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Physiological state of a microbial community in a biomass recycle reactor

A Konopka[1], T Zakharova[1,2], L Oliver[3], E Paseuth[1] and RF Turco[3]

[1]*Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA;* [2]*On leave from the Institute of Soil Science and Photosynthesis, Russian Academy of Science, Pushchino, Russia;* [3]*Department of Agronomy, Purdue University, West Lafayette, IN 47907, USA*

The transition in physiological state was investigated between a carbon-limited chemostat population and microbes growing very slowly in a biomass recycle reactor. The mixed microbial population was metabolizing a mixture of biopolymers and linear alkylbenzene sulfonate, formulated to represent the organic load in graywater. Biomass increased 30-fold during the first 14 days after a shift from chemostat to biomass recycle mode. The ratios of ATP and RNA to cell protein decreased over the first days but then remained constant. The specific rate of CO_2 production by microbes in the reactor decreased 6-fold within 24 h after the shift, and respiratory potentials declined 2–3 fold during the first 7 days. Whereas chemostat cultures used equal proportions of organic carbon substrate for catabolism and anabolism, the proportion of organic substrate oxidized to CO_2 rose from 62 to 82% over the first 8 days in a biomass recycle reactor, and eventually reached 100% as this reactor population exhibited no net growth. Biomass recycle populations removed from the system and subjected to a nutritional shift-up did not immediately initiate exponential growth. The physiological state of cells in the biomass recycle reactor may be distinct from those grown in batch or continuous culture, or from starved cells.

Keywords: biodegradation; biomass recycle reactor; physiological state; graywater

Introduction

Continuous-flow bioreactors with a 100% recycle of biomass can operate at faster rates than batch or conventional continuous-flow systems because they contain higher levels of biocatalyst. Although these systems have found use as empirical solutions to applied problems [5,17,25], there is relatively little information on the physiological state of the microbes. Several studies on pure cultures in biomass recycle reactors have concluded that growth proceeds through several discrete phases [6,26]. We have investigated the use of the biomass recycle system containing a mixed microbial community for treatment of biopolymers and surfactants found in graywater (wastewaters produced from galley, scullery, laundry, shower, and lavatory sinks) [14]. Via analyses of physiological indicators (biochemical composition and metabolic activity), we found differences between the microbial populations in continuous-flow systems without biomass recycle (chemostats) and in biomass recycle reactors.

The purpose of this study was to systematically investigate the temporal dynamics in microbial physiological state after a chemostat was switched to biomass recycle mode. Physiological state encompasses the biochemical composition of the biomass (a product of the past history of the culture), and its metabolic potential [21] (the key to understanding how the organisms may respond to future changes in the environment). Previous work on microbes that were grown very slowly ($<5\%$ μ_{max}) in biomass recycle reactors has placed most emphasis on the increased energetic costs of growth [22] and the cell cycle [6]. In contrast, we have placed more emphasis on the reactivity of the microbial population, because of its relevance to environmental transients (such as changes in nutrient quality or quantity, and the influx of toxic agents) that may occur in biotechnological applications. Furthermore, the reactivity of slowly growing microbes in reactors may prove a useful model for understanding the physiology of microbes in nature that have grown at very slow rates but must respond to temporal fluctuations in nutrient supply. The results of this study suggest that physiological state at very low growth rate may not be a simple extension of principles derived from experiments done in chemostat cultures.

Correspondence: A Konopka, Purdue University, Department of Biological Sciences, West Lafayette, IN 47907, USA
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Methods

Biomass recycle cultures

The original inoculum for biomass recycle reactors was activated sludge from the aerator of the West Lafayette, IN, municipal treatment plant. For the experiments described here, 10 ml of inoculum was transferred from an established biomass recycle reactor into a continuous-flow bioreactor (0.58 L; CYTOLIFT glass airlift bioreactor, Kontes, Vineland, NJ, USA), operating at a dilution rate of 0.11 h^{-1}. The temperature was maintained at 28°C. The rate of sterile medium addition was controlled by a Gilson Minipuls 2 peristaltic pump (Gilson Inc, Middleton, WI, USA). Culture aeration and agitation occurred by passage of 5–6 L min^{-1} of sterile, humidified air through the culture. The dissolved oxygen tension in the culture fluid remained above 80% of saturation throughout the experiments. Five to ten volume

changes of medium were run through the continuous culture. This was adequate for these cultures to reach a steady-state with respect to biomass level.

Biomass recycle operation was initiated by pumping the culture fluid at a rate of approximately 1 L min^{-1} across a Millipore Minitan ultrafiltration system (Millipore Corp, Bedford, MA, USA). The system contained two membrane plates (polysulfone membranes with 100 000 nominal molecular weight limit; total surface area = 120 cm^2). The culture was drawn with a Masterflex model 7549–30 peristaltic pump (Barnant Co, Barrington, IL, USA) via a submerged tube. After passage across the ultrafiltration membranes, the culture was returned to the bioreactor via a submerged glass inlet. The cell-free culture eluate was drawn from the ultrafiltration system via a Gilson Minipuls 2 peristaltic pump. The rates of feed medium addition and ultrafiltrate removal were manually set to equal rates. The total volume of culture in the biomass recycle reactor system (reactor plus recycle loop) was 720 ml.

The medium feed contained the following components: 9.3 mM NH_4Cl, 3 mM NaCl, 2 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 0.25 mM $MgSO_4$, 5 μM $CaCl_2$, 14 μM $FeCl_3$, 23 μM disodium ethylenediaminetetraacetate, and 26 μM sodium citrate. The pH was 7.5. The medium contained 87 mg of commercial laundry detergent and 1 ml of SL7 trace element solution [3] per L. The organic substrates per L of medium were 400 mg starch (Argo, Englewood Cliffs, NJ, USA), 150 mg gelatin (Difco, Detroit, MI, USA), and 17 mg linear alkylbenzene sulfonate (LAS, 80% by weight, Sigma Chemicals, St Louis, MO, USA). The commercial laundry detergent (Dutch brand, Dial Corp, Phoenix, AZ, USA) contained 10% LAS by weight. Therefore, the actual amount of LAS in the medium was 21.5 mg L^{-1}.

Nutrient shift-up experiments were performed by inoculating samples from the bioreactor into batch cultures (50 ml medium in a 250-ml flask). The medium contained all of the inorganic components of the reactor feed medium + 0.2% casamino acids as an organic substrate. These cultures were inoculated to an initial $OD_{600\,nm}$ of 0.05–0.1 and incubated at 28°C on a shaker water bath at 100 rpm. Culture OD values were measured as a function of time.

Analytical methods

Five-day biochemical oxygen demand (BOD) [2] was assayed on ultrafiltrate samples from the biomass recycle reactor. Total carbohydrate in the ultrafiltrate or culture supernatant (samples from the reactor were centrifuged at 7000 × *g* for 10 min) was determined by the anthrone method [10]. The method of Lowry *et al* [16] was used to measure soluble protein in the ultrafiltrate or culture. Dissolved organic carbon in ultrafiltrate samples was determined in a DC190 carbon analyzer (Teckmar-Dohrmann, Cincinnati, OH, USA). All values presented are the means calculated from analysis of triplicate samples. The coefficients of variation for these samples were typically <15%.

Carbon dioxide gas in the off-gas from the reactor was measured with an Anarad AR600-R infrared gas analyzer (Anarad, Inc, Santa Barbara, CA, USA). Gas from the reactor was dried by passage through anhydrous $CaSO_4$, and entered the instrument at a flow rate of 2 L min^{-1}.

For analyses of dry weight and organic carbon content of biomass, 10 ml of culture was centrifuged in dried, tared centrifuge tubes at 7000 × *g* for 10 min, and the cell pellet was lyophilized on a Lyph-Lock 12 freeze drier (Labconco Corp, Kansas City, MO, USA). The dried pellets were weighed to determine dry weight. The lyophilized cells were then analyzed in a NA1500 Series 2 CNS analyzer (Fisons Instruments Inc, Beverly, CA, USA) to determine the fraction of organic C in biomass.

Microbial biomass in the reactors was monitored by measuring the optical density (OD) in a 1-cm cell at 600 nm on a Gilford 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH, USA). If the OD of a sample was >0.4, the sample was diluted with a solution containing the mineral salts components of the medium so that the measured value was between 0.1 and 0.4. The measured OD was divided by the dilution factor to produce an estimated biomass value for the culture. The turbidity values were converted to biomass units using the following experimentally-determined conversion factors. An optical density of 1.0 corresponded to 260 mg bacterial protein L^{-1}, 625 mg dry weight L^{-1} and 23 mmol C L^{-1}.

Cellular protein and total carbohydrate were measured as described by Herbert *et al* [10]. ATP was extracted from cells and analyzed as described in Cook *et al* [7]. The reported values are means calculated from analysis of triplicate samples. The coefficients of variation were usually <15%. Viable and direct microscopic counts were made by making appropriate dilutions of the samples in XBM mineral salts solution [13]. For viable plate counts, subsamples were plated onto TSA agar medium and incubated at 28°C. Direct microscopic counts were made using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA, USA), with a Zeiss standard research microscope at a magnification of 400× under phase contrast optics. The proportion of actively respiring cells was microscopically assayed after incubation with the tetrazolium dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences, Inc, Warrenton, PA, USA), as described in Konopka *et al* [15]. Three nutrient amendments were made to the incubation: (i) no organic substrates added; (ii) 0.2% casamino acids + 4 mM maltose; or (iii) 0.2% casamino acids + 4 mM maltose + 100 μM LAS. Maltose and casamino acids were used as lower molecular weight surrogates for the presumed products of exoenzyme hydrolysis of the starch and protein macromolecules that were in the nutrient feed.

Oxygen consumption by washed resting cell suspensions was measured with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA). A total volume of 1.5 ml of washed resting cells (approximately 3×10^8 cells ml^{-1}) were incubated at 28°C, and the rate of endogenous respiration was monitored. An individual test substrate was added and oxygen consumption was monitored over the next 5 min. To measure respiratory potential (that is, the rate achieved when cells were provided a non-limiting substrate concentration) the concentrations of added test substrates were either 2 mM maltose, 3 mM glucose, 100 μM LAS or 2 g casamino acids L^{-1}. These concentrations were sufficient to produce the maximum (saturated) rates of oxygen consumption.

Results

After a continuous flow bioreactor was switched to biomass recycle operation, the bacterial biomass in the reactor increased 36-fold (from 0.31 to 11.2 g dry weight L^{-1}) over the next 14 days (Figure 1). The total cell density at the end of the experiment was 6×10^{10} cells ml^{-1}. The data set could be fit to a linear function with a slope of 33.33 mg dry weight L^{-1} h^{-1} ($r^2 = 0.98$). For the 720-ml system, this corresponded to the production of 24 mg biomass h^{-1}. The linear increase in biomass indicates a continuous decrease in the apparent instantaneous specific growth rate (μ) (calculated as slope/(mg dry weight L^{-1})) from 0.1 h^{-1} just after the switch to biomass recycle mode to 0.003 h^{-1} after 14 days. Nutrient shift-up experiments indicated that μ of 0.29–0.5 h^{-1} on gelatin or maltose occur when growth rate is not restricted by substrate concentration (unpublished data).

The proportion of cells which reduced the tetrazolium dye CTC was determined (Figure 2). A low proportion of CTC-reducing cells (4%) was found even in continuous culture mode if no organic substrates were added to the incubation (data not shown). If organic substrates related to the feed constituents (that is, maltose and casamino acids) were added, relatively high proportions of active cells were found during the first 3 days of biomass recycle operation. At later times, <20% of the cells appeared to be metabolically active.

The concentrations of organic substrates in the ultrafiltrate were low both in chemostat cultures, and after the transition to a biomass recycle mode. Five-day BOD analyses were <2 mg L^{-1} in all samples. Total carbohydrate concentrations in the ultrafiltrate ranged from 0.1 to 1.2 mg L^{-1} over the 14 days of the experiment and protein was undetectable in the ultrafiltrate. In contrast, the concentration of soluble carbohydrate in the reactor increased after the switch to biomass recycle mode, and was greater than 100 mg L^{-1} (Figure 3). However, because the biomass level in the reactor was >100 mmol C L^{-1} after day 2, the extracellular polysaccharide was only 1% of the microbial C in the reactor. Increased concentrations of protein were measured in the reactor supernatant, but the maximum concentrations were eight-fold lower than the total carbohydrate levels.

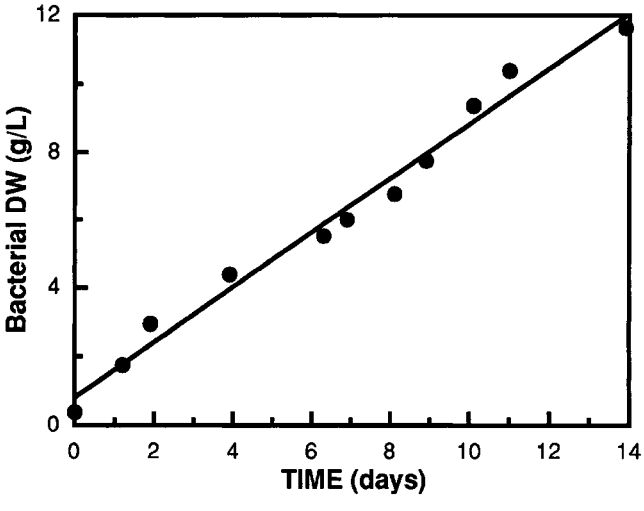

Figure 1 Changes in biomass (●) after a shift from continuous culture to biomass recycle mode.

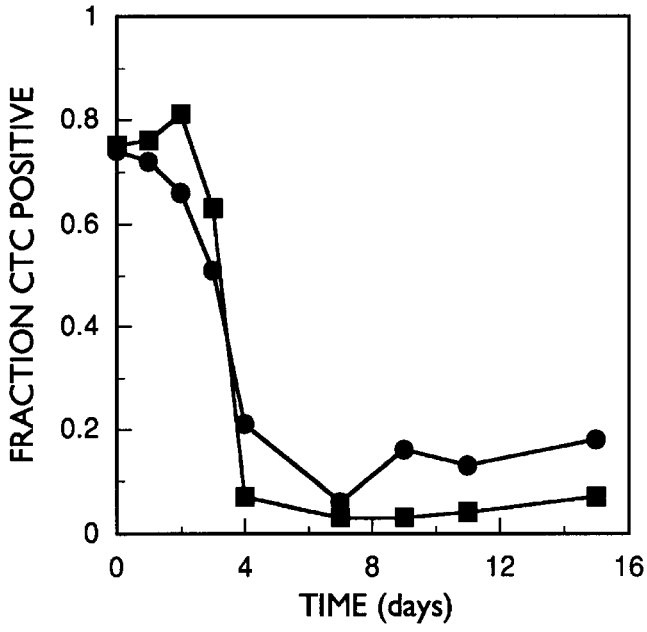

Figure 2 Change in the fraction of active cells (as assayed by the reduction of CTC) after a shift from continuous culture to biomass recycle mode. Cells were provided with (●) 0.2% casamino acids + 4 mM maltose or (■) 0.2% casamino acids + 4 mM maltose + 100 μM LAS as electron donors.

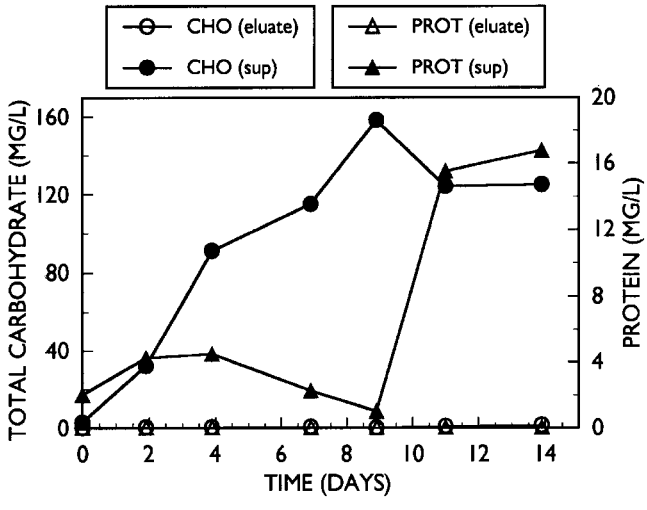

Figure 3 Concentrations of carbohydrate (CHO) or protein (PROT) found either in the culture supernatant (sup) or in the ultrafiltration membrane eluate (eluate) after the reactor was switched from continuous culture to biomass recycle mode.

There were biochemical changes in the microbial biomass (Figure 4). The ratio of ATP : protein declined by 40% on day 2 and then remained relatively constant over the next 12 days. The ratio of RNA : protein decreased by about 33% over the first 7–9 days, but did not decline further.

The growth responses to a nutrient input of casamino acids (Figure 5) or maltose (data not shown) were similar.

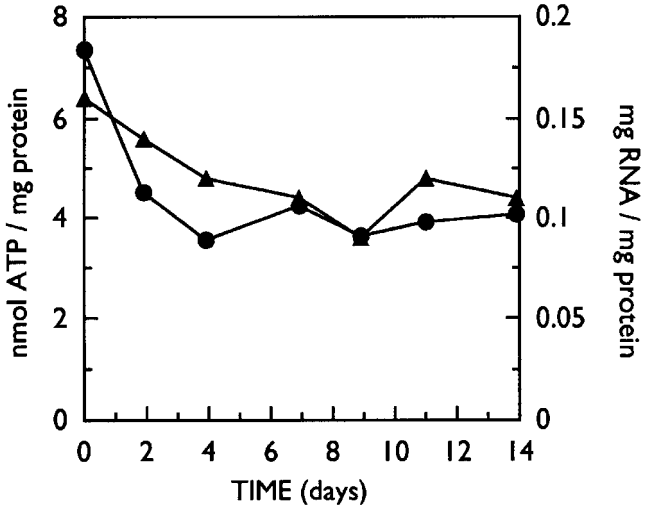

Figure 4 Biochemical changes after the switch from continuous culture to biomass recycle mode. The amount of ATP (●) or RNA (▲) is normalized to microbial biomass (expressed as the amount of particulate protein).

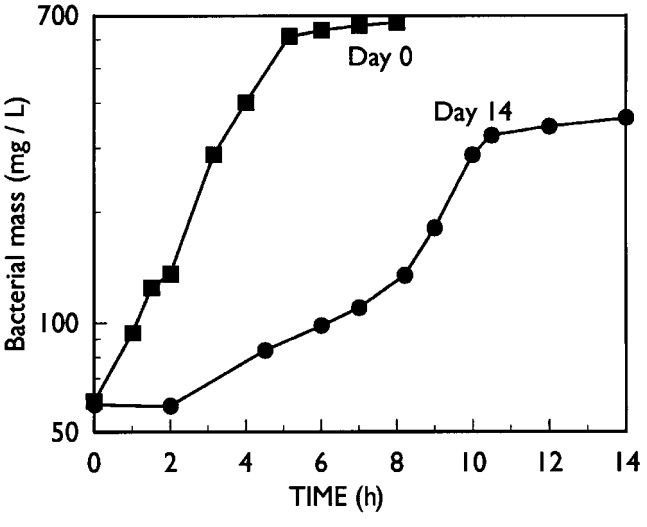

Figure 5 The kinetics of batch culture growth from samples removed from (■) the continuous culture or (●) after 14 days operation in biomass recycle mode. Samples from the cultures were inoculated into mineral salts medium that contained 0.2% casamino acids.

Samples removed from chemostat cultures and inoculated into batch culture with casamino acids or maltose began exponential growth (μ = 0.46 h^{-1}) immediately without a lag. Cultures initiated after 4 and 8 days of biomass recycle operation had lag periods of 0.5 and 3 h, respectively. Samples removed on day 14 of biomass recycle operation had more complex growth kinetics: a lag from 0–2 h, a slow growth phase from 2–8 h, and lastly an exponential growth rate similar to the other cultures. The apparent yield in this culture was only 50% of the one inoculated from a chemostat culture.

There was also a transition in metabolic potential (Figure 6). The respiratory potentials on low molecular weight substrates (maltose and casamino acids) derived from the two primary organic substrates in the feed medium (starch and gelatin) declined by a factor of 2–3 fold during the first 7

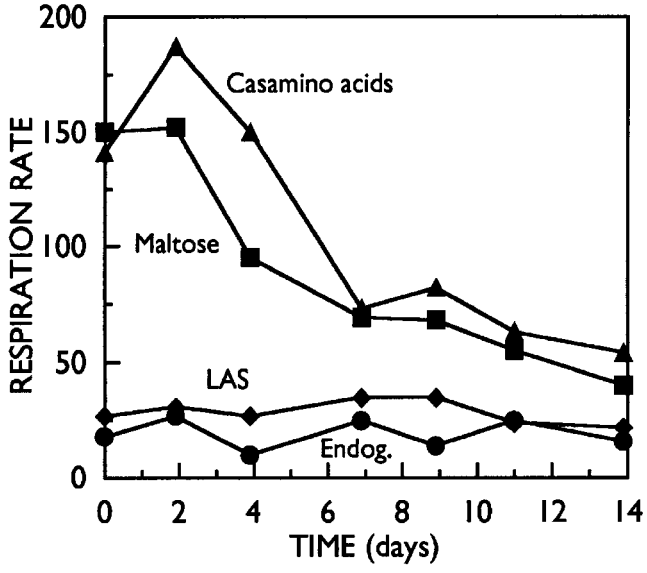

Figure 6 Changes in the potential respiration rates (μg O_2 mg^{-1} protein h^{-1}) of samples from the biomass recycle reactor. Respiration rates were measured (●) in the absence of organic substrate (endogenous), or after the addition of (▲) casamino acids, (■) maltose, or (◆) linear alkylbenzene sulfonate (LAS).

days. Biomass recycle reactors have been run as long as 74 days, and the potential respiration rates did not decline further beyond what was observed in this experiment [15]. The respiration rates (μg O_2 mg^{-1} protein h^{-1}) on LAS showed no trend with time and were relatively constant in these samples (mean = 29 ± 5 (s.d.)), as were the endogenous respiration rates (mean = 19 ± 6 (s.d.)).

In addition to analyses of respiratory potential on samples removed from the reactor, we assessed metabolic rates within the reactor by constructing a mass balance of carbon, in a separate experimental run. The feed rate and composition of the medium generated a flux of 2350 μmol organic C h^{-1} into the reactor. The flux of dissolved organic C via the ultrafiltrate was always about 60 μmol h^{-1} (<3% of the C flux into the system). During this reactor run, the rate of biomass accumulation (Figure 7a) was 490 mg dry weight L^{-1} day^{-1} from 0 to 220 h (r^2 = 0.98). This rate was converted to a flux of C into bacterial biomass from periodic parallel analyses of dry weight and organic C content (Figure 7b). This flux was related to the rate of CO_2 production calculated from the rate of gas flow and concentration of CO_2 in the off-gas. Whereas during chemostat operation the fluxes of C to CO_2 and biomass (equal to the product of dilution rate and steady-state biomass level) were approximately equal, about 80% of organic substrate was oxidized to CO_2 during the first 2 h after the shift to biomass recycle. The biomass-specific rate of CO_2 evolution was similar to the chemostat operation for these first 2 h, but then rapidly fell 4-fold and remained relatively constant during the remainder of the experiment (Figure 7c). From 2–200 h the flux to CO_2 slowly rose from 63 to 85% of the total C input. The biomass level in the reactor did not rise after this time. Thus, virtually all of the organic C was apparently catabolized to CO_2.

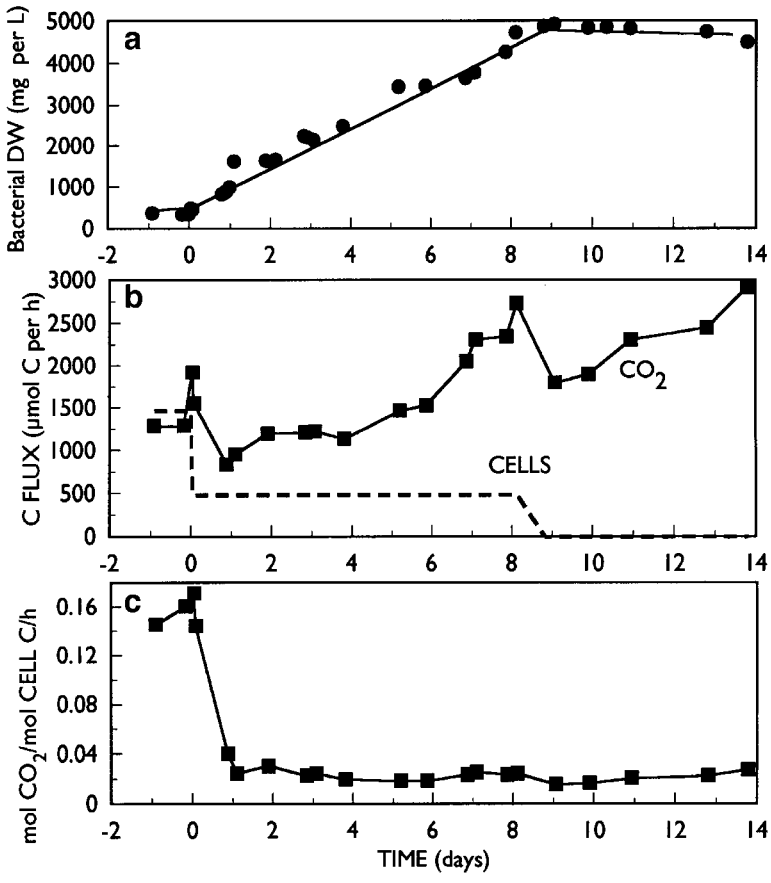

Figure 7 Fate of organic carbon fed into a biomass recycle reactor. The system was switched from continuous culture to biomass recycle mode at t = 0 h. (a) Change in biomass. (b) The rate at which organic substrate fed to the reactor was used for catabolism to CO_2 (■) or biomass synthesis (–––) by calculation from linear regression of turbidity data. (c) The rate of CO_2 evolution in the reactor, normalized to the amount of bacterial biomass, expressed as mol C.

Discussion

Studies on pure cultures grown in biomass recycle reactors have concluded that growth proceeds through several discrete linear phases (domains) [6,26]. The linear (rather than exponential) increase in biomass found in these studies as well as in ours indicated that the population's specific growth rate continuously decreased, as expected if the constant rate of substrate influx must support an ever increasing amount of biomass in the reactor. Muller and Babel [18] attained a stable plateau in the biomass level of *Alcaligenes eutrophus* in biomass recycle reactors. They interpreted this to mean that all of the substrate flux was used to provide energy for maintenance demands. We observed an apparent plateau in one of our 14-day runs (Figure 7). However, in earlier long-term experiments [15] our mixed cultures exhibited unstable plateaus followed by further growth. Thus, there may be more complex interspecies dynamics occurring in mixed systems.

The analyses of C flux to biomass *vs* CO_2 before and after a shift from chemostat to biomass recycle operation are consistent with the conclusions of others [18,22] that growth at very low rates is energetically more expensive than at μ near μ_{max}. This can be observed either as a decrease in growth yield, or as an increase in the fraction of organic substrate oxidized to CO_2.

One issue in dispute is whether cultures growing at extremely low rates contain a homogeneous population of slowly growing cells, or a heterogeneous population of growing and non-growing cells [18,22,23]. There was a large decrease in the proportion of respiring cells in the biomass recycle system, as assayed with CTC. There is a concern that the results represent a methodological artifact, due to the low respiratory rate of the populations. However, extending the incubation period to 3 h did not alter the results (data not shown).

Although the CTC results imply a reduction in active cells, the biochemical and metabolic analyses do not support this view. ATP contents were within a factor of 2 of values reported for batch cultures growing at μ_{max} [12]. We found that the ATP : protein ratio declines at $\mu < \mu_{max}$ in continuous cultures [14]; therefore, these ATP contents are consistent with a system in which the majority of the cells are active. The 2–3 fold decreases in RNA content and respiratory potential are also consistent with a shift in physiological state of a population of active cells, rather than a large decrease in the proportion of metabolically active

cells. Therefore, our current view is that the biomass recycle system contains a substantial proportion of metabolically active cells.

If these results are compared to those typical of starved cells, the physiological state in the biomass recycle reactor is substantially different. Starved cells of various bacterial species have been reported to have 10-fold lower biomass-specific ATP contents [27], 4–5-fold lower RNA contents [4,9], and 10-fold decreases in endogenous respiration rates [4,8,20] compared to growing cells. Thus, in the biomass recycle reactor certain physiological indicators are present in lower quantities than in nutrient-limited chemostats, but are poised at much higher values than those found in starved cells.

The other physiological change we found was a period of slow or no growth after nutrient additions. The measurements of respiratory potential indicated that the cells can immediately metabolize organic substrates, but growth is delayed. This phenomenon is similar to that observed for starved *Vibrio* sp S14 [1]. When exponentially-growing cells are subjected to a nutritional shift-up an immediate response is the synthesis of ribosomes to the level necessary to support the higher growth rate [19]. Our results and those of Albertson *et al* [1] suggest that the phenomena may be more complex. Under conditions of very slow or no growth, there may be selection for processes that maintain viability over those that determine rapid growth, and therefore the growth response to nutrient inputs is rather slow.

The physiological state of microbes is highly relevant to understanding their ecology and their application in biotechnology. Research in microbial physiology over the last 50 years suggests several broad categories of physiological state: (1) unrestricted growth, at the μ_{max} for the limiting substrate [19]; (2) nutrient-limited growth in which $\mu < \mu_{max}$ [24]; and (3) no growth (starvation or stationary phase conditions) [11]. Studies at near-zero growth rates in biomass recycle reactors raise the issue as to whether their physiological state is merely a special case of nutrient-limited growth, or if they represent a distinct, fourth category. We are conducting further studies with pure bacterial cultures to resolve this issue.

Acknowledgements

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