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GROWTH OF THE BLUE-GREEN ALGA *ANACYSTIS NIDULANS* DURING WASHOUT FROM LIGHT- AND CARBON DIOXIDE-LIMITED CHEMOSTATS

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1. Introduction

For microorganisms growing in continuous-flow culture systems, the organism's specific growth rate reaches a maximum at the critical dilution rate, D_{crit}. D_{crit} is a good approximation to the maximum specific growth rate, μ_{max}, for the organism under the prevailing conditions provided that the concentration of the growth-limiting substrate in the inflowing medium is considerably greater than the organism's saturation constant for the limiting nutrient. At dilution rates above D_{crit} steady-state conditions are unobtainable and, in theory, the organism should grow at μ_{max} during the transient conditions which occur during washout from the growth vessel. Washout kinetics have been used to determine μ_{max} values [1–3] according to the relationship:

$$\mu_{max} = \frac{(\ln x_2 - \ln x_1)}{(t_2 - t_1)} + D$$

where x_1 is the initial steady state organism concentration at time t_1 and x_2 is the organism concentration at time t_2 after an instantaneous change in the dilution rate to a value $D > \mu_{max}$ at t_1.

Some attention has been paid to the factors which may influence the μ_{max} values determined during washout; for example, wall growth in the culture vessel [4] may lead to erroneously high values. Moreover the technique depends on the organism's immediate capacity to grow at its maximum rate during washout and in some cases this does not seem to be true. Jannasch [2] described some experiments in which the organisms either ceased to grow or washed out at rates in excess of the μ_{max} rate suggesting that cell lysis and death was responsible for accelerating the rate of culture washout. It has also been proposed that non-growing culture washout could be due to population density effects [5]. It has been shown for *Escherichia coli* that growth at μ_{max} may not always be physiologically attainable in the first stages of culture washout, particularly for large changes in the dilution rate [6,7].

This communication describes the pattern of growth of the blue-green alga, *Anacystis nidulans*, during washout from various steady state cultures with either light or carbon dioxide as the growth-limiting nutrient.

2. Materials and Methods

The organism used in this study was *A. nidulans* (strain number 625, Indiana University Culture Collection and obtained from N.G. Carr) and stock cultures were maintained as previously described [8]. The details of the light-limited chemostat, growth media, inoculum growth and growth conditions were as previously described [9] except that the cultures were illuminated from two sides by a total of 18 8W Warm White fluorescent tubes placed 10 cm away from the culture vessel.

A similar system was used for the carbon dioxide-limited chemostat with the addition of pH monitoring and controlling facilities (EIL Model 9150 pH controller) which maintained the culture at pH 7.5. In most experiments the culture was illuminated by 12 8W Warm White fluorescent tubes but in some experiments additional illumination was used. The

growth medium was a modified Kratz and Myers' Medium C [9,10] supplemented with sodium bicarbonate to give a final concentration of 0.42 g l^{-1}. Under these conditions carbon dioxide (bicarbonate) was the limiting nutrient.

Culture washout was established by a stepwise increase in the dilution rate to a value greater than the expected maximum specific growth rate. There was no concomitant saturation of the growth-limiting nutrient either by the addition of excess bicarbonate or by an increase of the incident light intensity.

Culture absorbance was measured in a Unicam SP600 spectrophotometer in 1 cm light path cuvettes at 600 nm. Samples for cell number determination were fixed in an equal volume of 2.5% (v/v) gluteraldehyde solution and the cell number was determined in a Model ZBI Coulter Counter.

3. Results

The growth of *Anacystis nidulans* during washout from three different initial steady state cultures for both light-limited and carbon dioxide-limited chemostats was measured in terms of culture absorbance and cell number. None of the cultures, assessed by either absorbance or cell number, showed the theoretical pattern of immediate and continuous exponential growth at a rate equal to μ_{max} after changing to a culture washout dilution rate. Furthermore difference existed between the washout curves for absorbance and cell number for the same culture.

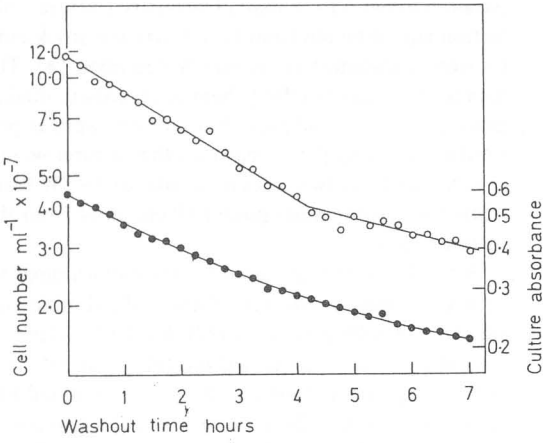

Fig. 1. The washout curves in terms of cell number (○) and absorbance (●) for a carbon dioxide-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At $t = 0$ the dilution rate was instantaneously increased to 0.27 h^{-1}.

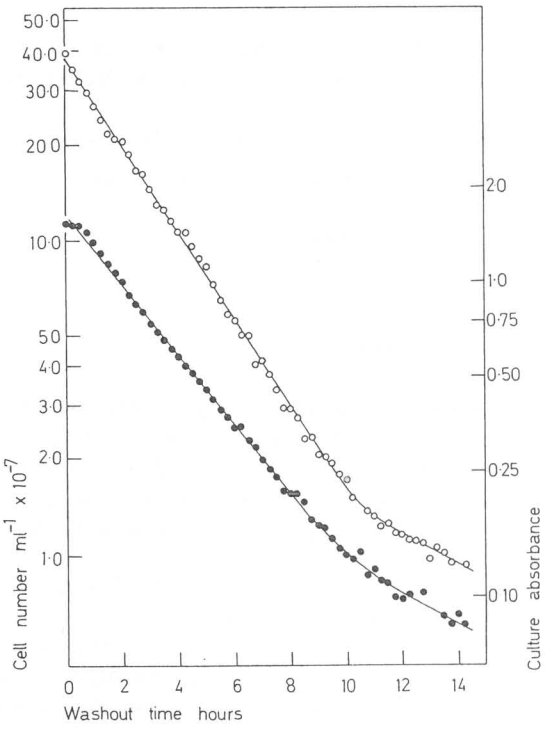

Fig. 2. The washout curves in terms of cell number (○) and absorbance (●) for a light-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At $t = 0$ the dilution rate was instantaneously increased to 0.37 h^{-1}.

Cell number washout curves for both limitations were biphasic showing a marked change in the organism's specific growth rate over a relatively short period of time during washout growth (Figs. 1 and 2). In all cases the specific growth rate of the first phase, μ_1, was less than the specific growth rate of the second phase, μ_2 (Table 1), where the rates were determined from the coefficients of regression line analysis. For both growth-limiting conditions the values of μ_1 were variable, declining with decreases in the initial growth rate of the culture prior to washout. Indeed, within the limits of experimental error,

TABLE 1

The values of transient washout parameters from various initial steady-state light-limited and carbon dioxide-limited cultures

Culture limitation	Initial steady-state culture conditions			Transient washout parameters					
				Washout dilution rate (h^{-1})	Absorbance		Cell number		
	Dilution rate (h^{-1})	Absorbance A_{600nm}	Cell number $ml^{-1} \cdot 10^{-7}$		Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-1}-$	Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-1})$	Length μ_1 phase (min)
Light	0.03	1.54	39.6	0.37	0.10	0.26	0.05	0.26	615
Light	0.08	0.92	23.0	0.32	0.14	–	0.06	–	>390
Light	0.15	0.54	9.3	0.31	0.16	0.24	0.11	0.23	285
CO_2	0.03	0.59	11.6	0.27	0.09	0.17	0.03	0.17	255
CO_2	0.10	0.35	5.0	0.28	0.11	0.16	0.11	0.16	215
CO_2 [a]	0.17	0.33	3.0	0.28	0.16	0.20	0.15	0.22	220

[a] Higher incident light intensity than preceding CO_2-limited cultures.

the μ_1, values were the same as the dilution rates of the preceding steady-state cultures (Table 1). The specific growth rates of the second phase of washout were independent of the initial growth rate and hence the μ_1 phase and were equal to μ_{max} values for the given growth conditions as determined in closed culture experiments. The different μ_2 values shown in Table 1 were the result of the different growth conditions used, particularly the incident light intensity. The length of the μ_1 phase, that is the time taken to reach growth at a μ_{max} rate, depended on the type of growth limitation. For carbon dioxide-limited cultures the μ_1 phase lasted just over 200 min and seemed to be independent of the initial steady-state culture growth rate, although for the slowest growing initial culture the μ_1 phase was significantly longer at 255 min. For the light-limited culture the length of the μ_1 phase increased with declining initial culture dilution rate such that the length of the μ_1 phase for the culture with the slowest initial growth rate examined, $D = 0.03\ h^{-1}$, was approx. 2.5 times longer than that for the fastest growing culture where $D = 0.15\ h^{-1}$.

The washout curves measured by culture absorbance were different to the pattern of the cell number washout curves. During the course of washout the specific growth rate increased gradually usually with no indication of a biphasic curve (Fig. 2) although washout from slow-growing light-limited cultures resulted in a pattern similar to the biphasic cell number washout curves (Fig. 1). The initial specific growth rates, μ_1, measured over the first 1 to 2 h were always less than μ_{max} and equal to or greater than the cell number μ_1 phase values (Table 1). For the slower growing cultures there was an immediate 3- to 4-fold increase compared with the steady state growth rate. By the time the cell number curves had reached μ_{max} values, the absorbance curves had also attained final μ_2 growth rates equal to the expected μ_{max} values for those conditions (Table 1). The adsorbance washout curves were different to the cell number curves probably due to the additional influence on the measurement of the population size due to changes in cell volume. Mean cell volumes determined from Coulter Counter measurements showed that cell volumes began to increase almost immediately after the shift to washout dilution rates, an effect which was most apparent with the slow growing cultures.

4. Discussion

The chemostat washout procedure adopted in these experiments was the conventional method used to determine μ_{max} by continuous-flow culture washout. Clearly, for *Anacystis nidulans* under these growth conditions, the initial stages of culture washout were not reliable for determining μ_{max} values. Two reasons

could account for the inability of *Anacystis nidulans* to grow at a μ_{max} value for the outset of culture washout both of which are likely to be applicable to washout μ_{max} determinations with other organisms.

Firstly it is normally assumed that simply by increasing the dilution rate of the growth system, the conditions within the growth vessel immediately, or at least very quickly, are optimised for growth at μ_{max} for the prevailing physico-chemical conditions. For growth systems in which the population's growth rate is controlled by the concentration of one growth-limiting substrate, an increase in the dilution rate has to ensure that the concentration of the growth-limiting nutrient rises rapidly to levels which do not restrict the population's growth rate. The time taken to establish these conditions depends on the dilution rate chosen for washout and also on the affinity the organism has for the growth-limiting substrate. Organisms with low saturation constants require a relatively small increase in the growth-limiting substrate concentration, hence a short period of washout time, before growth at or close to μ_{max} is possible. These constants for carbon dioxide (bicarbonate) or light have not been determined or reported for *Anacystis nidulans*; thus their effect on washout kinetics cannot be fully assessed at this stage. However, the delay in attaining μ_{max} growth rates could be circumvented if the cultures were saturated with the growth-limiting substrate at the same time as switching to the washout dilution rate. In an experiment to determine whether or not this was the principal reason for the long μ_1 phases observed during the washout of low dilution rate light-limited culture, a low biomass steady state culture at $D = 0.03\ h^{-1}$ was simultaneously switched to a washout dilution rate and the incident light intensity increased 4-fold. This culture still took about 550 min to reach growth at μ_{max} values compared with the 615 min for washout by the original method suggesting that possibly factors additional to changes in the concentration of the limiting nutrient influenced the length of the μ_1 phase.

The second reason for growth at sub-μ_{max} rates during the first phase of culture washout may be due to a more fundamental physiological reason. The classical "shift-up" closed culture experiments from nutritionally poor to rich growth environments showed that heterotrophic microorganisms maintained a growth rate which was characteristic of the poor medium for a fixed period of time after the shift-up before changing to an increased specific growth rate characteristic of the richer growth medium [11,12]. The initial post shift-up growth phase represents a transitional stage during which the organisms adapt to growth in the richer medium, particularly by increasing the rate of chromosomal replication (and ultimately cell division) by increasing the frequency of initiating new rounds of DNA replication. The length of post shift-up submaximal growth phase is equal to the sum of the time taken to complete one round of DNA replication and the time between the end of DNA replication and cell division – the C + D time [13,14]. Continuous-flow culture washout experiments are analogous to closed culture shift-up experiments and so the μ_1 phase is a measure of the C + D time. For carbon-dioxide limited cultures the C + D time was approx. 210 min and appeared to be independent of the initial steady-state culture growth rate, although the length of the μ_1 phase for the slowest growing initial culture was significantly longer, and compares with the previous C + D times determined in closed cultures of about 180 min [15,16]. In contrast for light-limited cultures the length of the μ_1 phase was dependent on the initial growth rate of the culture, suggesting that perhaps under these growth conditions some process involved in chromosome replication and/or cell division was growth-rate dependent. This possibility is strengthened by the inability to shorten the length of the μ_1 phase significantly by increasing the incident light intensity at the same time as switching to a washout dilution rate and is currently being examined in greater detail.

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