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A fattening factor to quantify the accumulation ability of microorganisms under N-starvation

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ABSTRACT

Many microorganisms can accumulate biomass in the form of lipids and polysaccharides, which can be used for biofuels, bioplastics, food and feed. Some innovative bioprocesses exploit the competitive advantage provided by such accumulation ability, mainly under N-starvation, to select high-accumulating strains against biological contaminants, by using uncoupled nutrient feeding. However, there is no general and easily comparable parameter available to compare biomass accumulation ability among different microbial strains, which could measure the competitive advantage. Here, a parameter termed "fattening factor" (η_x) is described to quantify such strain-specific biomass accumulation ability in bacteria, yeasts and microalgae. This parameter measures how many fold a microbial population can increase its biomass just as the result of accumulation. It is derived from considerations about the main metabolic aspects of cells' response to N-starvation, which induces variations in cell cycle, biomass production and biochemical composition. The fattening factor described here should be easily estimatable in N-starvation for every culturable microbial strain, by measuring the amount of accumulated biomass.

Introduction

Microorganisms are usually studied and cultivated under optimal growth conditions, in which they grow in exponential phase, by duplicating cell concentration at a rate determined by their maximum specific growth rate (μ_{max}). This is considered a balanced growth, in which cells maintain a constant biochemical composition [1,2], by doubling the biomass proportionally and simultaneously with cell number [1,3,4]. However, when at least one nutrient becomes exhausted, cell duplication stops, cells exit from the cell cycle and enter in the stationary phase [5–7]. This mode of growth is usually described by the empirical Monod's model, on the basis of which new cells and new biomass can be produced only while all nutrients are available in the cultivation medium [8,9]. In this conventional growth condition, new biomass can be produced indefinitely until all the nutrients are available, and the amount of producible biomass can be calculated as the product of consumed substrate (ΔS) and the yield factor $Y_{x/S}$ (strain-specific and function of environmental conditions). However, unlike the prediction of the Monod model, many microbial species can synthesize and accumulate a relevant amount of biomass in the absence of some nutrients, such as in nitrogen (N)-starvation, in which cell duplication is arrested (unbalanced growth). In this case, cells accumulate biomass, usually in form of polysaccharides and lipids, by increasing their cellular mass. Eukaryotic microalgae, yeasts and bacteria can accumulate large

Abbreviations: TAGs, triacylglycerides; PHA, polyhydroxyalkanoates; PHB, polyhydroxybutyrate; MMC, mixed microbial cultures; μ_{max}, maximum growth rate (d^{-1}); f_{PHA}^{max}, maximum ratio of PHA to active biomass (C-mol/C-mol); $\%_{LIP}$, lipids percent content (g/g); $\%_{CAR}$, carbohydrates percent content (g/g); $\%_{CAR,Acc}$, percent amount of carbohydrate accumulated (g/g); $\%_{LIP,Acc}$, percent amount of lipid accumulated (g/g); W_x, biomass (g); W_{LIP}, mass of lipids (g); W_{CAR}, mass of carbohydrates (g); W_{RNA}, mass of RNA (g); W_{DNA}, mass of DNA (g); W_{PROT}, mass of proteins (g); $W_{x,0}$, biomass at the beginning of N-starvation (g); W_{cell}, cell weight (g/cell); $W_{cell,min}$, minimum cell weight (g/cell); $W_{cell,max}$, maximum cell weight (g/cell); x(t), biomass concentration measured at a time t during N-starvatio; x_0, biomass concentration at the beginning of N-starvation (g/L); x_{max}, maximum biomass concentration attained during N-starvation (g/L); η_x, fattening factor (g/g); $\eta_{x,max}$, maximum fattening factor (g/g); η_{cell}, cellular fattening factor (g/g); Q_0, minimum nitrogen quota (g_N/g_x); Q_{max}, maximum nitrogen quota (g_N/g_x); $Q_{N,max}$, maximum molar nitrogen quota (N-mol/C-mol); $q_{N,mol}$, molar nitrogen quota (N-mol/C-mol); q_C, carbon quota (g_C/g_x); q_N, nitrogen quota (g_N/g_x); η_{mol}, molar fattening factor (C-mol/C-mol); α_{C,x_0}, carbon fraction in the initial biomass x_0 (C-mol/g_{x0}); α_{F,x_0}, fraction of functional biomass in the initial biomass (g_x/g_{x0}); α_{PHA}, fraction of PHA in the biomass (g_{PHA}/g_x); $\alpha_{C,P,HA}$, fraction of C in PHA (C-mol/g_{PHA}); $W_{0,i}$, initial mass for each i-component; W_j, mass of the accumulated j-compound; $\alpha_{C,}$i, carbon content for the i-compounds; $\alpha_{N,}$i, nitrogen content for the i-compounds.

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amounts of lipids in the form of triacylglycerides (TAGs) [7,10–13] and/or polyhydroxyalkanoates (PHAs) [14–17] and a large amount of polysaccharides (starch, glycerol) [7,10–12]. These microorganisms have received increased industrial interest for the development of biotechnological processes aiming at the production of biofuels, bioplastics, food and feed [18–23], by exploiting the accumulated PHA, polysaccharides and TAGs.

N-starvation is the condition most commonly used to induce the accumulation of these compounds, because its deficiency is easily implementable under industrial cultivations and because many microorganisms can survive for long periods, maintaining different metabolic pathways active [24–28]. The accumulation ability in N-starvation is also an ecological advantage that can increase microbial competitiveness (against unproductive contaminants) when exposed to fluctuating nutrient availability. This competitive advantage is increasingly exploited for the development of several biotechnological processes working with mixed microbial cultures (MMC), in which strains with higher accumulation ability for the target products (e.g. PHA) are selected during cultivation. In these processes, microorganisms are favored on the basis of their accumulation ability [29–33] by maintaining a selective environmental pressure using specific cultivation strategies based on the alternation of nutrients availability (uncoupled feeding) [34]. However, for the strains able to accumulate biomass under the unbalanced N-starvation, the biomass cannot grow indefinitely, but only up to a cert limit that depends on the accumulation ability of the specific strain. Thus, the quantification of this limit, namely of the maximum accumulation ability of microorganisms cultivated in N-starvation, is fundamental for the understanding and design of every biotechnological process exploiting microbial accumulation in N-starvation.

A widespread method of estimating accumulation ability is based on the measurement of the maximum percent amount (w/w) of a storage compound (e.g. TAGs or PHA). However, this method only measures the relative content of a certain compound, rather than its accumulation (i.e. accumulated mass under unbalanced growth). Also, the variation in such content is not easily linked with its accumulation. For microalgae, the Droop model (based on N quota, i.e. the N content inside the biomass) has been often used to model the biomass growth in N-starvation [35]. However, N quota has different limits, it can be used only indirectly to measure the maximum amount of accumulated biomass and requires the knowledge of the maximum N quota (attained in optimal growth) for the same strain. Moreover, the measurement of cellular N content requires instrumentation (e.g. a CHNS elemental analyzer) that is rarely available in many laboratories. In addition, N quota is often reported in terms of N-mol/C-mol, or N-mol/cell or pg N/cell and other forms that cannot be directly converted to the amount of biomass produced. Similarly, for bacteria accumulating PHA, the maximum accumulation has been usually reported in terms of f_{PHA}^{max} (C-mol/C-mol), as the ratio between the C-mol of PHA to the C-mol of functional biomass [36]. This parameter has different drawbacks: it is determinable only when the total amount of organic compounds accumulated, and their chemical formulae, are known and it requires the determination of the C content of the functional biomass and the determination of the fraction of the functional biomass. Thus, the determination of this latter parameter also needs the requirements of specific analytical instruments (e.g. CHNS elemental analyzer, chromatographs), and its linkage to biomass accumulation requires some assumptions which could not be valid for many microorganisms.

This work aims to describe an alternative approach to quantifying the accumulation ability of microorganisms. A “fattening factor” is described as a general term that can be easily measured in a conventional batch or fed-batch cultivation for every culturable strain. It is further described how the fattening factor can be integrated into the models previously used to predict biomass production under N-starvation, and a general behavior of cell growth size variation during N-starvation is proposed.

Materials and methods

Experimental data collection

Experimental data used to define and quantify the accumulation ability were collected from figures and tables reported in published studies by searching Google Scholar and Web of Science using as keywords: bacteria; microalgae; yeast; nitrogen starvation; accumulation; lipids; PHA, in different combinations. Data for biomass concentration (x (t)), and accumulated lipids and carbohydrates (% dry weight) were only from experiments performed in batch or fed-batch mode, in which a condition of N-starvation was attained. N-starvation is here defined as a condition in which N supply is absent, while the C and energy source (which are often furnished by the same substrate) are fully replete. The references for the articles used as sources of experimental data are reported with each figure.

Simulation for the variation of N and C molar content

The variation in C and N molar content during N-starvation was simulated by considering a typical initial microbial biomass composition: 60 % proteins, 15 % TAGs, 0 % PHB, 15 % carbohydrates and 10 % nucleic acids and an arbitrary initial mass (proteins + TAGs + PHB + nucleic acids + carbohydrates) $W_{x,0} = 1$ g. Starting from this point, the fattening factor (η_x – Eq. 1) was increased from 1 to 7.85, by simulating an increment in the mass (accumulation) of three different biomolecules (j-compounds): carbohydrates, TAGs or PHB.

$$\eta_x = \frac{\sum_i W_{0,i} + W_j}{\sum_i W_{0,i}} \tag{1}$$

where $W_{0,i}$ is the initial mass for each i-component ($W_{x,0} = \sum_i W_{0,i}$), while W_j is the mass of the accumulated j-compound (carbohydrates, TAGs or PHB). For each different η_x point, the carbon quota q_C (gC/gx) and the nitrogen quota (gN/gx) were calculated for the three different accumulated j-compounds with the following equations:

$$q_C = \frac{\sum_i W_{0,i}\alpha_{C,i} + W_j\alpha_{C,j}}{\sum_i W_{0,i} + W_j} \tag{2}$$

$$q_N = \frac{\sum_i W_{0,i}\alpha_{N,i}}{\sum_i W_{0,i} + W_j} \tag{3}$$

where the $\alpha_{C,i}$ and $\alpha_{N,i}$ indicate the C and N content respectively for the different i-compounds (carbohydrates, TAGs, nucleic acids and proteins) in the initial biomass, while $\alpha_{C,j}$ indicate the C content in the j-compounds accumulated (carbohydrates, TAGs or PHB). These values were fixed as follows:

- For C: proteins = 0.53 g_C/g; carbohydrates = 0.44 g_C/g; TAGs = 0.76 g_C/g; nucleic acids = 0.38 g_C/g; PHB = 0.56 g_C/g.
- For N: proteins = 0.16 g_N/g; nucleic acids = 0.16 g_N/g.

C and N content in nucleic acids were calculated based on the chemical formula of the nitrogenous bases, assuming 40 % of guanine + cytosine. C content in TAGs, carbohydrates and PHB was calculated based on the chemical formula of tripalmitin, starch and PHB ($[C_4H_6O_2]_n$) respectively. For proteins the average C content value calculated in [37] and the conventional 16 % for N were used.

The molar nitrogen quota (N-mol/C-mol) has been then determined as:

$$q_{N,mol} = \frac{q_N 12}{q_C 14} \tag{4}$$

Finally, for each η_x point, the η_{mol} was calculated as follows:

$$\eta_{mol} = \frac{Q_{N,max}}{q_{n,mol}} \quad (5)$$

where $Q_{N,max}$ is the maximum molar nitrogen quota (N-mol/C-mol).

Fattening factor estimated in terms of cell size variation

Experimental data about cell size (diameter, d) have been collected from experiments of cultivation carried out in N-starvation for heterotrophic bacteria, yeast and eukaryotic phototrophic microalgae. The diameters measured during N-starvation have been normalized with respect to those at the beginning of the N-starvation (d_0) to obtain a normalized size ($d(t)/d_0$).

Results and discussion

Definition of the fattening factor to quantify accumulation ability

The absence of N stops synthesis of proteins and nucleic acids in every cell, inducing exit from cell cycle and entery into a quiescent phase, i.e G_0 for eukaryotic cells [38–40]. Under N-starvation, the available substrate (source of energy and C) cannot be used to produce new cells, thus it induces an imbalance, resulting in the accumulation of metabolites.

This work aims to define a parameter to measure the whole accumulation ability of microorganisms during N-starvation. This has usually been measured in terms of mass percent content (w/w) of specific compounds, such as lipids ($\%_{LIP}$) or carbohydrates ($\%_{CAR}$) to the total biomass (W_x) (Eq. 6).

$$\%_{LIP} = \frac{W_{LIP}}{W_x}\ 100 \quad (6)$$

However, a percentage content alone is not a measurement of accumulation, as it only relates to a particular condition, while accumulation ability should measure mass variation with respect to a reference condition (in this case to balanced growth in N replete medium). The variation measured as the difference between the percentage value at a time point during starvation and that at the beginning of N-starvation (0) has been usually considered, e.g.:

$$\Delta\%_{LIP} = \%_{LIP}(t) - (\%_{LIP})_0 \quad (7)$$

but it should be noted that:

i) when the compound content increases during the time, the percentage content does not vary proportionally with the amount of accumulated compound, making comparisons difficult, especially for high accumulation values.
ii) the variation of the percentage content cannot be directly related only to the amount of one accumulated compound, because it changes with the variation in other cell compounds.

To overcome such limitations, an easy and comprehensive approach to assess microbial accumulation ability may be the measurement of whole biomass production attained during N-starvation in a batch or fed-batch cultivation [10,30]. In this condition, all the biomass produced can be considered 'accumulated biomass', because the balanced growth is hindered by the absence of N and the functional biomass (namely the sum of proteins, nucleic acids and membranes) should not increase.

Therefore, a 'fattening factor' can be defined as follows.

$$\eta_x(t) = \frac{x(t)V(t)}{x_0 V_0} \underset{V(t)=V_0}{\overset{for\ batch}{\Rightarrow}} \frac{x(t)}{x_0} \quad (8)$$

where x(t) is the biomass concentration measured during N-starvation, while x_0 is the biomass concentration at the beginning of the N-starvation. This factor can be determined by measuring the variation in the biomass concentration throughout a batch or fed-batch cultivation in which all nutrients are fully replete, except N, which is not included. Fed-batch is more appropriate when substrate inhibition may be an issue. The initial biomass (x_0) should come from optimal and balanced growth conditions and it is assumed that there is no cell lysis during the test. Therefore, if there is no preliminary information about cell survival of the tested strain under N-starvation, for the considered time of the experiments, cell survival throughout the test should be also assessed.

During batch cultivation in N-starvation, the biomass increases following a saturation curve, with an accumulation rate decreasing over time until reaching a plateau corresponding to the end of the accumulation (Fig. 1). Experimental data from microalgae (Fig. 1) and bacteria [41] indicate that this behavior is not due to absence of substrate, because accumulation ceases whether or not C and energy sources are still available [10,30,42–47]. This behavior indicates that the final biomass concentration attained under N-starvation is limited by an intrinsic species-specific maximum accumulation property [10,36]. This maximum biomass increment ($\eta_{x,max}$) can be measured during batch cultivation, for every species/strain, as the ratio between maximum biomass concentration attained at the plateau, with respect to the initial biomass concentration:

$$\eta_{x,max} = \frac{x_{max}}{x_0} \quad (9)$$

The physiological mechanisms at the basis of this "saturation" in accumulation are unknown [36]. A large part of the research investigating the accumulation ability of microorganisms has focused on the regulatory mechanisms that activate the synthesis of storage molecules, while less attention has been given to mechanisms that stop accumulation. It may be supposed that the maximum accumulation ability corresponds to saturation in the available space for storage [36], in turn related to a limit in cellular size increase. It is known that there is a minimum amount of biovolume required for cell survival, i.e. for essential functional biomass [48]. Therefore, it can be supposed that small cells (such as many bacteria with low accumulation ability) can have a limited space for accumulation because a large fraction of the whole cellular volume is occupied by the essential functional biomass. On the other hand, larger cells can have a larger fraction of biovolume occupied/occupiable by not essential biomass (storage compounds).

An overview of the maximum accumulation ability, determined for different yeasts and microalgae species, is reported in Fig. 2. On the base of different literature data [10,13,25,27,43,44,46,49–52], the median

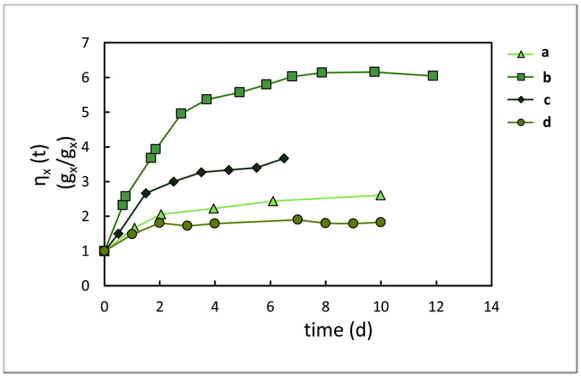

Fig. 1. Fattening factor ($x(t)/x_0$) as function of time during batch cultivation under N-starvation. The data come from previous studies conducted with different microalgae. Carbon and energy sources were not responsible of the cessation of the accumulation, because supplied in excess. References: (a) [46], (b) [47], (c) [44] and (d) [30].

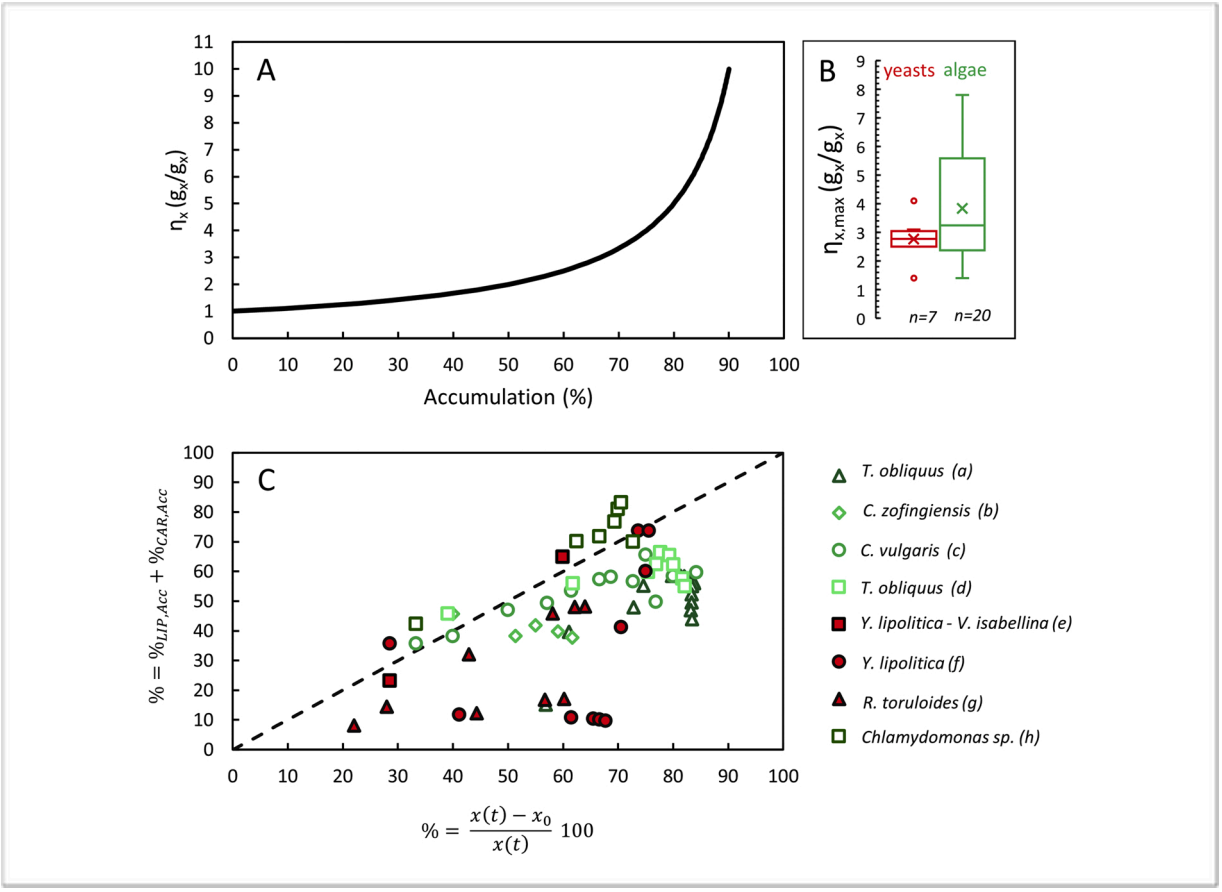

Fig. 2. A) Variation of the fattening factor (η_x) as function (Eq. 11) of the accumulated biomass as % (Eq. 10). B) Boxplot of the $\eta_{x,max}$ determined from data taken from previous studies investigating biomass growth during batch cultivation in N-starvation. C) Relation between the % of biomass accumulated measured from Eq. 11 and that estimated from Eq. 13, data reported have been taken from different previous studies about yeasts and microalgae. References: (a) [47], (b) [46], (c) [45], (d) [52], (e) [49], (f) [13], (g) [50] and (h) [44].

value $\eta_{x,max}$ found for yeasts is 2.78, while for microalgae is 3.25. The larger $\eta_{x,max}$ values have been found for microalgae, up to 7.8 for *Tetradesmus obliquus* [10]. However, it should be noted that, for some available data, there is relevant uncertainty in the measurement of the exact $\eta_{x,max}$, because of the uncertainty in the determination of the exact start point of N-starvation. Some data come from batch cultivations in which N was depleted during the time, and there was uncertainty in determining the exact time point in which N was over (due to the distance between experimental data points), which in turn gave uncertainty in establishing exactly the x_0. In addition, in the experiments carried out for yeasts, the $\eta_{x,max}$ attained corresponded to the exhaustion of glucose (C and energy source), suggesting that the true values have been underestimated [13,25,50]. Unexpectedly, data to determine η_x in bacteria were scarce. Only limited partial data on bacteria accumulating polyhydroxybutyrate (PHB) indicated a $\eta_{x,max}$ ranging from 1.6 (from 20 % to 50 % PHB) [53] to about 10 (from 0% to 90 % PHB) [41,54]. The paucity of the literature data indicates that this relevant aspect of microbial growth has been understudied.

The value of η_x can be related to the percentage amount of stored biomass accumulated, with Eqs. 10 and 11, with an expected behavior as shown in Fig. 2A.

$$\%_{Acc}(t) = \frac{x(t) - x_0}{x(t)}\ 100 \tag{10}$$

$$\eta_x(t) = \left(1 - \frac{\%_{Acc}(t)}{100}\right)^{-1} \tag{11}$$

Based on this relation, the fattening factor increases exponentially with respect to the percentage of accumulated biomass (Fig. 2A). Thus, values very close in terms of the percentage of accumulated biomass can correspond to significantly higher biomass increments. For instance, a shift from 87 % to 90 % (+ 3 %) as percent dry weight of the accumulated compound might seem negligible, but corresponds to a η_x increment from 7.7 to 10 (+ 30 % accumulated biomass).

Relationship between experimental data on biomass increase and compound accumulation

The experiments conducted in N-starvation usually measure only the fraction of one or a few accumulated compounds (e.g. TAGs, PHA or carbohydrates). Therefore, some differences can be found between data on biomass increase and compound accumulation. In principle, Eq. 10 can be expressed to include the contribution of the different microbial biomolecules in determining the whole accumulation. The biomass (W_x = x V) can be divided into the following main fractions:

$$W_x = W_{CAR} + W_{LIP} + W_{DNA} + W_{RNA} + W_{PROT} \tag{12}$$

corresponding to carbohydrates, lipids, DNA, RNA and proteins. The variation of the percentage of these fractions during starvation should take into account the possible degradation of N-rich compounds, which can be a result of autophagy, which during N-starvation is a relevant mechanism involved, mainly used to reallocate the limited N [55,56]. Autophagy is essential in yeast to complete the cell cycle and entering in

G_1/G_0 phase [57–59]. Proteins and RNA are usually the molecules mainly degraded because they are N-rich [56]. Different experimental data did not show any relevant release of N into the cultivation medium during N-starvation [13,51,55], and an almost constant protein concentration was found in the medium [25,56]. Functional biomass measured in bacteria accumulating PHA also remained almost invariant during N-starvation [54]. Therefore, the experimental data indicate that autophagy of N-rich molecules mainly involves N reallocation, rather than loss of functional biomass [25].

Consequently, it can be assumed that the variation of whole biomass concentration during N-starvation (W_x (t)) is mainly given by the accumulation of lipids and carbohydrates, while the autophagy of nucleic acids and proteins is not expected to induce relevant biomass variability. The whole initial biomass ($W_{x,0}$) can be considered as entirely composed of functional biomass because the accumulation is zero at this point. Consequently, the percentage of accumulated biomass can be calculated as follows:

$$\%_{Acc} = \frac{W_{CAR}(t) - W_{CAR,0} + W_{LIP}(t) - W_{LIP,0}}{W_{CAR}(t) + W_{LIP}(t) + W_{x,0}} 100 = \%_{LIP,\,Acc} + \%_{CAR,\,Acc} \quad (13)$$

where $\%_{LIP,Acc}$ and $\%_{CAR,Acc}$ indicate the percent amount of lipids and carbohydrates accumulated, respectively.

Among the experimental data collected, those in which both biomass concentration and percentage content of lipids (as TAGs) and/or carbohydrates were measured during N-starvation were used to compare the biomass increment with that expected based on the accumulated compounds (Eq. 11 and Eq. 13). As shown in Fig. 2C, for many points a good relationship was found, indicating that the biomass increase was mainly given by the increment of accumulated compounds. In contrast, for many other experimental points, there was a relevant underestimation of the accumulation percentages, namely, the determined increment in the accumulated compounds explained only a fraction of the whole biomass increase during N-starvation. This common issue was likely due to an analytical underestimation of the accumulated biomolecules. Indeed, in some cases, only lipids or only carbohydrates were analyzed and the accumulation of free sugars or other small metabolites was rarely considered, despite potentially being a relevant fraction of the biomass. For instance, free glucose accounted for 10 % of the biomass dry weight in *Neochloris oleoabundans* cultivated in N-starvation [60]. It should be emphasized that the assumption made about functional biomass, based on which its amount has been considered constant, cannot account for the difference between the model and the experimental values. In fact, in the case of a relevant decrease of functional biomass due to autophagy, this would have resulted in a higher percentage of lipids and carbohydrates, with respect to that expected. Namely, in this case, the experimental points would have been in the upper left region of the figure, instead of on the lower right region. Therefore, the factor responsible for the differences reported for some experimental data was likely due to a partial analysis of the biomolecules accumulated during N-starvation. These data indicate that many microbial strains, investigated during N-starvation, were often only partially analyzed for their whole accumulation ability.

Relationship between fattening factor and other parameters

The fattening factor (Eq. 8 and 9) can be linked to the N quota (q), which is usually used to model microalgae/phytoplankton biomass accumulation in N-starvation by using the Droop model [35,61]. As stated previously, it may be assumed that there is no relevant loss of N outside cells during N-starvation; the amount of internal N can be assumed constant, and the N quota (measured as g_N/g_x) will vary as a result of biomass accumulation ($C_xH_yO_z$), that dilutes N content inside the whole biomass. The maximum (Q_{max}) and minimum (Q_0) N quota can be obtained by measuring the internal amount of nitrogen (g_N/gx) at the beginning of N-starvation (N-replete state of growth) and at the end of the N-starvation, respectively. Consequently, the different N quotas (g_N/gx) attained during batch growth in N-starvation can be linked to the biomass concentration as: $Q_{max} = [N]/x_0$; $q = [N]/x(t)$; $Q_0 = [N]/x_{max}$, with [N] the concentration of biomass internal nitrogen at the beginning of N-starvation. Thus, the Droop model can be expressed as follows:

$$\mu = \mu_{max}\left(1 - \frac{Q_0}{q}\right) \rightarrow \mu = \mu_{max}\left(1 - \frac{x(t)}{x_{max}}\right) \quad (14)$$

Based on Eq. 14, this becomes equivalent to the logistic model [9] and can be linked to the fattening factor as follows:

$$\mu = \mu_{max}\left(1 - \frac{\eta_x}{\eta_{x,max}}\right) \quad (15)$$

The ratios Q_0/q, $x(t)/x_{max}$ and $\eta_x/\eta_{x,max}$ can all be considered a measurement of the degree of saturation with respect to the maximum storable biomass. From the Q_0 and Q_{max} of a strain, the maximum fattening factor can be obtained as follows:

$$\eta_{x,max} = \frac{x_{max}}{x_0} = \frac{Q_{max}}{Q_0} \quad (16)$$

However, these relations are valid only where the quota is measured as g_N/g_x. Instead, N quota has been often measured in term of N-mol/C-mol ($q_{N,mol}$) or N-mol/cell, so that $\eta_{x,max}$ cannot be obtained directly from these literature data. C content inside biomass is not constant during N-starvation, but may change due to the molecule (carbohydrate, TAG or PHA) accumulation. To measure how C and N content may change during N-starvation, a simulation has been performed by considering typical initial biomass composed of 60 % proteins, 15 % TAGs, 0 % PHB, 15 % carbohydrates and 10 % nucleic acids. The variation in C and N content was calculated using three different mass balances, each one considering the accumulation of only one compound: carbohydrates, TAGs or PHB. All the balances assumed no net degradation of the components during starvation. The results, reported in Fig. 3A, show that C content slightly decreases when carbohydrates alone are accumulated, due to their lower C content (44 %) compared to proteins (53 %) [62]. When PHB are accumulated the C content remains almost constant, because their C content (56 %) is similar to that of proteins. In contrast, TAG accumulation induces a relevant increase in C content, due to the TAG C content (76 %) [61] much higher than proteins. N quota calculated in terms of N-mol/C-mol ($q_{N,mol}$, Fig. 3 B) decreases faster for the accumulated compounds with higher C content (Fig. 3B). The $q_{N,mol}$ calculated at $\eta_x = 3.25$ (the median value for microalgae, Fig. 2B) is between 0.063 N-mol/C-mol (only carbohydrates) and 0.043 N-mol/C-mol (only TAGs), in agreement with the reported experimental values [63]. When these molar values were used to calculate the molar fattening factor (η_{mol}), differences were found with respect to the η_x, determined based on the mass (Fig. 3C), which were up to 16 % for carbohydrates, only 4% for PHB and up to 36 % for TAGs, due to the variability in the C content inside biomass.

In addition, in many studies, N quota has been measured in terms of N/cell [63,64], but these values cannot be used to estimate the η_x because N/cell is expected to vary mainly as a function of the cell cycle progression, without being affected by biomass accumulation. For this reason, this latter unit of measurement should not be used to assess biomass production due to accumulation (described further below).

The other microorganisms for which biomass production under N-starvation has been often modeled are the bacteria that accumulate PHA [36]. For these species too, an empirical relation has been used, similar to that used by the Droop and logistic model. In this latter case, the PHA quota (f_{PHA}) has been measured as a ratio of C-mol of PHA to C-mol of the functional biomass (measured as whole biomass minus PHA) [36]. The limit of PHA accumulation was determined by a relation similar to the relation used in the Droop model [36], such as f_{PHA} / f_{PHA}^{max}, where f_{PHA}^{max}

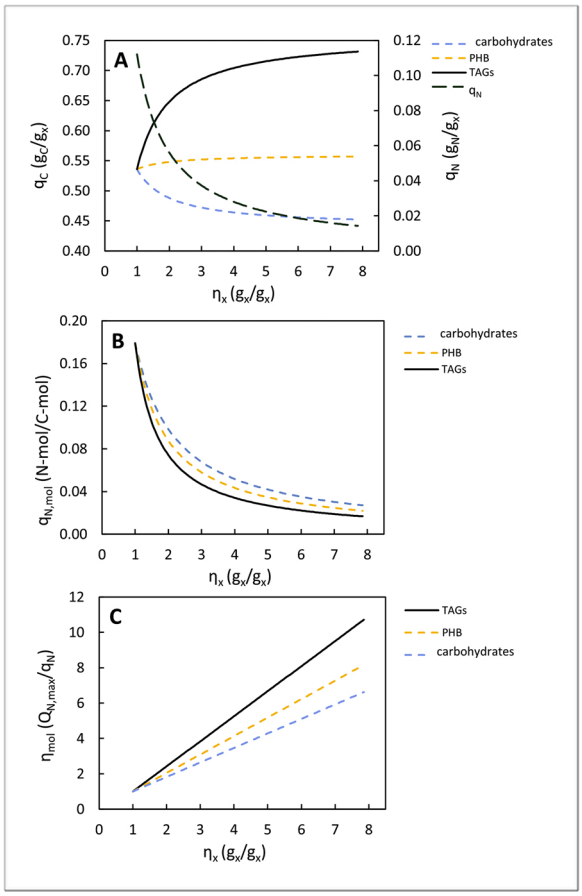

Fig. 3. Results of the simulation for biochemical composition variation of microbial biomass as the accumulation of TAGs, PHB and carbohydrates during N-starvation. A) Variation of N quota (q_N) and C quota (q_C), the latter calculated for TAG, PHB or carbohydrate accumulation. B) Variation of N quota ($q_{N,mol}$) calculated as molar ratio. C) Relation between the fattening factor calculated by mass ratio (Eq. 16) and by molar ratio (N quota calculated as molar ratio).

is the maximum amount of PHA storable. The η_x can be linked to the f_{PHA} with the following equation (Eq. 17), which includes the stoichiometric conversion factors.

$$\eta_x = \frac{x(t)}{x_0} = f_{PHA}\left(\frac{\alpha_{C,x_0}\ \alpha_{F,x_0}}{\alpha_{PHA}(t)\alpha_{C,PHA}}\right) \tag{17}$$

where α_{C,x_0} is the fraction (C-mol/g_{x0}) of C in the initial biomass x_0, α_{F,x_0} is the fraction (g_x/g_{x0}) of functional biomass in the initial biomass (this value could be considered = 1), $\alpha_{PHA}(t)$ is the fraction (g_{PHA}/g_x) of PHA in the biomass measured during N-starvation, while $\alpha_{C,PHA}$ is the fraction (C-mol/g_{PHA}) of C in PHA.

Relation between fattening factor and cell size

The biomass increase determining the η_x factor is related to the amount of biomass accumulated in G_0 phase, i.e. in absence of cell duplication. Thus, the η_x measures how much a microbial cell can increase its weight during N-starvation, with cell cycle stopped, and biomass production is only given by cell weight increase. From an ecological point of view, this difference at the cellular level is the key parameter that gives an ecological advantage to the cells under conditions of nutrient depletion, as the cells with a high content of accumulated biomass can use it as a source of energy to avoid death. Thus, a fattening factor directly related to the cell weight can be determined as follows:

$$\eta_{cell}\ (t) = \frac{W_{cell}\ (t)}{W_{cell,0}} \tag{18}$$

where $W_{cell,0}$ is the dry cell weight (g/cell) of a certain microorganism at the beginning of the N-starvation, while W_{cell} (t) is the dry cell weight attained at a specific time (t) during N-starvation. However, cell concentration does not always remain stable during N-starvation, and an increase in cell number may be registered before all cells exit from the cell cycle, due to progression of the cycle started before entering N-starvation [56,57,65].

Experimental data reported in some previous reports [7,51,65–69] indicated two main mechanisms, that induce two opposite effects (Fig. 4):

- *Cell size reduction*: cells under N-starvation do not always stop their cell cycle immediately, but those coming from active growth usually complete their cell cycle showing a corresponding decrease in average cell size (Fig. 4A). This decrease is mainly given by cytokinesis and the corresponding reduction in DNA and protein content. This mechanism is expected to be present in every microbial cell.
- *Cell size increase*: the accumulation of storage compounds causes cellular weight to rise (Fig. 4B). This mechanism is expected to be relevant only in the species able to accumulate large amounts of compounds.

Based on these two mechanisms, a general behavior to describe cell weight variation during N-starvation has been suggested and is proposed in Fig. 5. Depending on the extent and the relative kinetics of such mechanisms, a minimum point might be observed in which a minimum cell weight ($W_{cell,min}$) is achieved (Fig. 5) [51].

Microorganisms under N-starvation accumulate storage compounds up to a limited value (Fig. 1), so that a corresponding species-specific maximum cell size increase is expected. Cells should increase in mass until reaching their maximum cell weight ($W_{cell,max}$) under starvation and, consequently, its maximum cellular fattening factor ($\eta_{cell,max}$), as described in Eq. 19.

$$\eta_{cell,max} = \frac{W_{cell,max}}{W_{cell,min}} \tag{19}$$

On the basis of such mechanisms, the behavior of some microorganisms (Fig. 4A) that show a lower weight in N-starvation with respect to optimal growth ($\eta_{cell} < 1$) [65,66,70] can be explained as a result of a low accumulation ability (cell cycle progression prevailing). Instead, the behavior of other microorganisms (Fig. 4B), that achieve greater cell weights ($\eta_{cell} > 1$) [7,30,51], is given by their higher accumulation ability. The difference between $W_{cell,0}$ and $W_{cell,min}$ should depend on the specific cell cycle, which can vary between species, and even for the same species, depending on the growth conditions experienced before entering in N-starvation [40]. For instance, microalgae can perform different cell cycles, by which they can produce 2^n daughter cells, where n is typically between 1 and 4 [40]. This difference is usually induced by the amount of light (energy) supplied before entering N-starvation [40]. However, irrespective of the condition at the beginning of the starvation, the minimum cell size $W_{cell,min}$ should be a constant value for each species, corresponding to the minimum threshold size of a single cell at the beginning of the G_1/G_0 phase [71]. The behavior described in Fig. 5 can be explained because a reduction in cellular N quota (as N/cell) has usually been found for microalgae under N-starvation [63,64], in apparent contradiction with the previously described data indicating no loss of N into the culture media. The progression of the cell cycle for a limited phase, before entering G_0, dilutes the cellular N quota. However, the measurement of N quota as N/cell cannot consider the increase in biomass due to accumulation (and the resulting N dilution), and therefore should not be used for such purpose.

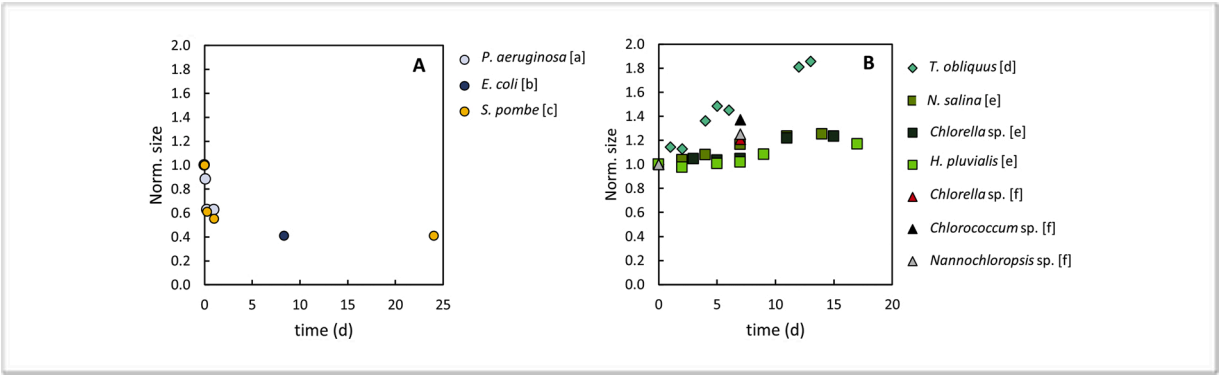

Fig. 4. Variation of the normalized size for heterotrophic bacteria and yeast (A) and phototrophic eukaryotic microalgae (B) during N-starvation. References: (a) [65], (b) [67], (c) [69], (d) [7], (e) [51], (f) [68].

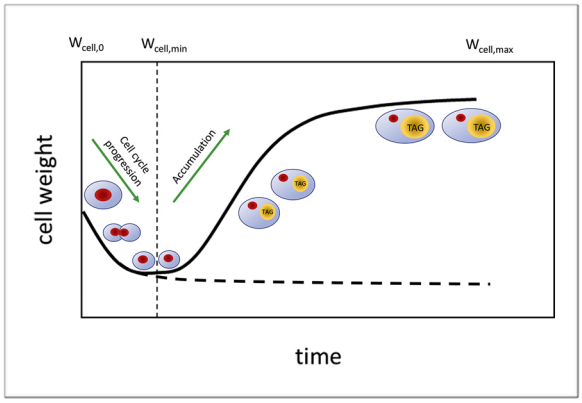

Fig. 5. Qualitative variation of microbial cell dry weight during N-starvation. Two mechanisms are involved: cell size reduction due to cell cycle progression and cell size increase due to the accumulation of storage compounds. Cell weight changes from initial cell weight ($W_{cell,0}$) to minimum cell weight ($W_{cell,min}$) and to maximum cell weight ($W_{cell,max}$).

Conclusions

The accumulation ability of microorganisms is fundamental for the development of several biotechnological processes and to predict their growth in nutrient-poor environments. The "fattening factor" described here allows a ready quantification of the mass accumulation ability in every culturable microbial species in batch or fed-batch cultures. The median value found for the maximum fattening factor is 2.78 for yeasts and 3.25 for eukaryotic microalgae, while for PHA-accumulating bacteria, preliminary data indicate values ranging from 1.6 to 10. The comparison of the expected fattening factor with respect to the evolution of accumulated biomolecules measured in previous reports indicated that in many of these studies the whole accumulation ability has been often underestimated. By using appropriate conversion factors, the fattening factor described here can be related to the N quota used in the Droop model and to the f_{PHA}^{max} previously used to model PHA accumulation. The fattening factor may be used for the quantification, comparison and prediction of biomass accumulation among different microorganisms under N-starvation growth conditions.

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