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# Effect of culture residence time on substrate uptake and storage by a pure culture of *Thiothrix* (CT3 strain) under continuous or batch feeding

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ABSTRACT

A pure culture of the filamentous bacterium *Thiothrix*, strain CT3, was aerobically cultured in a chemostat under continuous acetate feeding at three different culture residence times (RT 6, 12 or 22 d) and the same volumetric organic load rate (OLR 0.12 gCOD/L/d).

Cells cultured at decreasing RT in the chemostat had an increasing transient response to acetate spikes in batch tests. The maximum specific acetate removal rate increased from 25 to 185 mgCOD/gCOD/h, corresponding to a 1.8 to 8.1 fold higher respective steady-state rate in the chemostat. The transient response was mainly due to acetate storage in the form of poly(3-hydroxybutyrate) (PHB), whereas no growth response was observed at any RT. Interestingly, even though the storage rate also decreased as the RT increased, the storage yield increased from 0.41 to 0.50 COD/COD. This finding does not support the traditional view that storage plays a more important role as the transient response increases.

The transient response of the steady-state cells was much lower than in cells cultured under periodic feeding (at 6 d RT, from 82 to 247 mgCOD/gCOD/h), with the latter cells showing both storage and growth responses. On the other hand, even though steady-state cells had no growth response and their storage rate was also less, steady-state cells showed a higher storage yield than cells cultured under dynamic feeding. This suggests that in *Thiothrix* strain CT3, the growth response is triggered by periodic feeding, whereas the storage response is a constitutive mechanism, independent from previous acclimation to transient conditions.



## 1. Introduction

In wastewater treatment plants, bulking control is usually considered on the basis of competition for available substrates among floc-formers and filamentous microorganisms, based on respective rates of substrate uptake, which in turn reflects different growth rates. To model such competition, it has been proposed that floc-forming and filamentous microorganisms have different dependences on the kinetics of substrate uptake rate on substrate concentration, based either on steady-state or transient (unsteady) conditions [1].

Under steady-state conditions, Monod-type kinetics are applied (balanced growth), where floc-forming and filamentous microorganisms are supposed to have different profiles of growth kinetics, the latter being characterized from both lower $K_s$ and $\mu_{max}$. Thus, even though floc-formers have a higher specific growth rate than filaments at high substrate concentrations ($\mu_{max}$ strategy), filaments will have higher growth rates at low substrate concentration ($K_s$ strategy). As a consequence, the general theory on bulking control considers that continuous flow stirred tank reactors (CFSTR) are advantageous to filaments because of the low substrate concentration along the reactor volume [2]. On the contrary, plug-flow or batch reactors, where a substrate profile occurs along a distance or time, are favourable to floc-formers [3].

In the latter case, even if the whole process can be assumed as a steady-state, the biomass faces transient (unsteady) conditions. Indeed, owing to the concentration gradient of the substrate in the reactor and following back recirculation from settler to biological tank, the biomass alternately is subjected to high and low substrate concentrations. These separate zones are often created on purpose (for example, in contact-stabilization processes, basins with selector, sequencing batch reactors). Moreover, in most activated sludge processes, a true steady state condition is seldom possible owing to unavoidable fluctuations (flow-rate, feed composition, temperature), which are more or less relevant depending on process configuration (equalizer presence, plant size). Thus, a

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pseudo "steady-state" condition with several almost periodic disturbances is the most likely situation even in the presence of the CFSTR configuration.

Usually, biomass growth is not balanced under transient conditions [4]; i.e., cells are unable to instantaneously adapt their growth rate in a non-steady-state period. In fact, the RNA synthesis rate increases the most quickly, followed by the synthesis rate of proteins and/or DNA, and a lag phase is typically observed [5,6]. Indeed, with a sudden change in substrate concentration, cells require a period of adaptation/acclimation before showing an increase of their growth rate to the maximum value (so-called "growth response" phenomenon). On the other hand, other mechanisms of substrate consumption can be activated more quickly, i.e., the storage of the substrate in the form of a biopolymer, such as polyhydroxyalkanoates and/or polysaccharides. In fact, the storage response requires a short adaptation time compared to the growth response; for this reason, along a forced transient, a significant number of microorganisms are able to store substrates, reusing stored matter for subsequent growth. This fast mechanism of substrate uptake entails a competitive advantage on those microorganisms exhibiting storage response; consequently, the activated sludge will be characterized by storage activity [7]. Compared to floc-formers, filamentous microorganisms are supposed to have less storage ability, but this does not constitute a general knowledge for a wide range of known filamentous microorganisms under both balanced and unbalanced growth conditions [8,9].

The microbial composition of a microbial mixed culture (MMC), as well as the physiological state of any microorganism, affects biomass behaviour under both steady state and transient conditions. In addition, a mixed consortium is in turn steered by the operating conditions; e.g., the presence of an anaerobic selector to overcome filamentous bulking [10], culture residence time [11], and oxygen [12] or nutrient levels [13]. Owing to huge advancements in determining the microbial composition of MMC, most recent research has shifted towards using biomolecular techniques. In one example, the identification and quantification of *Thiothrix* spp., more specifically the ratio of *Thiothrix* spp. to total bacterial concentrations (TH/TB, %) by qPCR, was recently proposed as an indicator of bulking phenomena affecting effluent quality in a full-scale water reclamation plant [14]. However, the possibility to directly determine in a MMC the relationship between transient response and operating conditions for different species remains an open issue. Hence, pure culture studies can still contribute because the only thing that is considered is the effect of physiological adaptation of the microorganism, without any potential effect of possible microbial composition changes.

In recent years, several authors [15–19] have investigated the transient responses of several microbial species in pure culture by a combination of chemostat operation and short-term batch tests. A singular approach consisting of five continuous stirred tank reactors in series (5-SCR) or cascade was proposed by Atlic et al. [20] as process configuration solution to use the transient response of steady-state pure culture (*Cupravidus necator*), maximizing PHA production under nitrogen-limiting conditions. The cascade was structured with balanced biomass growth at first, followed by a growth-associated PHA synthesis and finally a non-growth associated synthesis of PHA under nitrogen-free in the last three reactors. Each of the three different metabolic states of *C. necator* strain and the transient states in between were analysed by metabolic engineering methods as useful tools for getting information about metabolic and physiological situations of the cells, and to predict well growth of this microorganism and related PHA synthesis [21,22].

Most cited studies have been conducted in cells cultured at low sludge retention time (SRT), typically 3 d or less (not the usual SRT for activated sludge processes). Very few studies have been performed using long culture retention times (RT) (>12 d), and they were limited to investigating only the transient response under periodic feeding either for floc-formers (*Amaricoccus kaplicensis*) [23] or filamentous microorganisms (*Thiothrix* CT3) [24]. For this reason, the filamentous microorganism *Thiothrix* strain CT3 has been used in this study to explore its behaviour under steady-state conditions and its transient response when maintained under high RT.

## 2. Materials and methods

### 2.1. The microorganism: Thiothrix *CT3*

The filamentous microorganism *Thiothrix* strain CT3 was isolated from a wastewater treatment plant located in the south of Italy; the microorganism utilized in this study is affiliated with the *Thiothrix* genus and related to *Thiothrix fructosivorans* [25,26].

Strain CT3 is an aerobic bacterium and its metabolism is rather versatile. In fact, it can be considered as a heterotrophic, autotrophic and mixotrophic microorganism if grown on carbon compounds, reduced sulphur compounds and on both organic and inorganic energy sources, respectively. Its maximum heterotrophic and autotrophic specific growth rate ($\mu_{max}$) is 2.5–2.7 $d^{-1}$ (measured on acetate) and 1.8 $d^{-1}$ (measured on thiosulfate), respectively [24–27].

Strain CT3 usually produces sheathed and long filaments (ranging from 100 to 500 μm) and it resembles a *Thiothrix* morphotype [28]. Normally, reproductive gonidial cells are at the margin of filaments; in addition, under mixotrophic and chemolithotrophic growth conditions, stored sulphur granules are usually observed.

### 2.2. Steady state response: chemostat runs

*Thiothrix* strain CT3 has been aerobically cultured as pure culture in a chemostat (CFSTR) under continuous acetate feeding at an organic load rate (OLR) of 0.12 gCOD/L/d.

The chemostat (1 L operating volume) was operated under aerobic conditions, temperature of 20 °C and pH 7.5. The chemostat medium was composed of a modified MSV mineral base exhibited in Table 1 [29]; Eikelboom vitamin solution (1% v/v) [30], and acetate as the sole carbon source.

The highest risks of microbial contamination came from sampling actions and dissolved oxygen (DO) probe maintenance and/or recalibration; owing to the long applied RT, the highest possible care to maintain axenic conditions for the culture was required. For simplicity and safety reasons, the chemostat control apparatus was simplified: only pH control (by acid solution addition) was adopted, whereas internal DO control and oxygen uptake rate (OUR) measurement were removed. Microscopic observations in phase contrast and after Gram staining were used for a periodic control of culture purity; in addition, in order to

**Table 1**
Mineral medium used for *Thiothrix* cultivation (modified from Williams and Unz, 1989).

| Substance | g/L |
|---|---|
| $CaCl_2 \cdot 2H_2O$ | 0.050 |
| $MgSO_4 \cdot 7H_2O$ | 0.100 |
| $K_2HPO_4$ | 0.110 |
| $KH_2PO_4$ | 0.085 |
| $Na_2EDTA$ | 0.002 |
| $FeCl_3 \cdot 6H_2O$ | 0.003 |
| $(NH4)_2SO_4$ | 0.500 |
| $NaHCO_3$ | 0.420 |