ENERGETIC IRRELEVANCE OF AEROBIOSIS FOR *S. CEREVISIAE* GROWING ON SUGARS

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Summary

The net benefit that *Saccharomyces cerevisiae* obtains from aerobiosis as compared to anaerobiosis has been studied. For this purpose yeasts with different respiratory capacities have been obtained by growing them in batch cultures on different substrates. Even with sugars with low catabolite repression effect, as is the case of galactose, aerobiosis increased the growth rate and the growth yield by less than twofold. These variations, which are much lower than the expected considering the actual oxygen utilization, indicate that either the amount of ATP produced in respiration is much lower than the theoretically expected or a much greater expenditure of ATP occurs in aerobic than in anaerobic growth. The results show that *S. cerevisiae* obtains only a slight benefit from aerobiosis when growing on sugars at the relatively high concentration prevailing in its natural habitats.

The inhibition of sugar consumption rate by aerobiosis (Pasteur effect) has also been studied, Pasteur effect was almost unnoticeable during growth on any tested sugar and very low during ammonia starvation. These results contrast with the general belief that Pasteur effect is a quantitatively important phenomenon in yeast. It is concluded that the relevant observations of Louis Pasteur have little relationship with the phenomenon that bears his name.

Introduction

It is generally accepted that the appearance of the ability to utilize oxygen was a critical step for cellular economy. If a facultative anaerobic cell shifts from sugar fermentation to complete oxidation it could produce up to 18 times more ATP per mol of catabolized sugar. As a consequence, the growth yield would increase since this magnitude is related to ATP production[1,2]. Also, if the rate of carbohydrate consumption remains constant, a much higher velocity of growth would be expected in aerobic conditions. However, if the rate of ATP production were equal in the presence of air as in its absence, a great decrease in carbohydrate consumption, what is known as Pasteur effect, would take place in aerobiosis. These situations are probably extreme ones. What should be expected in the shift from anaerobiosis to aerobiosis is an intermediate situation, i.e. an increased growth yield accompanying a somewhat increased growth rate and a decreased sugar consumption.

The aim of this work is to study the net benefit of aerobiosis as compared to anaerobiosis for *S. cerevisiae* when growing on sugars at the relatively high concentration prevailing in its natural habitats. For this purpose the above mentioned parameters have been measured in yeast with different respiratory capacities. These yeasts were obtained by growing them in batch cultures on galactose, maltose or glucose whose effects as nutritional repressors of respiration are well documented[3,4]. The results show that the increase of cellular yield and growth rate is very low even when yeast respiration is important, and that the Pasteur effect is nearly unnoticeable during growth and very low during ammonia starvation.

Materials and Methods

Yeast growth

Three different strains of *S. cerevisiae* were used: D665-1A and S288C (from the Cold Spring Harbor Course on Genetics), and S-13-Gal isolated for its ability to grow on a synthetic medium with galactose as only carbon and energy source from the strain 1724-14A (originally provided by Dr. HAWTHORNE, Seattle). The results found were similar with all of them. The results shown in this paper were obtained with S-13-Gal, which was grown in a synthetic medium as previously described[4] with glucose (2%), galactose (2%), maltose (2%) or ethanol (1%) as the only carbon and energy source.

Aerobic cultures had air as gaseous phase. 25 ml of media were placed in 250 ml flasks. This ratio was maintained when greater volumes of cultures were used (250 ml in 2500 ml flasks). The flasks were closed with cotton and incubated at 30 °C in a New Brunswick Scientific rotatory shaker Model G25 at 200 rpm. The tension of oxygen, which was measured with an Astrup Microequipment Radiometer in the presence of 1 mM KCN, remained constant throughout the logarithmic and stationary phase of growth and it was equal to that of the media without cells (ca. 140 mmHg). When growing anaerobically the cultures were previously saturated with nitrogen by bubbling this gas through the media for 15 minutes. The flasks were closed with rubber stoppers with a hermetic closure (Muller's valve) filled with sulfuric acid to avoid air diffusion into the cultures, and incubated as described above. During the sampling of anaerobic cultures nitrogen was continuously bubbled through the cultures.

Ammonium starvation

Exponentially growing cultures were centrifuged at 3,000 g × 5 min and the cells washed at room temperature with a NH_4^+ free medium with glucose as indicated in each experiment. The NH_4^+ free medium contained all the components present in the growth medium except that the 3.3 g of $(NH_4)_2HPO_4$ and the 0.001 g of $(NH_4)_2Fe(SO_4)$ were replaced by 5.9 g $K_2HPO_4 + 3H_2O$ and 0.001 g $FeCl_2$. Incubation proceeded as above in a rotatory shaker at 30 °C.

Analytical procedures

Growth of the cultures were followed turbidimetrically in a Klett–Summerson colorimeter using filter n°42. Growth yield was measured by determining the weight of yeast.

Total proteins were determined as described by JAYARAMAN *et al*[5].

Fermentation and respiration were measured manometrically at 30 °C by conventional methods[6] in aliquots of growing cultures or of starving suspensions, containing ca. 0.8 mg of yeast (dry weight). The increments in the manometers were followed for at least 1 hour. Oxygen consumption was measured in the presence of 20% KOH in the center well. When indicated, anaerobiosis was obtained by bubbling nitrogen for 15 minutes through the Warburg vessels. In the experiments of inhibition of respiration by cyanide a Clark type oxygen electrode was used. Aliquots containing ca. 0.8 mg of yeast (dry weight) were poured into the reaction chamber and O_2 consumption at 30 °C was followed.

Glucose, galactose and products of their catabolism were estimated in aliquots of the media filtered though Millipore filters 0.47 μm pore size, by conventional enzymic methods[7]. Maltose was estimated colorimetrically[8]. In all cases internal standards were used.

Reagents

Enzymes and nucleotides were from Boehringer (Mannheim, G.F.R.), yeast extract from Difco Laboratories (Detroit, Mich. U.S.A.). All other reagents were of analytical grade. Nitrogen contained less than 1 μg of oxygen/g nitrogen.

Results

Validity of the methods used to estimate the Pasteur effect

The Pasteur effect is presently defined as the inhibition of glycolysis by respiration and this meaning would be used throughout this article. However, it is important to note that the term "Pasteur effect" has been indiscriminately used in the literature with different meanings, namely the decrease of either sugar consumption or fermentation produced by aerobiosis with respect to anaerobiosis.

Glycolytic flux in yeast utilizing sugars can be

estimated, at least, by two procedures: a) Measurements of the rate of sugar disappearance from the medium. This procedure would be valid if, as it seems to be the case in growing yeast, the Embden-Meyerhof pathway is the predominant route of carbohydrate utilization[4]. The results in Table 1 indicate that this is also the case in NH_4^+ starved cells. In fact more than 80% of the sugar consumed goes through the glycolytic chain. Therefore this requirement is fulfilled by yeast in the experimental conditions used in this work.

b) Measurements of the rate of fermentation and respiration. This procedure is valid when the glycolytic chain is mainly used for catabolic purpose without major drainage for anabolic reactions. This is the case of growing[4] and NH_4^+ starved yeast (Table 1) since in both cases almost 80% of utilized sugar accumulated in the media as fermentation product.

The rate of sugar fermentation can be estimated manometrically if at least two requirements are met: 1) The major proportion of fermented sugar is channelled to pathways producing CO_2; 2) No pathways other than fermentation produce important amounts of CO_2. The first requirement is reasonably well met by yeast since, exception made of glyceric fermentation which accounts for less than 15% of the total sugar consumed, all other fermentative pathways produce one mol of CO_2 for each mol of fermentation products formed[4] (Table 1). The second condition is also met since the two other major pathways that produce CO_2 (respiration and the pentosephosphate shunt) would not significantly interfere with the manometric measurements. In fact the respiratory quotient of sugar is 1 and our previous results indicate that the pentosephosphate shunt accounts for only 2.5% of the total sugar consumed[9,10]. This contribution would be still lower during NH_4^+ starvation. The good experimental agreement between the CO_2 produced and the fermentation products accumulated in the media (Table 1) supports this conclusion.

The rate of sugar oxidation can be estimated from manometric measurements if most of the oxygen consumed by yeast is used in the respiratory chain. Although biosynthesis of unsaturated fatty acids and ergosterol utilize oxygen[11], their contribution to oxygen consumption is negligible[9,12], therefore the main consumer of oxygen seems to be the respiratory chain. This assertion is supported by the fact that inhibition of oxygen consumption by cyanide, a specific inhibitor of cytochromeoxidase[13], was higher than the 90% (results not shown).

All these facts indicate that, in the experimental conditions used throughout this work, the Pasteur effect can be estimated either from sugar consumption rates or from fermentation and respiration rates. The good agreement obtained using both methods gives further support to this conclusion.

Influence of aerobiosis on the growth rate, growth yield and the rate of sugars consumption.
No significant differences in the rate of growth or growth yield were observed in yeast growing aerobically and anaerobically on glucose (Fig. 1). The fact that glucose disappearance from the

Table 1
CO_2 production, O_2 consumption and fermentation products accumulated in the medium by *Saccharomyces cerevisiae* utilizing glucose during NH_4^+ starvation

Conditions	Glucose consumed (mmol/l.)	Growth yield (g dry yeast/l.)	CO_2 (mmol/l.)	Ethanol (mmol/l.)	Glycerol (mmol/l.)	Acetaldehyde (mmol/l.)	Acetate (mmol/l.)	O_2 (mmol/l.)
Aerobiosis	42±5	0.72±0.04	69±10	60±7	9.8±1.0	≤1	1±0.1	5.2±0.6
Anaerobiosis	44±3	0.72±0.08	70±11	63±5	13.7±0.8 ;	≤1	1.2±0.1	—

Yeast was grown with gluose as carbon source. At the middle of the exponential growth the culture was centrifuged, washed and resuspended at a cellular density of 1.8 g dry yeast/l. in the NH_4^+ free medium with ca. 1% glucose. 1.5 ml aliquots of this suspension were placed in 15 ml Warburg vessels and O_2 consumption and CO_2 production measured. As soon as CO_2 production stopped, the yeast suspensions were centrifuged and fermentation products measured in the supernatant. Mean values and standard deviation of four experiments are shown.

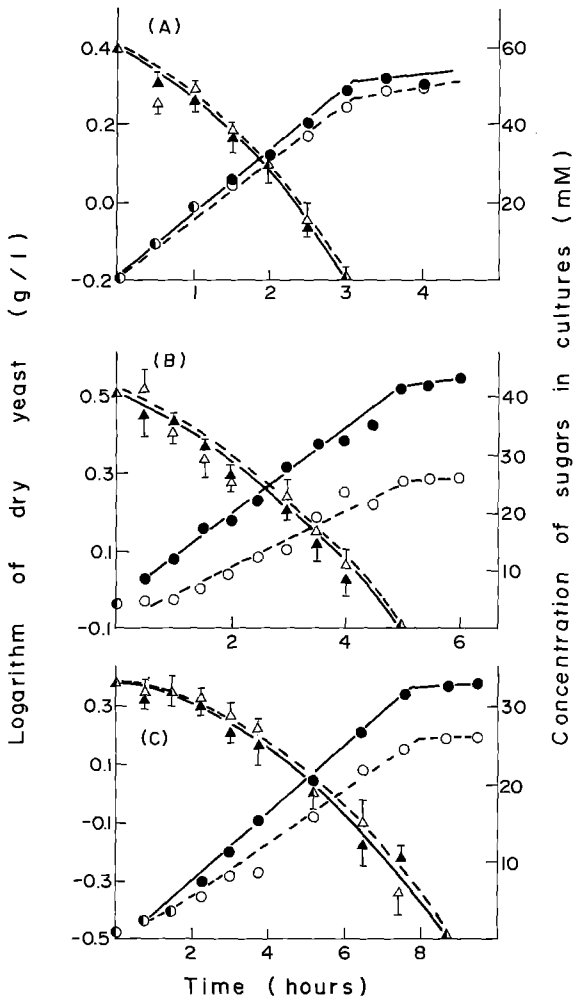

Fig. 1. Rate of growth, sugar consumption and growth yield of yeast growing under aerobic and anaerobic conditions on different sugars.

Cultures at the beginning of the exponential growth with glucose (A) galactose (B) or maltose (C) as carbon and energy source were separated into two aliquots. One aliquot was incubated aerobically at 30 °C with shaking, and the other one anaerobically under the same conditions. At the indicated times samples were taken, filtered through Millipore and growth and sugar concentration in the media determined. Mean values and standard deviation of four experiments are shown, Symbols: ●——● growth in aerobiosis; ○- - -○ growth in anaerobiosis; ▲——▲ sugar concentration in aerobic cultures; △- - -△ sugar concentration in anaerobic cultures.

medium occurred also at the same velocity (Fig. 1) indicates that glycolytic flux was similar in both conditions, in other words, that no Pasteur effect was observable during growth on this sugar. In cultures of galactose and maltose, although sugar disappearance from the media occurred at similar velocity, the growth rate was lower in anaerobiosis indicating that the actual rate of sugar consumption per mass of cells was somewhat higher in the absence of air and therefore that a small Pasteur effect was taking place during growth on these sugars (Fig. 1).

It can be calculated from data of Figure 1 that the generation time in galactose was 4.2 hours in anaerobiosis and 2.4 hours in aerobiosis, and that the growth yield was respectively, 58 and 25 g dry yeast/mol of sugar consumed. In the case of maltose generation times of 3, 4 and 2.7 hours took place and growth yield of 30 and 20 g dry yeast/mol of hexose consumed were respectively observed.

Pasteur effect in growing yeast calculated from manometric data

The rates of fermentation and respiration during exponential growth are shown in Table 2. Only ca. 30% of the galactose and 3% to 5% of the glucose and the maltose catabolized were oxidized. Glycolytic flux calculated from these data were similar in aerobiosis and in anaerobiosis indicating that the Pasteur effect was too small to be detectable by these methods.

Contribution of respiration and fermentation to ATP production

The response of a facultative anaerobic cell to the presence of oxygen would depend on the actual contribution of respiration to its energy metabolism. Production of ATP in fermentation can be easily determined since each mol of sugar transformed to ethanol, acetate, or acetaldehyde produces a net gain of 2 mol of ATP and no net gain of ATP takes place in the transformation of 1 mol of sugar to glycerol. The amount of ATP produced in respiration is more difficult to estimate since different phosphorylation efficiencies of the yeast respiratory chain have been reported. When catabolite repression occurs, only two phosphorylation sites seem to be operative[14] and a third site (site I) seems to appear when a complete development of the respiratory chain occurs[15].

In the calculations of ATP production shown in Table 3, two values are given corresponding to P/O ratios of 2 and 3. With either assumption it appears that yeast growing on galactose obtained from respiration as much as 85% of the total ATP produced in catabolism. In the case of yeast growing on glucose and maltose

Table 2
Pasteur effect in growing yeast calculated from manometric data

Substrate in the medium	Hexose consumption rate (mmol g protein^{-1} h^{-1})				
	Aerobiosis			Anaerobiosis	
	Fermented	Oxidized	Glycolytic flux[a]	Glycolytic flux[a]	Pasteur effect[b]
Glucose	39±4.4	1.1±0.1	40	42±1.0	1.05
Galactose	11±0.3	4.5±0.2	16	17±1.2	1.08
Maltose	28±1.0	1.4±0.1	29	26±3.0	0.90

Aliquots of exponentially growing cultures containing 0.8 mg of yeast (dry weight) were placed in Warburg vessels and O_2 consumption and CO_2 production were measured in the presence or in the absence of air. From these data, fermented and oxidized substrates have been calculated assuming that all CO_2 is produced in fermentation and that all O_2 is consumed in respiration (see the text), by substitution in the equations:
Fermentation: $C_6H_{12}O_6 \rightarrow 2CH_3{-}CH_2OH + 2CO_2$
Respiration: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$
[a] Glycolytic flux has been calculated by addition of the rate of sugar fermentation and oxidation.
[b] Pasteur effect is expressed as the ratio between glycolytic flux in anaerobiosis and in aerobiosis. Mean values and standard deviation of four experiments are shown.

about 30% and 50% of the ATP were obtained in this pathway.

Pasteur effect during ammonia starvation
Since in growing yeast a very small Pasteur effect was observable it appeared interesting to test the effect of NH_4^+ deprivation on this phenomenon. For this purpose yeasts, after growing on different substrates, were washed and resuspended in NH_4^+ free medium with glucose as carbon source, and the glycolytic fluxes were estimated in the presence or absence of air.

Yeast grown on glucose showed no significant differences between glycolytic fluxes in aerobiosis and anaerobiosis during an initial period of NH_4^+ starvation, but differences gradually appeared and were easily detected after 22 hours, that is when the relative contribution of respiration had increased noticeably (Table 4). Yeast grown on galactose or ethanol showed differences measurable from the beginning.

Table 3
Calculated rate of ATP production during growth in aerobiosis and anaerobiosis

Substrate in the medium	ATP production rate (mmol g protein^{-1} h^{-1})				
	Aerobiosis				
	Fermentation	Respiration	Total	% of the total (produced in respiration)	Anaerobiosis
Glucose	78	40 (29)	118 (107)	34 (27)	84
Galactose	22	162 (117)	184 (139)	88 (84)	34
Maltose	56	51 (37)	107 (93)	48 (40)	53

Data from Table 2 have been used. 2 mol of ATP are gained in the fermentation of 1 mol of hexose. P/O equal 3 and 2 (in brackets) in the respiratory chain have been assumed. With P/O = 2, 26 mmol of ATP would be produced in the oxidation of one mmol of sugar. 36 mmol of ATP would be formed with P/O = 3.

Table 4
Pasteur effect in yeast during NH_4^+ starvation calculated from manometric data

Yeast grown on	Time without NH_4^+ (h)	Glucose consumption rate (mmol g protein^{-1} h^{-1})				
		Aerobiosis			Anaerobiosis	
		Fermented	Oxidized	Glycolytic flux[a]	Glycolytic flux[a]	Pasteur effect[b]
Glucose	0	20.5±1.1	1.0±0.09	21.0	22.5±1.5	1.1
	3	12.0±0.5	1.0±0.1	13.0	17.0±0.8	1.3
	22	3.3±0.4	0.6±0.08	3.9	7.0±0.4	1.8
Galactose	0	6.5±0.2	3.5±0.1	10.0	16.0±2.1	1.6
	3	4.5±0.4	3.6±0.3	8.1	16.0±1.5	2.0
	5	2.8±0.4	3.3±0.3	6.1	13.0±1.7	1.8
Ethanol	0	3.0±0.2	3.2±0.4	6.2	8.0±1.0	1.3
	3	4.0±0.4	3.6±0.4	7.6	11.5±0.2	1.5
	5	4.0±0.3	3.5±0.4	7.5	11.5±0.7	1.5

Yeasts were grown on glucose, galactose or ethanol. At the middle of exponential growth cultures were centrifuged, washed and resuspended in the NH_4^+ free medium with 2% glucose. Aliquots of these suspensions were placed in Warburg vessels and oxygen consumption and CO_2 production were measured. From these data fermented and oxidized substrates have been calculated assuming that all CO_2 is produced in fermentation and that all O_2 is consumed in respiration (see the text), by substitution of the CO_2 and O_2 increments in the equations:

Fermentation: $C_6H_{12}O_6 \rightarrow 2CH_3\text{–}CH_2OH + 2CO_2$

Respiration: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$

[a] Glycolytic flux has been calculated by the addition of the rate of sugar fermentation and oxidation. [b] Pasteur effect is expressed as the ratio between glycolytic flux in anaerobiosis and aerobiosis. Mean values and standard deviation of four experiments are shown.

Similar results were found when, instead of manometric techniques, measurements of glucose consumption were used (results not shown).

Discussion

The actual benefit, in terms of ATP production, that a cell would obtain from aerobiosis would be dependent on the contribution of respiration to its energy metabolism. A cell with only aerobic catabolism in the presence of air would produce up to 18 times more ATP in aerobiosis than in anaerobiosis, assuming that alcohol fermentation is the only catabolic pathway in anaerobiosis (Table 5). Since yeast growing on galactose respires 30% of the total sugar utilized (Table 2) its theoretical benefit would be ca. 6 times (Table 5). According to this, important differences in the growth rate, growth yield and velocity of galactose consumption would occur depending on the presence or the absence of air. Much lower differences could be expected in the case of glucose and maltose whose contribution of respiration is only 3 to 5% (Table 5).

The results presented in this work show that, in batch cultures, even when *Saccharomyces cerevisiae* is growing on sugars with low catabolite repression effect as occurs in the case of galactose[3,4], the effect of aerobiosis on the above mentioned parameters is very small. In fact differences lower than twofold were found during growth on this sugar (Fig. 1). Two reasons at least can contribute to the explanation of these facts: a) The actual amount of ATP produced in respiration is lower than in vitro experiments indicate, as suggested by VON MEYENBURG[16]. b) A greater expenditure of ATP takes place in aerobic than in anaerobic growth as other results also seem to indicate[17,18]. Whatever it may be, it is clear that yeast shows a very weak response to aerobiosis in batch cultures, weaker than could be expected from its respiratory activity. Of course it cannot be excluded that yeast could show important responses under some special conditions, i.e. in

Table 5
Theoretical benefit of the aerobic versus the anaerobic catabolism of sugars in hypothetical facultative aerobic cells

Catabolic pathway	Aerobiosis		Anaerobiosis		Theoretical benefit[a]
	Catabolized sugar (mol)	ATP formed (mol)	Catabolized sugar (mol)	ATP formed (mol)	
Fermentation	0	0	1	2	
Respiration	1	36 (26)	0	0	
Total	1	36 (26)	1	2	18 (13)
Fermentation	0.7	1.4	1	2	
Respiration	0.3	11 (8)	0	0	
Total	1	12 (9)	1	2	6 (5)
Fermentation	0.9	1.8	1	2	
Respiration	0.1	3.6 (2.6)	0	0	
Total	1	5.4 (4.4)	1	2	2.7 (2.2)
Fermentation	0.97	2.0	1	2	
Respiration	0.03	1.1 (0.8)	0	0	
Total	1	3.1 (2.8)	1	2	1.5 (1.4)

Cells with different contribution of fermentation and respiration to sugar catabolism have been supposed (see the text). For the calculation of ATP produced in fermentation 2 mol of ATP per mol of sugar fermented has been assumed. If P/O = 3 or P/O = 2 (in brackets) are assumed 36 and 26 mol of ATP would be respectively formed in the oxidation of 1 mol of sugar. [a] Theoretical benefit is expressed as the ratio between total ATP formed in aerobiosis and in anaerobiosis.

glucose-limited chemostat cultures as demonstrated by VON MEYENBURG[16], and ROGERS and STEWART[18]. What our results suggest is that yeast is scarely affected by O_2 when it is growing at relatively high concentrations of sugars, as those prevailing in its natural habitats. The fact that similar results were found with three different strains, strongly suggests that this is the general behaviour of *S. cerevisiae*.

Although very moderate, the Pasteur effect is greater in yeast during NH_4^+ starvation than during growth. While growing yeast showed an almost unnoticeable Pasteur effect, changes of the glycolytic flux by a factor of nearly two occurred in the absence of NH_4^+ source (Table 2 and 4). In these cells a relationship between respiratory capacity and Pasteur effect seems also to exist. This conclusion is based on the fact that NH_4^+ starved yeast grown on galactose or ethanol, in which the contribution of respiration is relatively high, showed Pasteur effect as soon as it was deprived of NH_4^+ (Table 4), while yeast grown on glucose showed Pasteur effect only after several hours under NH_4^+ starvation, that is when the relative contribution of respiration had increased noticeably (Table 4).

The results shown in this paper contrast with a biochemical axiom: The great economical benefit of aerobiosis for the facultative aerobic cells. Among the effects that aerobiosis produces on cellular metabolism the Pasteur effect has been perhaps the most extensively studied. Although scattered reports in the literature[16,18,19,20,21] could have conveyed the idea that the Pasteur effect, as is now defined, is very low in batch cultures, there exists indeed a widespread belief that large changes of glycolytic flux occurs in the transition from aerobiosis to anaerobiosis and viceversa (see among others KREBS[22]; RAMAIAH[23]; RACKER[24]; SOLS[25]; TEJWANI[26]). This belief has been in part caused by the use of the term "Pasteur effect" to designate two different phenomena without clear awareness of their differences: the action of oxygen producing a decrease in the fermentation products; and the action of oxygen lowering the rate of sugar catabolism. Although both effects of oxygen can occur simultaneously, they can also occur separately and indeed their values can be different (see Table 4).

Another reason that probably conveyed the idea of important changes of the glycolytic flux

in the shift aerobiosis-anaerobiosis could be an erroneous interpretation of Pasteur's results[27]. Pasteur observed that in anaerobiosis the growth rate of yeast was up to 100 times lower than in aerobiosis; the fermentation of sugar was higher; and the growth yield per amount of sugar consumed was up to 15 times lower. He did not mention differences in the rate of sugar consumption, and if his results are analysed in the light of the actual knowledge no apparent reasons exist to think that large differences occurred during his experiments. Since the ATP yield per mol of sugar catabolized can be no more than 18 times higher in aerobiosis than in anaerobiosis, a rate of growth as much as 100 times faster suggests that, in Pasteur's experiments, not only the rate of sugar consumption was not much faster in anaerobiosis but that it could have been even lower than in aerobiosis. Very likely the slow growth was due to difficulties in the biosynthesis of unsaturated fatty acids and ergosterol that requires oxygen at concentrations that, in the experimental conditions of Pasteur, were probably not present[11,28]. The differences in the yeast yield observed by Pasteur are consistent with the fact observed in microorganism that a decrease in the growth rate is accompanied with a decrease in cellular yield[2]. It seems therefore that no objective reasons exist to postulate a close relationship between the relevant observations of Pasteur and the phenomenon that now bears his name.

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