofar that for example $Zn^{++}$ when added at advanced stage has no influence, whereas if included in the medium from the beginning it shows a marked effect; 2) there is a formation of self-stimulating and self-inhibiting substances, the formation of which depends on inoculum size and trace element supply, and the action of which depends very markedly on the stage of culture development(3,4).

The latter phenomenon appeared very significant to us in the elucidation of the mechanism of the described effects of inoculum size and of trace elements. The following observations are considered to be of importance:

(1) When after increasing stages of culture development of Aspergillus oryzae, inoculated with a large inoculum, mycelium in equal quantity is used to start fresh cultures, one can observe that the ability to grow on this medium (expressed as rate of growth in the linear phase) first decreases, then increases and reaches in fact higher values than the original culture inoculated with conidia. Furthermore, small amounts of culture filtrate from these same stages of growth added to the cultures at the stage of inoculation showed that the conidial inoculum is now strongly stimulated, whereas in the case of the mycelial inocula from various stages of culture development this stimulation can be observed only with mycelium from relatively young cultures(5). This experiment

certainly shows that self-stimulating substances are produced and released into the medium, and that its action is most pronounced if these substances act at early stages of growth.

(2) Under a great variety of conditions large- and small-inoculum cultures from both A. oryzae and P. chrysogenum were tested for the effect of culture filtrate on conidial and mycelial inocula, and in the vast majority of cases one could observe that self-stimulating substances were present in very early stages of culture development if there was abundant growth in advanced stages, and conversely self-inhibitory principles could be detected if growth of the cultures at advanced stages was delayed(3,4).

It seemed to be of considerable interest to examine the nature of these substances, and while we have not yet made a great deal of progress in this line of research we consider that these substances are of a chelating nature.

One of the most interesting observations regarding the effect of chelating agents has been observed in the previously described system, with A. oryzae where Cu stimulated the growth rate of a small-inoculum culture much more strongly than that of a large one. If in this medium maltose is now autoclaved together with the other components of the substrate the stimulatory effect of Cu is completely abolished. A similar phenomenon was again observed with P. chrysogenum(4). Furthermore, it had been known to us that traces of $Cu^{++}$ can inhibit the formation of amylase in A. oryzae very considerably, and that caramelization of the sugar reversed this effect. We have shown that through caramelization chelating agents are formed which have a particularly high affinity for $Cu^{++}$ and less for $Fe^{+++}$, $Mn^{++}$, $Zn^{++}$.

## 6. Efficiency of metabolism of carbon and nitrogen sources in Aspergillus oryzae.

That the foregoing phenomena are of industrial significance is obvious, since rate of metabolism and yield are the very criteria an industrialist in arguing upon. To be aware of factors influencing rate and yield is to be considered a first command. Of no less importance is the knowledge of the mechanisms of any such effects since it is then possible to improve fermentation not so much by trial and error but by specifically aimed experiments.

To give a full explanation to the phenomena previously described is impossible, but attempts to understand them involved also some energetic considerations. Thus we may wonder whether a microbial culture growing faster and/or showing up a higher yield of cell material utilize the available energy source more efficiently than a culture with lower rate and yield.

With Aspergillus oryzae it could be shown that low maximum yield of cell material was coupled with a relatively faster turnover of the energy source(6). As shown in Fig. 6 the ratio of carbohydrate metabolized to cell material formed is at all stages higher for the culture giving a lower maximum yield of cell material, which in the present case is the small-inoculum culture. Thus it has been possible to produce an overall inefficient cell-synthesizing culture merely by changing inoculum size. This inefficiency seems to be due partly to the organism's switching over to an alcoholic fermentation, but there may in addition be also inefficient coupling between the energy-yielding and energy-requiring (synthesizing) reactions.

The inefficiency of cell synthesis in the previous case is expressed also in nitrogen metabolism(7). The extreme is represented in Fig. 8 where it can be seen that under conditions of strong agitation in submerged culture the carbon-inefficient culture is also inefficient in turning inorganic nitrogen into cellular material, since not only is nitrogen content of cell matter low, but there is also an excretion of organic nitrogenous compounds exceeding considerably this property in the corresponding carbon-efficient culture. This kind of inefficiency is thus allied to a property which we find normally associated with autolysis.

## 7. Efficiency of metabolism in Aspergillus oryzae and its relation to amylase production.

The above observations bring us to another complex of phenomena typical for Aspergillus oryzae, i. e. amylase production. In a great many of investigations we could show that in liquid substrates, be it in stationary or submerged form, this fungus showed very considerable amylase production during the phase of autolysis, examples of which are shown in Fig. 8 (8). The previously discussed case now shows that a culture which is

prone to autolysis (as is shown by the very rapid decrease of mycelium content in the inefficient culture as compared with the other) forms amylase also during the actual phase of growth to a considerably higher degree than the efficient cell-building culture.

This property is best shown in Fig. 9, where the specific rate of amylase production (amylase/MDW) is plotted against MDW. Thus at any stage of culture development (indicated by mycelium content) the inefficient cell-synthesizing culture produced specifically more amylase than the efficient one.

The previous example shown should not create the impression that high yield of amylase and high (specific) rate of production is always to be found in small-inoculum cultures. Environmental factors can switch the effects to a complete reversal. Fig. 10 for example shows amylase production on a synthetic substrate adsorbed onto the expanded clay mineral vermiculite. It is obvious that a gradual reduction of inoculum size brings about gradual reduction of rate and maximum yield in amylase production.

8. **Metabolism** activities in surface and submerged cultures.

In the formation of an extracellular enzyme like the present one (α-amylase) a number of phenomena do occur which are very difficult to explain, but the observations on inoculum size effects may give us a start in this respect as shown in the arguments below. Thus it is rather interesting to compare for example submerged with surface cultures under conditions giving commercially interesting yields of amylase. Under submerged culture conditions the maximum yield of cell material is reached after 38 - 48 h (Fig. 12) and the maximum yield of α-amylase a little later (82 - 94 h)(9). In surface culture α-amylase production can start very late, i. e. significant increases in the present case take place only after about 64 h; the maximum yield (which is considerably higher than in submerged culture) is reached after 160 - 180 h. Apart from the fact that higher yields are always obtained in stationary cultures one can calculate also that the actual rate of amylase formation in the approximately linear phase of production is quite high. The observations pose the question whether not much oxygen is needed during the phase of amylase formation. Suggestions

to this being so were obtained from observations on amylase formation on wheat bran and on vermiculite. More specifically directed experiments in stationary and shaken cultures showed in fact that access of oxygen could be very drastically reduced (closure of conical flasks with plastic membrane or reduction of the cotton wool plug to a small fraction of the normal size) without affecting amylase production under these conditions. And yet in deep-culture conditions a reduction of the aeration rate from 900 to 300 ml/l, a rate still far in access to the conditions existing in stationary cultures, reduces amylase production very considerably.

That the case of stationary culture and submerged culture is not just a question of poorer or better exchange of gases or poorer or better contact of the cells with the substrate can be shown in the folbwing: Shaken cultures in the vast majority of cases give much lower rates of production and maximum yields of amylase than stationary cultures under the same conditions. Earlier on we have shown that the conditions for good or poor growth in filamentous fungi is laid down at early stages of culture development. For amylase production in A. oryzae the same seems to be the case. If conditions are chosen such that there is no specific effect of inoculum size on amylase production in stationary culture, there is then usually still a considerable effect in shaken culture, i. e. amylase production under these conditions can now be considerably increased if inoculum size is increased (Fig. 12 (10). We interprete these phenomena to the effect that under shaken culture conditions with small inoculum in the present substrate the organism has been unable to establish at very early stages of culture development a micro climate conducive to a conditioning of the mycelium endowed with good amylase-forming ability, in view of the fact that through the shaking action any excreted substances are diluted immediately. In a still culture, on the other hand, metabolic compounds will be present in higher concentrations in the immediate vicinity of the mycelium (and therefore also inside the hyphae). If now we imagine that the property for good or poor amylase formation is laid down already at early stages of culture development and that this conditioning takes place through

self-produced substances, it will follow that on the one hand inoculum size can affect the culture differently in surface culture than in shaken culture, on the other hand the explanation for the different behaviour of the shaken culture (under conditions of equal rates of exchange of gases as for stationary cultures) would seem to be due to its property of establishing a micro climate in early culture development different from the one obtained in stationary incubation.

That the micro climate in early stages of culture development is important, and that the mould acquires then certain properties which are not extinguished so easily later on is shown also by the following. A. oryzae, when cultivated for short periods in submerged culture maintains the property regarding amylase formation (i. e. poor production) acquired during this phase, since incubation in stationary form beyond this submerged pre-incubation phase does not improve amylase production (Fig. 13). Conversely pre-incubation in stationary form followed by submerged cultivation results in cultures with higher amylase-producing abilities (Fig. 14) than incubation in submerged form during the whole culture cycle(10).

9. Energetics in anaerobic and aerobic metabolism.

91. Alcoholic fermentation

After all these hard-to-explain phenomena of kinetics and energetics it would seem advisable to return to some better defined and easier-to-comprehend systems.

Probably the best understood metabolic phenomenon is alcoholic fermentation of yeast. One can expect that this organism in a well defined substrate with glucose as C-source produces 2 moles of alcohol and 2 moles of carbon dioxide from 1 mole of hexose. Besides alcohol and carbon dioxide cell material is formed and it is clear that the extent of cellular synthesis is dictated by the extent of useful energy released through the fermentation process. This yield of energy is firmly established to be 2 moles of ATP per mole of glucose fermented, the equation being normally written as follows:

$$C_6H_{12}O_6 + PO_4^{---} + 2\ ADP \rightarrow 2CH_3\text{-}CH_2OH + 2CO_2 + 2ATP$$

It is also well established that for each mole of ATP produced 1o g of cell material can be synthesized, so that the following equations can be established, where "$CH_2O$" is a symbol for the formation of 3o g of cell material.

$$9\ C_6H_{12}O_6 + 18\ PO_4^{---} + 18\ ADP \rightarrow 18\ CH_3\text{-}CH_2OH + 18\ CO_2 + 18\ ATP$$

$$C_6H_{12}O_6 + 18\ ATP \rightarrow 6\ "CH_2O" + 18\ PO_4^{---} + 18\ ADP$$

---

$$1o\ C_6H_{12}O_6 \rightarrow 18\ CH_3\text{-}CH_2OH + 18\ CO_2 + 6\ "CH_2O"$$

1800 g → 828 g + 792 g + 18o g

1oo g → 46 g + 44 g + 1o g

The industrialist is only little interested in cell material produced under the present conditions. Could one now try to increase yield of alcohol at the expense of cellular material? Obviously one method to be considered could consist in the re-use of cell material once formed and preventing any further increase in cell material by limitation of nutrients. Letting aside the contamination problems involved in the re-use of cell material, it will have to be appreciated that the stability of the zymase complex is not indefinite. With complete lack of nutrients and energy decay of cell material will invariably set in, because of the widely reversible nature of enzyme reactions. The next step would then appear to be to add just enough nutrients enabling the cells to keep up their organized state. This would then effectively constitute what is usually called maintenance energy. While in industrial ethanol production this concept does not seem to have been used seriously, there is a good example of industrial fermentations known in gluconic acid production from glucose by Aspergillus niger. By re-using pre-formed and concentrated mycelium from gluconic acid fermentation over several cycles the nitrogenous components of the medium could be reduced drastically, thus limiting further increase in cell material and increasing yield of gluconic acid to about 1oo % of the fermented sugar(11).

Regarding alcoholic fermentation by yeast it has been shown recently that under certain conditions a re-use of cell material combined

with a limitation of yeast growth is now possible, although it is not a limitation by nitrogen sources. This process, in the case of brewing, has already been established in industry in a number of places(12). It is of interest to realize that, paradoxically as it may seem at first sight, limitation of growth is obtained by anaerobiosis. The explanation to this phenomenon is seen in the fact that continued propagation of yeast under complete exclusion of air will result in a very drastic drop of growth rate until a virtual stand-still is obtained if small inocula are used. It is now thought that this reduction of growth is due to establishment of a very low redox potential which in turn results in the yeast's inability to synthesize some essential steroids. The process, which is operated continuously, consists in propagating a strongly flocculating yeast in a tower-type fermenter. The upward flowing mash passes an infinite number of different fermentation stages, representing in fact a multi-stage fermenter with almost 100% yeast return, accomplished by its strong settling tendency (Fig. 15). The amount of surplus yeast produced is very small which explains the higher yields of alcohol obtained. The limitations of growth are obtained by the extreme anaerobiosis in the fermenter. In order to enable the yeast to make use of maintenance energy a small amount of oxygen has in fact to be introduced into the fermenter.

One point to be conscious about if the energy of the dissimilatory process is not used for cell synthesis is that it will be released as heat, which in the case of the tower fermenter can be quite large in view of the extremely high content of yeast. This may be advantageous in regions with low ambient temperature; it is disadvantageous, however, in tropical regions, particularly in arid areas.

Looking for ways and means to increase yield of ethanol one might also consider those organisms which intrinsically have an inefficient metabolism in so far that they deviate a still smaller fraction of the energy source than yeast to the synthesis of cell matter and yet have an equally high or even higher specific fermentation rate (alcohol production per unit time and unit cell weight). Such an organism is known in fact and occurs predominantly in tropical countries, i.e. Zymomonas mobilis (Pseudomonas lindneri). This bacterium produces only one mole of ATP per mole glucose fermented, and if one considers further that it produces on

average only about 8 mg cell dry matter per mole of ATP (13), as compared with about 1o mg for yeast, the following balance equation can be established:

$$C_6H_{12}O_6 + PO_4^{---} + ADP \rightarrow 2\ CH_3\text{-}CH_2OH + 2\ CO_2 + ATP$$

$$o.o45\ C_6H_{12}O_6 + ATP \rightarrow o.27\ "CH_2O" + PO_4^{---} + ADP$$

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$$1.o45\ C_6H_{12}O_6 \rightarrow 2\ CH_3\text{-}CH_2OH + 2\ CO_2 + o.27\ "CH_2O"$$

188.1 g → 92 g + 88 g + 8.1 g

1oo g → 49 g + 46.7 g + 4.3 g

showing in principle the higher yield of alcohol that could be obtained as compared to a normal yeast fermentation. In an undisturbed fermentation, where full use is made of the fermentation energy for cell synthesis, the rate of fermentation in the case of Zymomonas does not fall behind that of yeast as a result of the considerably lower generation time of the bacterium. Practically speaking there are a number of considerations to be taken into account before applying the Zymomonas fermentation industrially, i. e. growth of Zymomonas at somewhat higher pH values than yeast, thus rendering the fermentation prone to contamination, the lower alcohol tolerance of Zymomonas, the inability of Zymomonas to ferment various disaccharides.

92. Aerobic metabolism of yeast on carbohydrates.

While in fermentation it is desirable to repress biomass formation as far as possible, the reverse is naturally the case in the manufacture of baker's yeast or fodder yeast. Here the question we pose is: Can the yield of cell matter still be further increased? It would seem that after the operation of this process over more than a century has shown that under ideal conditions the yeast dry weight to be obtained is about 5o % of the sugar utilized, it would not be possible to gain much here. Nevertheless, after so much detailed knowledge has by now accumulated on the mechanism of dissimilation and assimilation it is interesting to make a few comparisons.

Yeast has been shown to use the citric acid cycle under aerobic conditions together with the electron transfer reactions typical for

mammalian cells, thus producing a total yield of 38 moles ATP per mole glucose oxidized. If about half of a sugar molecule is converted into an equal weight of cell material and the other half is fully oxidized to carbon dioxide and water, we conclude that 19 moles of ATP where necessary to produce 9o g of dry yeast. The yield of cell matter per mole ATP ($Y_{ATP}$) thus is about 4.7 g as compared to 1o g under anaerobic conditions. From this point of view the aerobic process is to be considered inefficient, and one keeps wondering whether there can be much hope to increase this efficiency. At least one should not be surprised to find aerobic micro-organisms endowed with a better efficiency of energy utilization. It would seem likely that the ATP pool of the cells under aerobic conditions is higher than under anaerobic conditions in view of the vastly greater number of ATP generated per mole of glucose metabolished. And yet the rate of multiplication is increased only by a factor of 2 as compared with the anaerobic process (see Fig. 16). It would appear that under these conditions the rate of ATP production is so high that it cannot sufficiently quickly be made use of in cellular synthesis, and a large proportion of ATP decays with the generation of heat.

93. Aerobic metabolism of yeast on hydrocarbons.

The previous discussion referred to the use of carbohydrate as source of energy and carbon. What is the situation now if more strongly reduced substrates are used? Considering that the calorific value is higher, one would also expect the yield of biomass to increase.

One can expect this to occur only, of course, in the case of an aerobic fermentation process. In the case of Klebsiella aerogenes (Aerobacter aerogenes) under anaerobic conditions the yield of ATP per mole of substrate utilised is smaller with mannitol (2.5) than in the case of glucose or fructose (3.o)(13). It would seem in this case that some energy is necessary to oxidize the substrate in order to make it comparable to the normal state of oxido-reduction of the cell material which is approximately that of carbohydrate.

Substrates as strongly reduced as hydrocarbons could not at all be made use of under anaerobic conditions or without suitable H-acceptor, as there is no free energy available in the reduction of hydrocarbon to the oxido-reduction stage of carbohydrate with concomitant release of $H_2$.

Under aerobic conditions it has now been established in a number of different laboratories that yeast is able to produce approximately an equal amount of biomass as weight in paraffins utilized(14,15,16). This balance equation could be written approximately as follows if we assume complete oxidation of the substrate (octadecane) to carbon dioxide and water.

$$C_{18}H_{38} + 19.5\ O_2 \rightarrow \underset{\text{cell mass}}{3\ "CH_2O"} + 10\ CO_2 + 11\ H_2O$$

254 g + 624 g → 240 g + 440 g + 198 g

100 g + 246 g → 95 g + 173 g + 78 g

Here again we may ask the question whether this yield coefficient could be expected to be further increased or whether it compares favourably with the processes previously discussed. For this purpose we could examine the mechanism of breakdown of hydrocarbons and of energy generation.

It seems quite well established now that the initial step of hydrocarbon oxidation is an oxygenation of the methyl group(17). There does not seem to be any phosphorylation involved in this step. After oxidation of the alcohol thus produced to a carboxylic acid via aldehyde the further oxidation would occur via β-oxidation with the generation of "active acetate" (acetyl-CoA) fragments as shown below:

$$R\text{-}CH_2\text{-}CH_3 + \text{red. Coenz.} \xrightarrow[O_2]{\text{oxygenase}} R\text{-}CH_2\text{-}CH_2OH + H_2O + \text{oxid. Coenz.}$$

$$R\text{-}CH_2\text{-}CH_2OH + \text{oxid. Coenz.} \rightarrow R\text{-}CH_2\text{-}CHO + \text{red. Coenz.}$$

$$R\text{-}CH_2\text{-}CHO + NAD + CoA + ATP \rightarrow R\text{-}CH_2\text{-}CO\text{-}CoA + NADH_2 + ADP + PO_4^{---}$$

$$R\text{-}CH_2\text{-}CO\text{-}CoA + FAD \rightarrow R' - CH = CH - CO\text{-}CoA + FADH_2$$

$$R' - CH = CH - COOH + H_2O \rightarrow R' - CHOH\text{-}CH_2\text{-}CO\text{-}CoA$$

$$R' - CHOH\text{-}CH_2\text{-}COOH + NAD \rightarrow R' - CO\text{-}CH_2\text{-}CO\text{-}CoA + NADH_2$$

$$R' - CO\text{-}CH_2\text{-}COOH + CoA \rightarrow R' - CO\text{-}CoA + CH_3CO\text{-}CoA$$

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$$RCH_2\text{-}CH_3 + O_2 + 2NAD + FAD + 2CoA + ATP \rightarrow R'\text{-}COCoA + CH_3COCoA + 2NADH_2 + FADH_2 + ADP + PO_4^{---}$$

The Coenzymes NAD and FAD will be regenerated from the corresponding reduced forms by oxidation with oxygen. The required Co-enzyme A will be regenerated after utilization of Acetyl-CoA.

The utilization of hydrocarbons thus can be reduced to a utilization of acetate, and it would now seem a reasonable procedure to estimate what the yield of biomass can be expected to be by aerobic utilization of acetate. Considering that for each acetate utilized in the tricarboxylic acid cycle 12 ATP will be generated, and assuming now a utilization of acetate for cell synthesis with the same degree of efficiency as yeast does with carbohydrates under aerobic conditions, we could construct the following equations:

$2\ NADH_2 + O_2 + 6\ ADP + 6\ PO_4^{---} \rightarrow 2\ H_2O + 6\ ATP$ ) from dehydrogenation of

$FADH_2 + 1/2\ O_2 + 2\ ADP + 2\ PO_4^{---} \rightarrow H_2O + 2\ ATP$ ) hydrocarbons to generate 1 acetate

$CH_3\text{-}CO\text{-}CoA + 2\ O_2 + 12\ ADP + 12\ PO_4^{---} \rightarrow 2\ CO_2 + 2\ H_2O + 12\ ATP + CoA$

$CH_3\text{-}CO\text{-}CoA \xrightarrow{12.7\ ATP}$ 2 "$CH_2O$" cell mass

A total of 2o ATP could thus be generated by removal of one mole of acetate from the hydrocarbons and oxidizing it to $CO_2$ and $H_2O$. This is more than required for the formation of 6o g (2 "$CH_2O$") of biomass from another mole of acetate. A balance equation for the utilization of octadecane based on the above theoretical considerations is shown below, where for simplicity the reactions with CoA are omitted.

$C_{18}H_{38} + O_2 + 2\ NAD + FAD + ATP^{*} + 2\ H_2O \rightarrow$
$CH_3\text{-}(CH_2)_{14}\text{-}COOH + CH_3\text{-}COOH + 2\ NADH_2 + FADH_2 + ADP + PO_4^{---}$

$CH_3\text{-}(CH_2)_{14}\text{-}COOH + 7\ NAD + 7\ FAD + 14\ H_2O \rightarrow 8\ CH_3\text{-}COOH + 7\ NADH_2 + 7FADH_2$

$9\ NADH_2 + 3\ FADH_2 + 8.5\ O_2 + 43\ ADP + 43\ PO_4^{---} \rightarrow 17\ H_2O + 9\ NAD + 3\ FAD + 43\ ATP$

$3\ CH_3\text{-}COOH + 6\ O_2 + 36\ ADP + 36\ PO_4^{---} \rightarrow 6\ CO_2 + 6\ H_2O + 36\ ATP$

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*One ATP is considered to be necessary here to produce fatty acid - CoA from the aldehyde.

$6\ CH_3\text{-}COOH + 72\ ATP \rightarrow 12\ "CH_2O" + 72\ ADP + 72\ PO_4^{---}$

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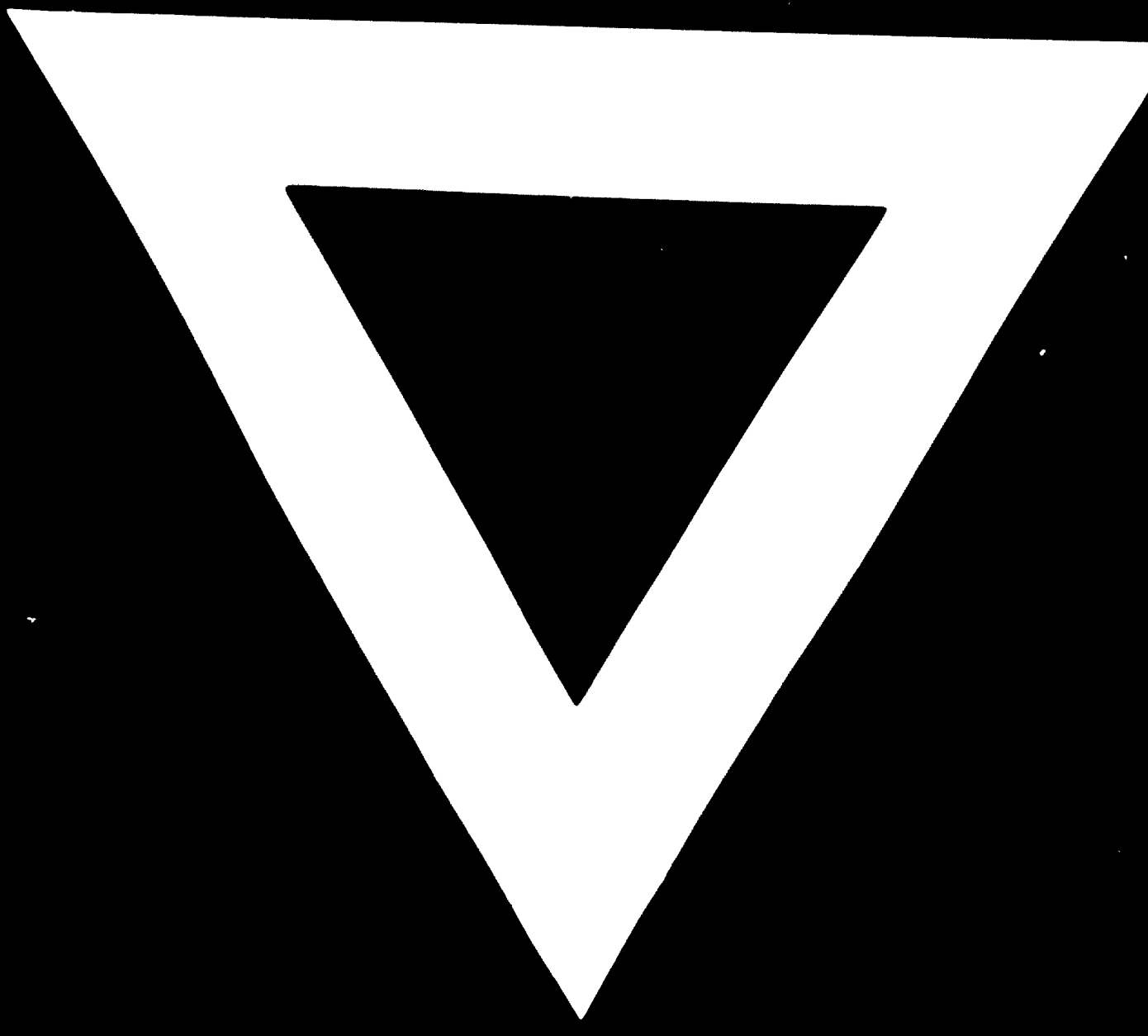
| | | | | | |
|---|---|---|---|---|---|
| $C_{18}H_{38}$ | + 15.5 $O_2$ | → | 12 "$CH_2O$" | + 6 $CO_2$ | + 7 $H_2O$ |
| 254 g | 496 g | → | 360 g | 264 g | 126 g |
| 100 g | 195 g | → | 142 g | 104 g | 49 g |

This balance equation, compared with the one based on actual observation, shows that yeast does not work in practice as efficiently as has been expected. Certainly more detailed investigations will be necessary to clarify fully the energy-yielding as well as the energy-requiring reactions. However, there seems to be a tendency towards more inefficient utilization of the energy source if the ATP yield per unit equivalent of C substrate increases. Thus, for yeast the following sequence can be established:

| type of metabolism | ATP yield per C atom of substrate | Biomass (g) per ATP |
|---|---|---|
| alcoholic fermentation | 0.33 | 10 |
| complete oxidation of sugar by oxygen | 6.33 | 4.7 |
| complete oxidation of hydrocarbons (octadecane) by oxygen | 3.3 | 1.69 |

The situation may not be as discouraging as it seems at first sight, since there are reports on very efficient utilization of hydrocarbons by bacteria, i. e. the Chinese Petroleum Company (Taiwan) indicated that the yield of biomass can be double that of hydrocarbons utilized (18). This in fact is not out of reach at all, and it would seem worthwhile to investigate whether yeast might not be able to do the same.



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