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The Substrate Carbon Consumption and Metabolite Production to Describe the Growth of *Geotrichum candidum* and *Penicillium camemberti* on Glucose and Amino Acids

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Summary

Amino acids had previously been characterized based on their ability to be assimilated as carbon sources by *Penicillium camemberti* and *Geotrichum candidum*. For each microorganism, three groups of amino acids have been characterized, leading to four different metabolic behaviours. To describe those recorded during *P. camemberti* growth on an amino acid and glucose, an unstructured model had previously been developed, based on the sequential consumption of both carbon substrates; glucose first, followed after its exhaustion by the selected amino acid. Only the part of the amino acid assimilated as a carbon source for cellular biosynthesis was considered in the model, which had to be deduced from the total amino acid consumption. To avoid the use of such an indirect parameter, ammonium was considered in this work, which was produced after amino acid deamination and corresponded to the release of the excess nitrogen, since amino acids contain excess nitrogen in relation to their carbon content in fungi. The model, therefore, involved substrate carbon consumption, ammonium production, as well as biomass yield on the carbon substrate, $Y_{X/S}$, and biomass yield on the produced ammonium, $Y_{X/P}$. The model proved to describe satisfactorily the various metabolic behaviours recorded during *P. camemberti* and *G. candidum* growth on an amino acid and glucose.

Key words: batch culture, carbon substrates, *Geotrichum candidum*, *Penicillium camemberti*, unstructured models, biomass yield

Introduction

Amino acids had previously been characterized based on their ability to be assimilated as carbon sources by *Penicillium camemberti* and *Geotrichum candidum*, both fungi involved in the ripening of soft white cheese. This characterization was carried out in the presence of glucose (*1*,*2*), a primary carbon source which is not relevant in terms of food microbiology. However, no clear response can be expected from a more relevant system like lactate/amino acid, owing to the simultaneous assimilation of both substrates, lactate and the selected amino acid, as carbon and energy sources by the fungi (*3*,*4*).

Three groups of amino acids were characterized for each microorganism (*1*,*2*,*5*), leading to four different metabolic behaviours. The first group was only used as

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nitrogen sources by *G. candidum*, with glucose being the carbon and energy source, while amino acids from the second group were dissimilated by *G. candidum* and *P. camemberti*; namely, they were used as energy supply by oxidation into CO_2 during stationary phase after glucose depletion, and the corresponding nitrogen was released as ammonium. Both species exhibited diauxic behaviour during growth on the third group of amino acids, since they can be assimilated as carbon sources, in addition to their assimilation as nitrogen sources, but only after glucose depletion. *P. camemberti* showed a clear differentiation between the assimilated and the dissimilated carbon from the last group of amino acids; the carbon from the amino acid was assimilated, while the carbon from glucose was dissimilated.

A simple unstructured model had previously been developed to describe diauxic growth (*6*). The consumption kinetics of both carbon substrates was described using a modified Verhulst model (*7,8*), without involving the key enzyme responsible for the degradation of the second carbon substrate, the selected amino acid (arginine). Growth kinetics was deduced from the sequential consumption of both carbon substrates, since biomass yield on carbon substrate was found to be constant (*6*):

$$\frac{dx}{dt} = -Y_{X/S} \cdot \frac{ds}{dt} - Y_{X/Z_C} \cdot \frac{dz_c}{dt} \qquad /1/$$

where s and z_c are glucose concentration and amino acid concentration, respectively, used as carbon source, $Y_{X/S}$ and Y_{X/Z_C} are the biomass yield on glucose and biomass yield on amino acid used as carbon source, respectively.

The model was also previously improved to describe the behaviour recorded during *P. camemberti* growth on amino acids from other groups (*9*):

$$\frac{dx}{dt} = -\left(1 - \frac{Y_{P/Z,0}}{Y_{P/Z,f}}\right) \cdot Y_{X/S} \cdot \frac{ds}{dt} - Y_{X/Z_C} \cdot \frac{dz_c}{dt} \qquad /2/$$

where $Y_{P/Z,0}$ and $Y_{P/Z,f}$ are the initial and final ammonium yield on the amino acid, respectively.

Glucose was therefore taken into consideration during *P. camemberti* growth on amino acids used as nitrogen source, while amino acid consumption was considered during growth on acids used as carbon source with only glucose being dissimilated for energy supply.

Only the part of the amino acid used as a carbon source was considered in the model (*6,9*), and it was obtained by subtracting the part of the amino acid used as nitrogen source during the first growth phase in the case of diauxic growth from the total amino acid consumption. To avoid the use of such an indirect parameter, ammonium was considered in this work, instead of the part of the amino acid used as a carbon source. Indeed, amino acids contain excess nitrogen in relation to their carbon content in fungi in general, leading to the release of ammonium after amino acid deamination during their metabolization as C and N sources, corresponding to the excess nitrogen (*10,11*), which can increase the pH (*12*).

From this, the growth model (Eq. 2) becomes:

$$\frac{dx}{dt} = -\left(1 - \frac{Y_{P/Z,0}}{Y_{P/Z,f}}\right) \cdot Y_{X/S} \cdot \frac{ds}{dt} + Y_{X/P} \cdot \frac{dp}{dt} \qquad /3/$$

where p is the ammonium concentration.

It can also be observed that the considered logistic (Verhulst) function needed a negligible residual substrate concentration to lead to a relevant solution from a mathematical point of view (*13*). The involvement of a metabolite (ammonium) in the model allowed to avoid this problem.

Materials and Methods

Microorganisms

The commercial strains *Geotrichum candidum* Geo17 and *Penicillium camemberti* LV2 (Danisco, Dangé St Romain, France) were used. Freeze-dried spores were stored at 7 °C.

Media

The media used contained (in g/L): glucose (Merck, Darmstadt, Germany); one of the following amino acids: threonine 10.5 (TGlc medium), lysine 6.5 (KGlc medium), glutamic acid 10.5 (EGlc), or arginine 4 or 9 (RGlc) (Acros Organics, New Jersey, USA); inorganic phosphates (Pi) KH_2PO_4 25 mmol/L and $NaH_2PO_4 \cdot H_2O$ 25 mmol/L (*14*); and trace elements (in mg/L): Mg 25, Fe 20, Ca 18, Zn 4.5, Mo 2 and Cu 1.3 (*14*) chelated with a solution of EDTA (ethylenediaminetetraacetic acid; 585 mg/L). The pH of the medium was adjusted to 4.6 with 6 mol/L HCl, before it was sterilised at 121 °C for 20 min.

Culture conditions

Batch fermentations were carried out in a 3-litre laboratory-made glass-blown fermentor (*15*). The fermentor was filled with 2 L of sterilized culture medium. Temperature was maintained at 25 °C. The broth was continuously aerated at constant airflow of 13.0 L/h (*i.e.* 6.5 L of air per litre of medium per h). This rate was obtained with the help of a mass flow controller GFC17 (Aalborg, New York, NY, USA) and the broth was stirred magnetically at 850 rpm. Inoculation of the culture medium was carried out by an aseptic addition of spore suspension (initial density of $4 \cdot 10^5$ spores/mL). Spores were left to rehydrate approx. 1 h in the sterilized medium at room temperature before inoculation.

The bioreactor was equipped with a sterilizable combination pH glass electrode (Ingold, Paris, France). The system contained also an aseptic recirculation loop allowing an on-line measurement of turbidity at λ=650 nm (*15*). Turbidity was calibrated from dry mass measurement of biomass at the end of culture. Total biomass and broth turbidity were linearly correlated up to a concentration of 5 g/L (the correlation coefficient was 0.98 determined in duplicate experiments) (*15*). Carbon dioxide in the off-gas was also monitored on-line by an IR detector Rubis 3000 (Cosma, Igny, France) after desiccation in a column of calcium chloride. In order to detect any gas leak in the system, the flow rate at the outlet of the IR detector was measured by a Pelton wheel flowmeter S110-3 (McMillan Co., Georgetown, TX, USA).

Analyses

After centrifugation of the samples, the following components were determined spectrophotometrically in

the supernatants: glucose by the phenol-sulphuric acid method for total sugars (*16*), ammonium by the Nessler method (*17*) and the chosen amino acid was deduced from the measurement of the primary α–amino groups $N_{\alpha NH_2}$ by the trinitrobenzenesulphonic acid (TNBS) method (*18*).

Fitting procedure

Origin software v. 7.5 (Microcal Software Inc., Northampton, MA, USA) was used for the fitting of all parameters.

Model development

The well-known logistic or Verhulst relation, previously applied to describe lactic acid production (*7,8*), was applied in this work to describe metabolite production:

$$r_P = \frac{dp}{dt} = p_0' \cdot p \cdot (1 - \frac{p}{p_f}) \qquad /4/$$

where p_f is the final ammonium concentration and p_0' is the ratio between its initial volumetric rate $r_{P,0}$ and its initial concentration p_0.

This logistic relation can be directly solved resulting in the following solution:

$$p = \frac{p_0 \cdot p_f \cdot e^{p_0' \cdot t}}{p_f - p_0 + p_0 \cdot e^{p_0' \cdot t}} \qquad /5/$$

with p_0 being the initial product concentration, and e exponential function.

Ammonium production is directly linked to amino acid assimilation as carbon and energy source, since it corresponds to the release of the excess nitrogen. To account for the time lag before the beginning of the second available carbon and energy source consumption (amino acid), an additional parameter t_P was added to Eq. 5 (*6*):

$$p = \frac{p_0 \cdot p_f \cdot e^{p_0' \cdot (t - t_P)}}{p_f - p_0 + p_0 \cdot e^{p_0' \cdot (t - t_P)}} \qquad /6/$$

Since substrate (glucose) consumption kinetics exhibited similar shape as production kinetics, the logistic or Verhulst model (*7,8*) can be applied to substrate consumption, giving after integration the following (*6*):

$$s_0 - s = \frac{s_0 \cdot s_{res} \cdot e^{s_0' \cdot t}}{s_0 - s_{res} + s_{res} \cdot e^{s_0' \cdot t}} - s_{res} \qquad /7/$$

where s corresponds to the glucose concentration; s_0 and s_{res} are the initial and the residual glucose concentrations, respectively; and s_0' is the ratio between its initial volumetric rate $r_{S,0}$ and its initial concentration s_0.

To take into account a possible time lag before the beginning of the substrate consumption, an additional parameter t_S was added to Eq. 7 (*6*):

$$s_0 - s = \frac{s_0 \cdot s_{res} \cdot e^{s_0' \cdot (t - t_S)}}{s_0 - s_{res} + s_{res} \cdot e^{s_0' \cdot (t - t_S)}} - s_{res} \qquad /8/$$

It should be observed that low t_S values were expected from the fitting of the glucose consumption time courses. Glucose consumption (Eq. 8) and ammonium production (Eq. 6) were then introduced in the above model (Eq. 3) to describe growth.

The use of the amino acids only as a nitrogen (and energy) source (*1*) during growth led to the absence of ammonium production, and therefore to a nil initial ammonium yield on an amino acid yield, $Y_{P/Z,0}$. The term $\left(1 - \frac{Y_{P/Z,0}}{Y_{P/Z,f}}\right)$ in Eq. 3 was therefore equal to one. Moreover, since threonine and lysine were not used as carbon sources for biosynthesis, no ammonium was produced during growth, leading to a nil $Y_{X/P}$ yield. From this, Eq. 3 became:

$$\frac{dx}{dt} = -Y_{X/S} \cdot \frac{ds}{dt} \qquad /9/$$

Growth kinetics can be then easily derived by introducing the substrate consumption time course (Eq. 8) into Eq. 9:

$$x = x_0 + Y_{X/S} \cdot \left(\frac{s_0 \cdot s_{res} \cdot e^{s_0' \cdot (t - t_S)}}{s_0 - s_{res} + s_{res} \cdot e^{s_0' \cdot (t - t_S)}} - s_{res} \right) \qquad /10/$$

The values of the parameters s_0, s_{res}, s_0' and t_S were deduced from the fitting of the substrate consumption time course (Eq. 8); while the biomass yield on carbon substrate, $Y_{X/S}$, corresponded to the linear regression of experimental data of biomass concentration *vs.* substrate carbon consumption.

In the case of diauxic growth, the use of the selected amino acid only as a nitrogen source, with glucose being the carbon source, led to the absence of ammonium production, and then a nil value for the $Y_{P/Z,0}$ yield. After glucose depletion, the use of the amino acid as a carbon (and nitrogen) source resulted in ammonium production, and then a non-nil $Y_{P/Z,f}$ yield. The term $\left(1 - \frac{Y_{P/Z,0}}{Y_{P/Z,f}}\right)$ in Eq. 3 was therefore equal to one, leading to the following expression:

$$x = x_0 + Y_{X/S} \cdot \left(\frac{s_0 \cdot s_{res} \cdot e^{s_0' \cdot (t - t_S)}}{s_0 - s_{res} + s_{res} \cdot e^{s_0' \cdot (t - t_S)}} - s_{res} \right) + Y_{X/P} \cdot \frac{p_0 \cdot p_f \cdot e^{p_0' \cdot (t - t_P)}}{p_f - p_0 + p_0 \cdot e^{p_0' \cdot (t - t_P)}} \qquad /11/$$

The use of amino acids as carbon (and nitrogen) sources (with glucose being the energy source) led to the production of ammonium and hence a constant $Y_{P/Z}$ yield throughout the growth. The term $\left(1 - \frac{Y_{P/Z,0}}{Y_{P/Z,f}}\right)$ was therefore nil and Eq. 3 became:

$$\frac{dx}{dt} = Y_{X/P} \cdot \frac{dp}{dt} \qquad /12/$$

Growth kinetics can then be easily derived by introducing the substrate carbon consumption time course (Eq. 6) into Eq. 11:

$$x = x_0 + Y_{X/P} \cdot \frac{p_0 \cdot p_f \cdot e^{p_0' \cdot (t - t_P)}}{p_f - p_0 + p_0 \cdot e^{p_0' \cdot (t - t_P)}} \qquad /13/$$

The values of the parameters p_0, p_f, p_0' and t_P were deduced from the fitting of ammonium production time course (Eq. 6).

Results and Discussion

Some representative amino acids from the different groups previously characterized for *P. camemberti* and *G. candidum* were chosen. Threonine was chosen among the amino acids used only as nitrogen source by *G. candidum* (*5*), and lysine was chosen among the amino acids used as nitrogen source and also dissimilated by *G. candidum* (*5*) and *P. camemberti* (*1*), namely used for energy supply by oxidation into CO_2 during stationary phase, after glucose depletion.

Some amino acids lead to diauxic growth, since after glucose depletion they can be used as nitrogen and carbon sources; arginine was chosen among them. A difference should however be noted between growth kinetics recorded for *G. candidum* and *P. camemberti*. Indeed, a continuous and sequential use of both carbon substrates, glucose and a particular amino acid, was recorded during *P. camemberti* growth (*1*); while after glucose depletion, a clear stationary phase was recorded before the assimilation of the chosen amino acid as both carbon and nitrogen source by *G. candidum* (*5*). The higher level of enzymatic activity of *P. camemberti* if compared to *G. candidum* (*19,20*) can account for this difference in behaviour.

P. camemberti exhibited a clear differentiation between the assimilated and the dissimilated carbon for the last group of amino acids, including glutamic acid (*1*).

Glucose and ammonium time courses are displayed in Figs. 1a–e for the used amino acids, as well as the corresponding fits using Eqs. 8 and 6. As observed, the model matched experimental glucose consumption and ammonium production data. The corresponding parameter values are collected in Table 1. It can be noted that ammonium production during *P. camemberti* growth on lysine and glucose was not fitted, due to the total uncoupling between growth and ammonium production (Fig. 1b). Indeed, ammonium release during stationary state resulted from lysine dissimilation for energy supply (*1*).

In the case of a clear differentiation between the assimilated and the dissimilated carbon, as was the case during *P. camemberti* growth on glutamate and glucose, the latter was only dissimilated for energy supply and was therefore not involved in the growth model (Eq. 12). Its consumption was therefore not fitted (Fig. 1e).

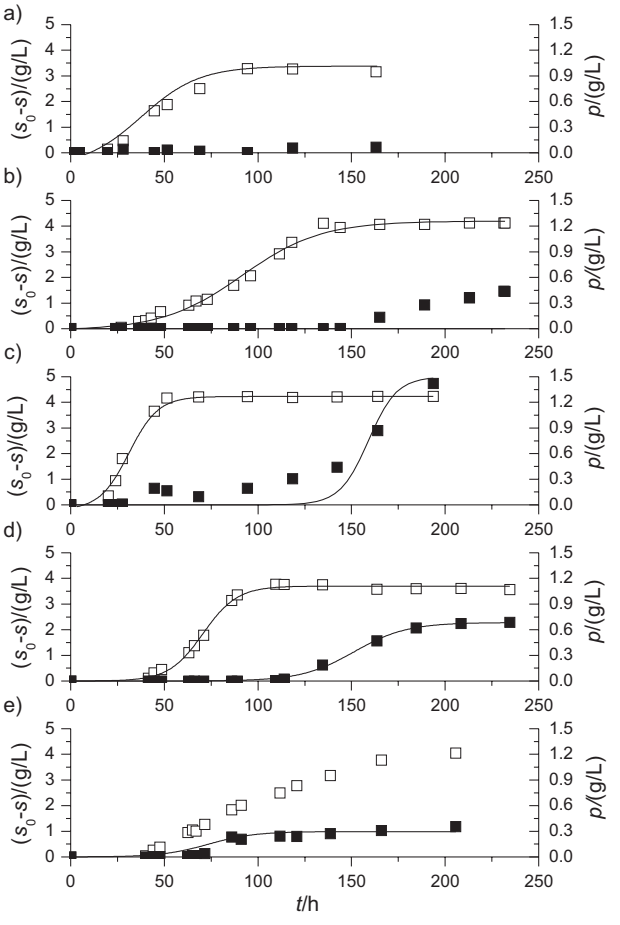

Fig. 1. Experimental (symbols) and calculated (continuous lines) time courses for carbon substrate (glucose) consumption, s_0–s (□) and ammonium, p (■) concentrations recorded during growth of *G. candidum* on threonine (a), *P. camemberti* on lysine (b), *G. candidum* on arginine (c), *P. camemberti* on arginine (d) and *P. camemberti* on glutamate (e)

Biomass concentration was plotted *vs.* ammonium production in Figs. 2a–e. This plot appears to be an interesting tool to characterize amino acid metabolic behaviour. Indeed, growth on amino acids used only as nitrogen source was characterized by an absence of ammonium production (Fig. 2a), or by a total uncoupling with growth leading to a nil value of the $Y_{X/P}$ yield in the case of amino acid dissimilation during stationary

Table 1. Optimised parameters given by the glucose consumption (Eq. 8), ammonium production (Eq. 6) and growth (Eqs. 10, 11 and 13) models for batch cultures of *G. candidum* and *P. camemberti* growing on a given amino acid- and glucose-based media, as described in the Materials and Methods section

	Medium*	s_0 g/L	s_{res} g/L	s_0' h^{-1}	t_S h	$Y_{X/S}$ g/g	p_0 g/L	p_f g/L	p_0' h^{-1}	t_P h	$Y_{X/P}$ g/g	R^2
Gc	Thr	3.94	0.56	0.065	9.9	0.31	–	–	–	–	–	0.92
Pc	Lys	4.25	0.055	0.047	0	0.47	–	–	–	–	–	0.96
Gc	Arg	4.43	0.20	0.128	7.1	0.47	0.008	1.50	0.132	82.6	0.79	0.96
Pc	Arg	3.70	0.002	0.109	0.001	0.75	0.0002	0.68	0.071	40.3	0.98	0.99
Pc	Glu	–	–	–	–	–	0.0008	0.30	0.081	0.8	11.05	0.96

*All media contained the selected amino acid and glucose
Pc=*Penicillium camemberti*
Gc=*Geotrichum candidum*

phase (Fig. 2b). When the amino acid can be assimilated as a carbon source in addition to being used as a nitrogen source, ammonium production during the second phase of growth leads to low $Y_{X/P}$ yields during this phase, 0.79 and 0.98 g/g (Table 1) for *G. candidum* (Fig. 2c) and *P. camemberti* (Fig. 2d), respectively. Contrarily, high $Y_{X/P}$ yield (11.05 g/g; Table 1) was recorded in the case of an assimilation of the carbon from the amino acid by *P. camemberti*, with only the carbon from glucose being dissimilated (Fig. 2e).

Biomass yield on glucose, $Y_{X/S}$, is also displayed in Fig. 2.

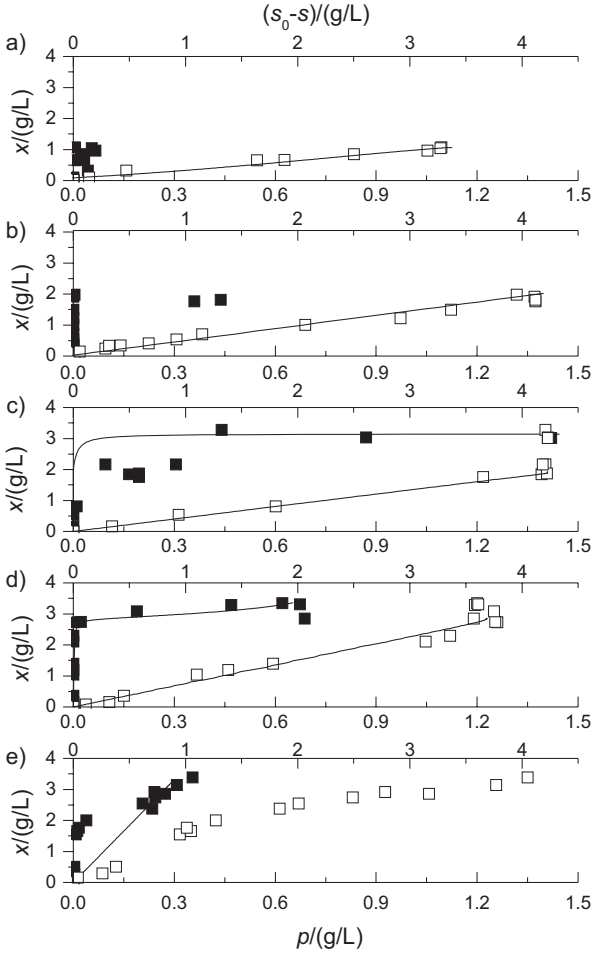

Fig. 2. Experimental (symbols) and calculated (continuous lines) biomass yield on carbon substrate $Y_{X/S}$ (□) and biomass yield on ammonium $Y_{X/P}$ (■) recorded during growth of *G. candidum* on threonine (a), *P. camemberti* on lysine (b), *G. candidum* on arginine (c), *P. camemberti* on arginine (d) and *P. camemberti* on glutamate (e)

Considering the parameter values given by the fitting of glucose consumption and ammonium production time courses (Fig. 1), biomass data were calculated and compared to experimental data in Figs. 3a–e. As observed, the model matched experimental data until stationary phase. It should be observed that to optimize the fitting (correlation coefficients always above 0.9), $Y_{X/S}$ and $Y_{X/P}$ yields were optimized during growth fitting and then displayed in Fig. 2 to be compared to experimental data for validation.

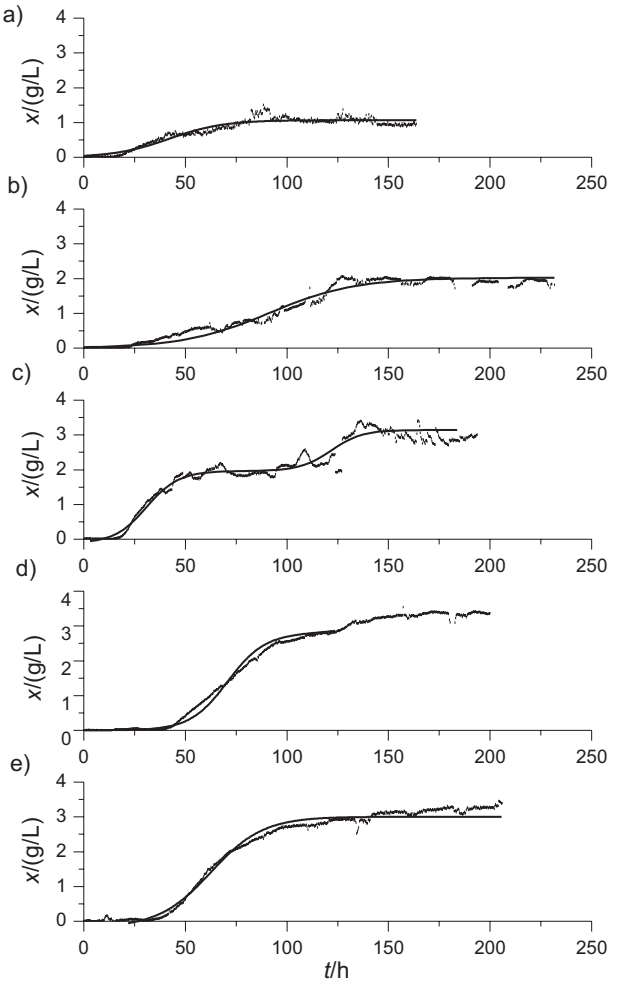

Fig. 3. Experimental (symbols) and calculated (continuous lines) biomass concentration time courses recorded during growth of *G. candidum* on threonine (a), *P. camemberti* on lysine (b), *G. candidum* on arginine (c), *P. camemberti* on arginine (d) and *P. camemberti* on glutamate (e)

In the absence of amino acid assimilation as a carbon source for biosynthesis leading to an absence of ammonium production during growth, only the carbon substrate was involved in the growth model (Eq. 10). The low *G. candidum* growth recorded on threonine as a nitrogen substrate (Fig. 3a) accounted for the low biomass on glucose yield $Y_{X/S}$ (0.31 g/g; Fig. 2a), if compared with the growth recorded during *P. camemberti* culture on lysine as a nitrogen source ($Y_{X/S}$=0.47 g/g; Fig. 2b). It can be noted that both species led to similar $Y_{X/S}$ yield (about 0.5 g/g for *G. candidum* (5)) during growth on amino acids from the second group, namely dissimilated for energy supply during stationary phase, as was the case for lysine. As expected, low values were recorded for the parameter t_S (Table 1), which characterized the time lag before the beginning of the carbon substrate consumption, since glucose was the sole 'usable' carbon substrate for biosynthesis, *viz.* 0 and 9.9 h during *P. camemberti* growth on lysine and *G. candidum* growth on threonine, respectively.

Amino acid assimilation as carbon sources resulted in ammonium production, which was therefore involved in the growth model, leading to Eq. 11 in the case of

diauxic growth and to Eq. 13 in the case of a clear differentiation between the assimilated and the dissimilated carbon, owing to the dissimilation of glucose for energy supply.

The use of glutamate as a carbon source during *P. camemberti* growth led to ammonium production from the beginning of growth (Eq. 13), leading to a low value of the time lag parameter t_P (0.8 h; Table 1). Contrarily, amino acid assimilation as a carbon source after glucose depletion led to a concomitant production of ammonium, and then high values of the time lag parameter t_P (Table 1), while the corresponding t_S values were low (7.1 and 0.001 h for *G. candidum* and *P. camemberti* respectively; Table 1), owing to the assimilation of glucose during the first phase of growth. In the case of diauxic growth, examination of the parameter values can characterize the difference in metabolic behaviour between *G. candidum* and *P. camemberti*, since the clear stationary phase between the two growth phases recorded during *G. candidum* culture led to a clearly higher t_P value (82.6 h) if compared to that recorded during *P. camemberti* growth (40.3 h; Table 1). The clear second *G. candidum* growth phase led also to a higher amount of amino acid assimilated (*1,5*), resulting in a higher amount of ammonium produced (1.5 g/L) if compared to *P. camemberti* (0.7 g/L; Table 1).

Conclusion

The plot of biomass concentration *vs.* ammonium production appeared to be an interesting tool to characterize amino acid metabolic behaviour, since it varied from an absence or a total uncoupling of growth in the case of only nitrogen source amino acids to high biomass yield on ammonium in the case of amino acid assimilation with the carbon from glucose being only dissimilated.

Ammonium production and substrate consumption time courses were successfully described by means of a Verhulst model modified by adding a parameter that accounted for a possible time lag before the beginning of carbon substrate consumption, namely the used amino acid (leading to ammonium release) or glucose. Considering the parameter values given by the fitting of glucose consumption and ammonium production time courses, biomass data were calculated and were found to match experimental data until stationary phase for the four observed metabolic behaviours.

Nomenclature

P, p	the metabolite, ammonium, and its concentration (g/L)
p_0'	the ratio between the initial ammonium rate $r_{p,0}$ and its initial concentration p_0
r	volumetric rate (g/(L·h))
S, s	the carbon source, glucose, and its concentration (g/L)
s_0'	the ratio between the initial glucose consumption rate $r_{s,0}$ and its initial concentration s_0
t	time (h)
t_P, t_S	time lag before the beginning of the substrate consumption (h)
X, x	biomass and its concentration (g/L)
$Y_{X/S}$	biomass on glucose yield (g/g)
$Y_{X/P}$	biomass on ammonium yield (g/g)
Y_{X/Z_C}	biomass on C source amino acid yield (g/g)
Z, z	the nitrogen source, the considered amino acid, and its concentration (g/L)

Subscripts

C	carbon
0	initial
f	final
P	ammonium
max	maximum
res	residual
S	glucose

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