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Kinetics of Nitrate- and Nitrite-removal by *Rhodotorula glutinis*: Determination of a Reaction Mechanism

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In this work, the kinetics of nitrate- and nitrite-removal during the growth of the yeast *Rhodotorula glutinis* is addressed. More specifically, five different reaction mechanisms are defined as possible candidates to describe the system behaviour at the scale of the industrial process, i.e. unstructured modelling of transient biomass and nutrient concentrations in the pseudo-homogeneous liquid phase. System behaviour in a isothermal Batch reactor is simulated by varying the initial nitrate and nitrite concentrations. In conclusion, a sequence of experimental runs is defined to identify the reaction mechanism more capable to follow the behaviour of the system.

1. Introduction

Nitrate- and nitrite-removals represent a fundamental step in the water treatment industry. Denitrification is typically obtained through a biological process where specialized microorganisms are able to reduce nitrate and nitrite to molecular nitrogen. However, nitrate and nitrite may also be removed by yeast assimilation to eventually produce ammonium ion. In this context, the continuous search for a more sustainable economy demands for alternative microorganisms capable to assimilate nitrate and nitrite in a more convenient way. For this reason, in this work the attention is focused on *Rhodotorula glutinis*, a yeast with acknowledged capability of NOx assimilation (Hipkin, 1989; Smith, 1992).
*Rhodotorula glutinis*is well-known to produce carotenoids (Aksu and Eren, 2007) and remove pollutants as crude glycerol from biodiesel plants (Saenge, Cheirsilp, Suksaroge and Bourtoom, 2010), bio-sorption of uranium (Bai et al., 2010), and phenols from olive mill wastewater (Bozkoyunlu and Takac, 2014). However, to the best of authors' knowledge a kinetic analysis for the growth of *Rhodotorula glutinis* was never addressed before in the literature, except by Eren and Aksu (2007) who found a Monod and Haldane dependences from glucoseand ammonium ion concentrations, respectively. However, in that work substrate yield was not provided so that stoichiometry could not be determined, while NOx removal was not investigated. Actually, it is worth noting that the kinetics of NOx removal thorugh microbial growth has never been investigated so far in the literature for any microorganism.
For these reasons, a kinetic analysis of nitrate- and nitrite-removal during the growth of the yeast *Rhodotorula glutinis* is performed in this work. More specifically, five different reaction mechanisms are defined as possible candidates to describe the system behaviour at the scale of the industrial process i.e. unstructured modelling of transient biomass and nutrient concentrations in the pseudo-homogeneous liquid phase. The hypothesized mechanisms differ in the source of nitrogen used as nutrient for biomass cultivation (being nitrate, nitrite or ammonium ion), and in considering or not the nitrate- and nitrite-reduction as growth-associated reactions. However, the general reduction pathway proposed for yeasts (Siverio, 2002), i.e. from nitrate to nitrite, and then ammonium ion by means of nitrate- and nitrite-reductase, is always respected. System behaviour in a isothermal Batch reactor is simulated by varying initial nitrate and nitrite concentrations. The value of the model parameters used in the simulations is taken from the literature and related to similar microorganisms,

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when available. On the other hand, the stoichiometry is determined by following the fundamental approach proposed by McCarty and Rittmann (2001), based on the selection of an empirical, chemical formula of the cell, and the partitioning of substrate between energy generation and microbial synthesis. In conclusion, a sequence of experimental runs maybe defined to discriminate the reaction mechanism more capable to follow system behaviour among the five proposed.

2. Modeling Section

The five reaction mechanisms of NOx removal by *Rhodotorula glutinis* hypothesised in this work are given in Table 1, where X represents the biomass, S the substrate (glucose), and N the sum of nitrate and nitrite. The differences among these five mechanisms are the nitrogen source for biomass growth, and the nitrate- and nitrite-reduction considered or not as growth-associated reactions. However, all these mechanisms share the general reduction pathway from nitrate to nitrite, and then ammonium ion, by means of nitrate- and nitrite-reductase, correspondingly.

Table 1: Reaction mechanisms for nitrate- and nitrite removal by yeast (stoichiometry in terms of mass).

Mechanism 1
$X + 1.31\ NO_3^- + 0.38\ O_2 + 2.42\ S + 0.03\ H^+ \xrightarrow{1} 2X + 0.57\ NO_2^- + 1.59\ CO_2 + 0.97\ H_2O$
$X + 0.41\ NO_2^- + 0.52\ O_2 + 0.03\ H^+ + 2.21\ S \xrightarrow{2} 2X + 1.3\ CO_2 + 0.85\ H_2O$
Mechanism 2
$X + 0.41\ NO_2^- + 0.38\ O_2 + 2.08\ S + 0.00885\ H^+ \xrightarrow{1} 2X + 1.12\ CO_2 + 0.78\ H_2O$
$0.75\ NO_3^- + 0.18\ S \xrightarrow{2} 0.56\ NO_2^- + 0.27\ CO_2 + 0.11\ H_2O$
Mechanism 3
$X + 0.48\ N + 0.22\ O_2 + 1.99\ S + 0.0089\ H^+ \xrightarrow{1} 2X + 0.99\ CO_2 + 0.72\ H_2O$
$0.43\ NO_3^- + 0.11\ S \xrightarrow{2} 0.32\ NO_2^- + 0.15\ CO_2 + 0.06\ H_2O$
$0.32\ NO_2^- + 0.31\ S + 0.01\ H^+ \xrightarrow{3} 0.13\ NH_4^+ + 0.46\ CO_2 + 0.06\ H_2O$
Mechanism 4
$X + 1.55\ NO_3^- + 0.22\ O_2 + 2.31\ S + 0.0089\ H^+ \xrightarrow{1} 2\ X + 0.75\ NO_2^- + 1.44\ CO_2 + 0.91\ H_2O$
$X + 0.16\ NH_4^+ + 0.24\ O_2 + 1.55\ S + 0.54\ HCO_3^- \xrightarrow{2} 2\ X + 0.72\ CO_2 + 0.77\ H_2O$
$0.33\ NO_2^- + 0.33\ S + 0.015\ H^+ \xrightarrow{3} 0.13\ NH_4^+ + 0.48\ CO_2 + 0.06\ H_2O$
Mechanism 5
$X + 0.16\ NH_4^+ + 0.24\ O_2 + 1.55\ S + 0.534 HCO_3^- \xrightarrow{1} 2X + 0.72\ CO_2 + 0.77\ H_2O$
$0.47\ NO_3^- + 0.11\ S \xrightarrow{2} 0.35\ NO_2^- + 0.17\ CO_2 + 0.07\ H_2O$
$0.35\ NO_2^- + 0.34\ S + 0.01\ H^+ \xrightarrow{3} 0.14\ NH_4^+ + 0.5\ CO_2 + 0.07\ H_2O$

More specifically, in the first mechanism *Rhodotorula glutinis* is assumed to be able to use both nitrate and nitrite as nitrogen source for its own aerobic growth, albeit with different kinetics. For this reason, Mechanism 1 consists of two growth associated reactions, where the first one may be seen as the nitrate-reductase reaction producing the intermediate nitrite eventually followed by its assimilation. On the contrary, Mechanism 2 is based on the assumption that only nitrite may be used as nitrogen source for the growth of the microorganisms. Thus, the possibility to produce nitrite by nitrate-reduction is provided by the second reaction considered in Table 1 for this mechanism. In Mechanism 3, nitrate and nitrite may be indifferently used as nitrogen source for the growth of *Rhodotorula glutinis*, with the same assimilation kinetics. In this case, nitrate- and nitrite-reduction are considered as parallel reactions competing for NOx consumption with the final production of ammonium ion, which is not assimilated. The logical possibility to use ammonium ion as nitrogen source for biomass growth is taken into account in Mechanism 4. In this case, microbial growth is also

associated to nitrate-reduction to nitrite, which is eventually consumed by the nitrite-reductase reaction to produce ammonium ion. Finally, the case of ammonium ion as the exclusive nitrogen source for biomass growth is taken into account in Mechanism 5. Here, as in Mechanism 4, nitrate- and nitrite-reductase with the final production of ammonium ion are also considered.
Basically, the five reaction mechanisms detailed above represent different combinations of biomass growth with nitrate- and nitrite-reductions. In particular, the substrate plays always the role of a consumed reactant in any single reaction of any mechanism, while oxygen is consumed only by biomass growth reactions. Biomass is present in all reactions as well: as a reactant/product in the growth reactions or as the catalyst in nitrate- and nitrite-reductions, due to its enzymatic content.
The five reaction mechanisms were separately adopted for the simulation runs of a isothermal Batch reactor at varied initial concentrations for nitrate and nitrite, while keeping an excess of dissolved oxygen. The corresponding material balances and initial conditions are:

$$\frac{dC_i}{dt} = \sum_{j=1}^{NR} \gamma_{i,j} r_j C_i = C_i^0 \quad @ \quad t = 0 \tag{1}$$

where $\gamma_{i,j}$ represent the stoichiometry (given in Table 1, for each single reaction mechanism) of the i-th reagent/product in the j-th reaction.
All the non-elementary reaction rates in each single mechanism written in Table 1 are considered as generally depending from substrate, biomass, and nitrate/nitrite or ammonium ion concentrations, i.e. $r_j = f_j(C_S, C_X, (C_{NO_2^-} or\ C_{NO_3^-})\ or\ C_{NH_4^+})$ for any j. The dependence from oxygen concentration is neglected, since an excess of this nutrient is assumed to be fed to the reacting system. Moreover, any reaction rate is assumed as the product of single dependences from species concentrations. This way, in all the reaction rates (either growths or nitrate- and nitrite-reductions) substrate dependence is always of the Monod type, while a first-order power law is constantly used for the biomass. This latter choice is mandatory for the biomass growth rate if one wants to simulate the exponential-phase under the unlimited supply of nutrients.
Only biomass growths in Models 4-5 depend from ammonium ion concentration: in these cases, a limitation at relatively low and high concentration values is considered by means of the Haldane kinetic expression. On the contrary, the dependence of the different reaction rates from nitrate and nitrite concentrations changes from growth to nitrate- and nitrite-reductions: for nitrate concentration, a Monod expression is used in biomass growth and nitrate-reduction rates, while poisoning (i.e. inhibition at high concentrations) is assumed for the nitrite-reduction reaction rate; for nitrite concentration dependences, Haldane and Monod expressions are used in biomass growth and nitrite-reduction rates, correspondingly, while poisoning is assumed for the reaction rate of nitrate-reduction.
The system of ordinary differential Eqs (1) embedding the stoichiometry of the Mechanisms 1-5 reported in Table 1 is numerically solved as an initial value problem by means of the Reaction Engineering Lab module of the Comsol 3.4 software. A specific value must be necessarily assigned to every kinetic parameters involved to obtain a numerical solution. Regarding this, since it's not available in the literature, the specific stoichiometry shown in Table 1 for each single reaction mechanism is determined by following the fundamental approach proposed byMcCarty and Rittmann (2001), based on the selection of an empirical, chemical formula of the cell (i.e. $C_5H_7O_2N$), and the partitioning of substrate between energy generation and microbial synthesis. On the other hand, the kinetic parameters of the various Monod, Haldane and poisoning expressions used for the reaction rates in Table 1 are taken from the corresponding technical literature. In particular, the maximum specific rate and the half-velocity constants of the Monod dependence from substrate and ammonium ion concentrations used for the rates of biomass growth are specifically related to *Rhodotorula glutinis* (Eren and Aksu, 2007). The values of all the other parameters are taken from the literature when available, even if related to other microorganisms, being either yeasts different from *Rhodotorula glutinis* or even bacteria. In particular, the parameters related to nitrate and nitrite dependences in biomass growth as well as reduction rates are arbitrarily assigned in this work, since not available in the literature for any microorganism.
The system of Eqs. (1) is numerically solved in adimensional form by defining constant reference values for all the dependent and independent variables: time (τ) is scaled with the inverse of the maximum specific rate for the growth of *Rhodotorula glutinis*, while species concentrations $(\theta_i)$are referred to the corresponding initial values (for the Nox, the sum of initial nitrate and nitrite content is used).

3. Results and discussion

The simulation results in adimensional fashion are now reported and discussed. The focus is the definition of a practical strategy to identify (through direct comparison with measurements once these will be available) the reaction mechanism out of the five given in Table 1 capable to better describe system behaviour. For this reason, the simulation results corresponding to a reactor feeding of only nitrate (i.e. without nitrite and ammonium ion) are first considered in Figure 1.

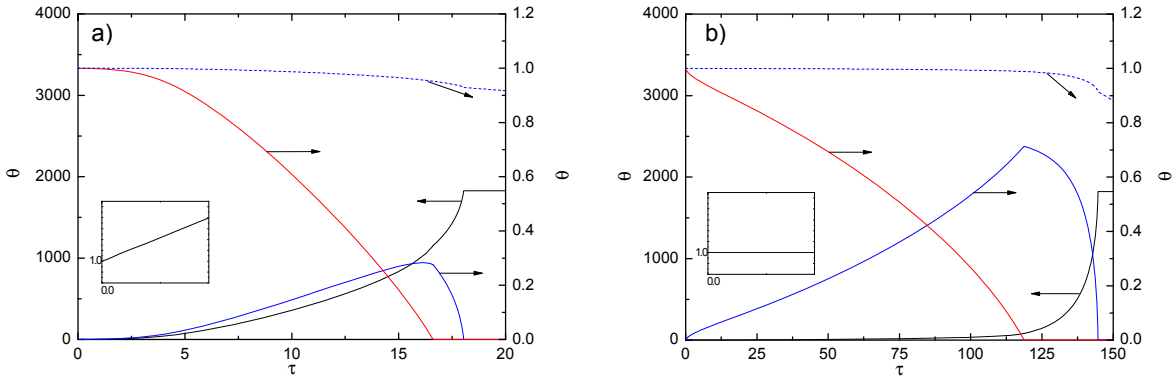

Figure 1: Temporal profiles of species concentration according to reaction mechanisms 1 (a) and 2 (b) when feeding only nitrate as nitrogen source for biomass growth.

Here, the temporal profiles of the reacting species concentrations are reported for the reaction mechanisms 1 and 2.Even if not initially fed to the Batch reactor system, the intermediate nitrite is first formed and then consumed, and biomass growth stops when nitrite (later than nitrate) is no longer available. This is valid for both mechanisms 1 and 2. However, biomass growth is not exactly the same for the two reaction mechanisms, as highlighted by the insets in Figure 1: biomass growth starts from the very beginning of the reaction system only in Mechanisms 1, while a delay is predicted by Mechanism 2. Clearly, this is due to the initial absence of nitrite which is the only nitrogen source considered in Mechanism 2: in this case, before biomass growth may start, first nitrite needs to be formed by the nitrate-reductase reaction. In Mechanism 1, on the contrary, biomass growth is sustained by the initial presence of nitrate and may start even when nitrite is initially absent.

This feature does not exclusively belong to the two reaction mechanisms considered in Figure 1: a close analysis of the reaction systems reported in Table 1 reveals that, the same initial biomass growth when only nitrate is initially fed may be obtained through Mechanisms 1, 3 and 4, while an initial delay is predicted by reaction Mechanisms 2 and 5. Moreover, this feature does not depend from the specific reaction rate kinetic expressions adopted for the simulations, briefly described in the previous section due to page limitation.

Along these lines, when only nitrite is initially fed to the reactor (i.e. without nitrate and ammonium ion), for Mechanisms 1-3 biomass growth starts immediately, while an initial delay may be predicted for Mechanisms 4-5. This is shown in Figure 2, where the comparison among the temporal profiles of the reacting species concentrations are reported for Mechanisms 1 and 4, for instance.

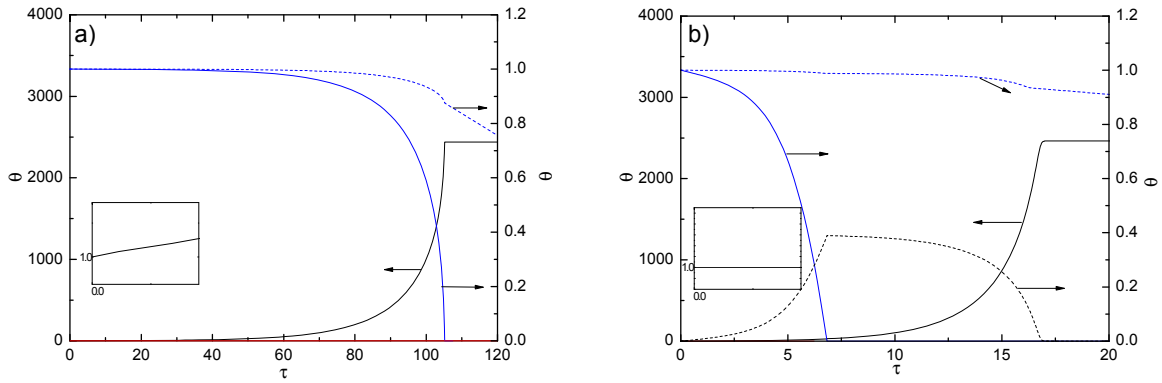

Figure 2: Temporal profiles of species concentration according to the reaction mechanism 1 (a) and 4 (b) when feeding only nitrite as nitrogen source for biomass growth.

In these simulations, nitrate is always absent (i.e. not initially present neither formed in any reaction) for all the mechanisms reported in Table 1, since the general reduction pathway proposed for yeasts (Siverio, 2002) is always respected.
These results suggest a way to identify experimentally the reaction mechanism out of the five given in Table 1 capable to better describe system behaviour, as reported in the flowsheet of Figure 3.

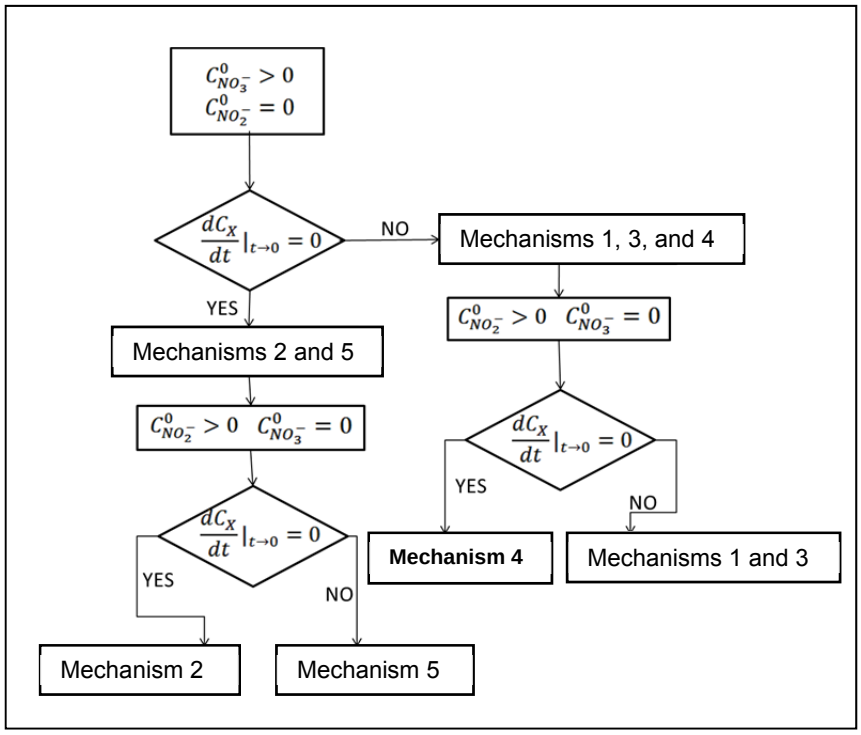

Figure 3: Flowsheet displaying the strategy to identify (through direct comparison with measurements once these will be available) the reaction mechanism out of the five given in Table 1 capable to better describe system behaviour.

If measured data on biomass growth will show an initial delay when feeding the reactor with only nitrate (i.e. nitrite and ammonium ion are absent), attention will be focused only to Mechanisms 2 and 5, while the reaction mechanisms 1, 3 and 4 should be dropped. In such a case, the further discrimination between Mechanisms 2 and 5 may be obtained through experimental runs by feeding only nitrite to the reactor (i.e. nitrate and ammonium ion are absent): as shown in Figure 4, when only nitrite is initially present in the system as nitrogen source for cell cultivation, Mechanism 2 predicts an initial biomass growth while a delay is obtained for Mechanism 5.

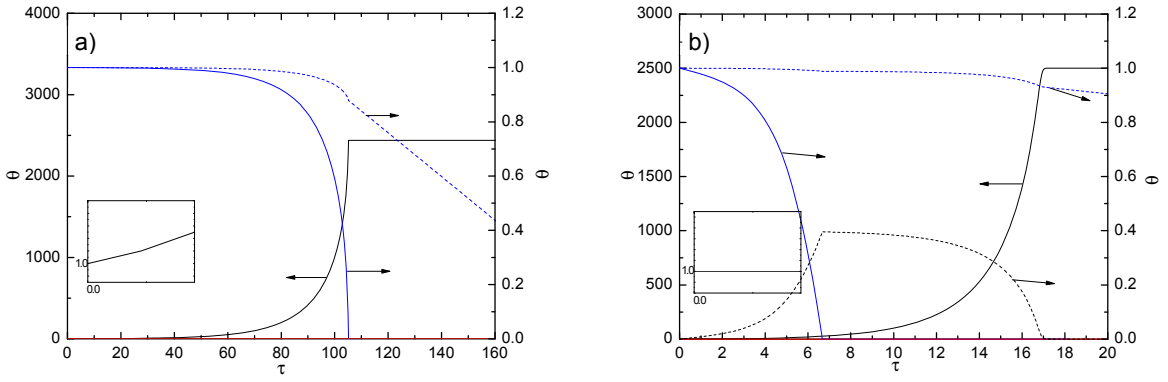

Figure 4: Temporal profiles of species concentration according to reaction mechanisms 2 (a) and 5 (b) when feeding only nitrite as nitrogen source for biomass growth.

On the other hand, the experimental runs when only nitrite is fed to the reactor will help also the further discrimination among mechanisms 1, 3 and 4. A close look to the reactions reported in Table 1 for these three mechanisms reveals that, in this case, only mechanism 4 shows an initial delay for biomass growth (see Figure 2), while in mechanisms 1 and 3 biomass growth starts immediately.

4. Concluding remarks

In this work, the modelling of kinetics for nitrate- and nitrite-assimilation by yeast *Rhodotorula glutinis* is addressed. Five different reaction mechanisms are hypothesized. These mechanisms represent different combinations of biomass growth with nitrate- and nitrite-reductions, but the general reduction pathway proposed for yeasts (i.e. from nitrate to nitrite, and then ammonium ion by means of nitrate- and nitrite-reductase, correspondingly) is always respected. For this reason, all these reaction mechanisms are possible candidates to describe the system behaviour at the scale of the industrial process i.e. unstructured modelling of transient biomass and nutrient concentrations in the pseudo-homogeneous liquid phase. Batch simulations at various initial compositions of the nutrient medium are performed in order to support the selection of the reaction mechanism more capable to describe system behaviour, through direct comparison with measurements once these will be available. In conclusion, a sequence of experimental runs maybe defined. More specifically, by means of only two experimental runs, one with only nitrate and the other one with only nitrite as limiting nutrient fed to the reactor as nitrogen source for biomass growth, it is possible to discriminate among reaction mechanisms 2, 4, 5, and 1-3. The further discrimination between mechanisms 1 and 3 is necessarily based on best fitting results, and, as such, depends on the specific kinetic expressions used for the reaction rates.

Acknowledgments

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