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ORIGINAL PAPER

Modeling growth, substrate consumption and product formation of *Penicillium nalgiovense* grown on meat simulation medium in submerged batch culture

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Abstract *Penicillium nalgiovense* is the most widely used starter mold for cured and fermented meat products. The development of a biomass film on the surface of these products prevents a large degree undesirable growth of various fungal contaminants and contributes to the ripening process with production of metabolites. This work presents an attempt to model the growth of *P. nalgiovense* and to relate it to substrate consumption and product release. Because of the extremely complex nature of the meat product fermentation, submerged culture was employed in a bioreactor system that enabled on-line monitoring, using a meat simulation medium, which contained peptones and lactate as carbon, nitrogen and energy sources. The unstructured model presented is based on a partial association of substrate assimilation and product formation with growth. Experimentally derived values for peptones and lactate were compared with model-derived values and their proportions corresponding to growth associated parts, used for biosynthesis, and non-growth associated parts, used for maintenance. The model was applied for the products ammonia, carbon dioxide and protons. Both peptones and lactate were used mainly for biosynthesis (85 and 80% of the total amounts provided, respectively). Assimilation of lactate and ammonia formation from amino acid metabolism resulted in a proton exchange, which was mainly growth associated. The contribution of the growth associated mechanism to the total proton exchange was estimated to be 75% while the contribution of the non-growth associated mechanism increased during the growth phase and reached a maximum of 25%. For carbon dioxide production, the contribution of a maintenance mechanism was evident at 40 h, while production was growth-associated and remained such even at the end of fermentation at 168 h when growth rate was very low. The partially growth associated model showed good agreement with the experimental data and allows accurate determination of the proportions of substrates or products related to biosynthesis and cell maintenance.

Keywords *Penicillium nalgiovense* · Modeling · Biosynthesis · Maintenance · Meat simulation medium

List of symbols

k_1, k_2	growth- and non-growth-associated coefficients. k_1 is dimensionless, k_2 (h^{-1})
K	general term denoting the concentration of either lactate or peptones
F	air-flow rate ($l\ h^{-1}$)
h	proton concentration ($g\ l^{-1}$)
c	lactic acid concentration ($g\ l^{-1}$)
n	ammonia concentration ($g\ l^{-1}$)
p	carbon dioxide concentration ($g\ l^{-1}$)
P_{gas}	carbon dioxide concentration in the gaseous phase ($g\ l^{-1}$)

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P_L carbon dioxide concentration in the liquid phase (g l^{-1})
P_{exp} experimental total mass of produced carbon dioxide (g) $(P\exp) = F\int p\,dt$
P_{mod} model derived total mass of produced carbon dioxide (g) $(P\mathrm{mod}) = k_1 V_L(x - x_0) + k_2 V_L \int x\,dt)$
r_{Pgas} rate of carbon dioxide production in the gaseous phase ($r_p = dP_{gas}/dt$) (g l^{-1} h^{-1})
r_{P_L} rate of carbon dioxide production in the liquid phase (g l^{-1} h^{-1})
t time (h)
x biomass concentration (g l^{-1})
r_x growth rate (dx/dt) (g l^{-1} h^{-1})
V_{gas} Volume of gaseous phase (l)
V_L volume of liquid phase (l)
z peptones concentration (g l^{-1})

Introduction

The filamentous fungus *Penicillium nalgiovense*, a terto quaterverticillate species of the genus *Penicillium*, is a frequently used starter culture for mold-ripened foods [15]. *P. nalgiovense* was originally isolated from cheese [15] and cured meat products [7]. In Spain, France, Switzerland, Germany, Hungary, and some other European countries, dry sausages are usually ripened with molds. The development of molds on the surface of these sausages is required before they are considered as cured [16]. *P. nalgiovense* is the most widely used starter for cured and fermented meat products [7, 15]. It is usually applied as conidia on the surface of the product at the beginning of the ripening process, and it gives a typical homogeneous, white appearance and distinctive odor and flavor to fermented meat products. The fast and homogeneous development of the biomass film on the surface of the product prevents a large degree undesirable growth of various fungal contaminants [6] and contributes to the ripening process with production of proteases [8]. However, despite its widespread use, *P. nalgiovense* produces secondary metabolites like the antibiotic penicillin [5] and isocoumarins [12].

Literature information on *P. nalgiovense* is very limited and mainly focused on the ability of the fungus to produce penicillin and the development of genetic tools and strategies for the production of improved and safer starter strains [5, 6, 10, 11]. This work presents an attempt to model the growth of *P. nalgiovense* and to relate it to substrate consumption and product release. Because of the extremely complex nature of the meat product fermentation, the parameters of which are very difficult to be monitored, submerged culture was employed in a bioreactor system that enabled on-line monitoring and control. Experiments were conducted using a meat simulation medium proposed by Verluyten et al. [17], which contained peptones and lactate as carbon, nitrogen and energy sources.

Submerged culture has been the tool of choice in a number of studies on the physiology of various cheese starter cultures. For example, liquid media that simulated the aqueous phase of a Camembert cheese during ripening were employed in the studies of Boutrou et al. [3], Amrane et al. [2], and Adour et al. [1]. On-line monitoring of biomass in bioreactor cultures and off-gas analysis permitted the development of unstructured models by Amrane et al. [2], Couriol et al. [4], and Adour et al. [1], which described successfully the cellular metabolism of *Geotrichum candidum* and *P. camembertii*, the two fungal species that regulate the ripening of soft Camembert cheese.

In this study, *P. nalgiovense* was cultivated in a 10 l stirred tank bioreactor system that enabled on-line measurement of a large number of fermentation variables, e.g., pH, DOT (dissolved oxygen tension), off-gas O_2 and CO_2 partial pressures, and NH_4^+ concentration. Experimental data permitted the extraction of information on kinetics of biomass production, peptones and lactate assimilation, proton transfer, as well as ammonia and CO_2 formation. From this information, an unstructured model was developed based on a partial association of substrate assimilation and product formation with biomass production.

Materials and methods

Microorganism and medium

The strain *Penicillium nalgiovense* Laxa MP2, isolated from the surface of Greek fermented meat sausage, was used throughout this work. The culture was maintained on Mycological Agar plates, while freeze dried-spores were stored at 4°C. For bioreactor cultures, a meat simulation medium was used with the following composition (per liter), according to Verluyten et al. [17]: bacteriological peptone, 20 g; Lab Lemco, 16 g; yeast extract, 8 g; $MgSO_4{\cdot}7\ H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.038 g; lactic acid (sterilized separately), 5 g; NaCl, 40 g; $NaNO_2$, 0.005 g; and Tween 80, 1 ml. A stock solution of $NaNO_2$ (10 g l^{-1}) was sterilized separately by microfiltration (Acrodisc; Pall Gelman Sciences, Ann Arbor, MI). The amount of $NaNO_2$

added was representative of residual nitrite levels encountered in fermented sausage, since nitrite is rapidly depleted when added to the sausage batter [17]. Following sterilization, pH was adjusted at 5.8 with addition of 2 M NaOH.

Culture conditions

The stirred tank bioreactor used in this work was a New Brunswick Scientific BIOFLO 410, with a working volume of 10 l. The agitation system consisted of three disc turbine impellers, 8 cm in diameter, with six flat blades, operating at the stirrer speed of 200 rpm. The bioreactor was equipped with a polarographic oxygen sensor (Mettler Toledo, Urdorf, Switzerland), a pH probe (Ingold, Infit, England), and on-line facilities for off-gas analysis (EX-2000, New Brunswick Scientific, Edisin, NJ, USA), which included a paramagnetic oxygen analyzer and an infrared carbon dioxide analyzer. Process temperature was maintained at 25°C. The air-flow rate was maintained at 1 vvm (1 l of air per liter of reactor working volume per minute). The bioreactor was inoculated directly with spores the amount of which corresponded to an initial density of $2–3 \times 10^8$ spores per liter. The spores were collected aseptically from plates, incubated for 5 days at 25°C, and left to rehydrate for 1 h in the sterilized media at ambient temperature before inoculation.

Assays

Cell dry weight (biomass, x) was determined by filtering 10 ml of broth through pre-weighed glass fiber filters (grade GF/C, 4.25 cm, Whatman International, Maidstone, UK), washing and drying them in a microwave oven (15 min at low power), and leaving them in a desiccator for 24 h before reweighing. Lactic acid concentration was determined with the EnzyPlus D/L Lactic Acid kit by Diffchamb AB (Diffchamb, Sweden). The concentration of ammonium ions in solution was calculated using an ammonium electrode (Asea Brown Boveri/Kent Taylor 8002-8) as described earlier [14]. Total nitrogen was determined according to Hach et al. [9]. Peptone concentration was calculated by subtracting the ammonium ion concentration from the total nitrogen concentration. Proton concentration was calculated from pH measurements. Buffering capacities of the medium were measured by automatic titration with 2 M NaOH in the bioreactor, filled with sterile medium and in full operation under the particular culture conditions. On-line measurements of pH variations ensured accurate conversion into changes in proton concentration.

Fungal morphology

Fungal morphology in submerged culture was characterized by using an automatic image analysis system consisting of an Olympus microscope (Olympus, New Hyde Park, NY, USA) operated as phase contrast, a CCD camera (Sony, Cambridge, UK), a PC with a frame-grabber, and an image analysis software (SIS, Olympus, Germany). Within 30 h from inoculation, the young hyphae formed microscopic clumps. Mycelial 'clumps' are stable particles of intertwined filaments around a small core. They lack the characteristic compact structure of pellets, while they represent the main morphological type for many filamentous fungal fermentations. The preparation of the samples and the measurements were as described in an earlier publication [13]. A magnification of 100× was applied for measurements of mean perimeters of clumps (morphology parameter P, μm).

Mathematical modeling

The software MATLAB® 7.1 (The MathWorks Inc., Munich, Germany) was used for simulation and modeling.

Results and discussion

The bioreactor was inoculated with spores of *Penicillium nalgiovense*. Germination was apparent 12 h later and small mycelial aggregates in the form of clumps appeared around 30 h from inoculation. In later stages of fermentation, the form of mycelial clumps predominated over free filaments and a very small number of pellets. Mean perimeters of mycelial clumps ranged between 400 and 900 μm and declined steadily during the first half of fermentation, but appeared almost stable during the second half (results not shown). Biomass concentration reached 9 g l^{-1} at the end of fermentation (168 h).

Modeling aspects

The sources of carbon and nitrogen in the applied meat simulation medium are lactic acid and peptones, respectively. Both represent energy sources and are involved in both biosynthesis and cell-maintenance. It can be assumed therefore that assimilation of each is

partially associated with growth and can be expressed in the form of the following equation as:

$$-\mathrm{d}K/\mathrm{d}t = k_1\mathrm{d}x/\mathrm{d}t + k_2x, \quad (1)$$

where K is a term denoting the concentration of either lactate or peptones, x is the concentration of biomass, and k_1 and k_2 are growth- and non-growth-associated coefficients. Equation 1 can be rearranged as:

$$-\mathrm{d}K/(\mathrm{d}t \cdot k_1) = (\mathrm{d}x/\mathrm{d}t) \cdot (k_2x/k_1). \quad (2)$$

Both lactate consumption and the deamination of amino acids contribute to the concentration of protons, the uptake of which is partly associated with growth. Similarly, partly associated with growth can be the product of amino acid metabolism, ammonia. Thus, similar equations can be written to describe the relationship between biomass and proton concentration and, from the side of products, between biomass and the concentrations of ammonia and carbon dioxide:

$$-\mathrm{d}h/\mathrm{d}t = (k_1\mathrm{d}x/\mathrm{d}t) + k_2x, \quad (3)$$

where h is the concentration of protons, and

$$\mathrm{d}n/\mathrm{d}t = (k_1\mathrm{d}x/\mathrm{d}t) + k_2x, \quad (4)$$

where n is the concentration of ammonia, and

$$\mathrm{d}P_L/\mathrm{d}t = (k_1\mathrm{d}x/\mathrm{d}t) + k_2x, \quad (5)$$

where P_L is the concentration of dissolved carbon dioxide.

Since during the batch run, P_L is only a part of the continuously produced carbon dioxide which is then transferred to the head-space of the reactor and to the gas exit, the mass balance equation for carbon dioxide is:

$$V_L \times \mathrm{d}P_L - FP_{gas} \times \mathrm{d}t = V_{gas} \times \mathrm{d}P_{gas}, \quad (6)$$

where V_L is the liquid volume, F is the air-flow rate, V_gas is the volume of the gaseous phase (head-space of the reactor) and P_gas is the carbon dioxide concentration in the head-space.

Combining Eqs. 5 and 6, the following form is derived:

$$F \times P_{gas} + [(V_{gas} \times \mathrm{d}P_{gas})/\mathrm{d}t] = V_L \times (k_1\mathrm{d}x/\mathrm{d}t + k_2x). \quad (7)$$

If the carbon dioxide mass in the reactor head-space is neglected ($V_\mathrm{gas} \times \mathrm{d}P_\mathrm{gas}/\mathrm{d}t$), the growth rate of the organism can be derived from:

$$(F \times P_{gas} - V_L \times k_2x)/k_1 = \mathrm{d}x/\mathrm{d}t. \quad (8)$$

Equations 1–5 are first order differential equations with constant coefficients k_1 and k_2. These coefficients can be calculated by multiple linear regression of Eq. 7, while Eq. 8 can be solved with numerical integration. The logistic function applied was:

$$\alpha = \gamma + \frac{\beta - \gamma}{1 + (\frac{t}{\mathrm{d}+1})^e}, \quad (9)$$

where α is the concentration of produced biomass or metabolite, or the concentration of consumed substrate or protons. β, γ, d, and e are constants.

Simulation results

Figures 1, 2, 3, 4, 5, 6 show the plots corresponding to experimentally and model-derived data for biomass production, peptone consumption, lactate consumption, proton transfer, ammonia production, and carbon dioxide production, respectively. The relationship between biomass kinetics and the kinetics of peptones and lactate consumption, proton transfer, ammonia, and carbon dioxide production, for both experimental and model-derived biomass concentration values, is shown in Fig. 1. Application of the partially growth-associated model of Eqs. 1 and 5 showed good agreement with experimental data and, in particular, biomass prediction by carbon dioxide emission (Fig. 1d). The growth- and non-growth-associated coefficients k_1 and k_2 derived from multiple linear regression on Eqs. 1 and 5 are shown in Table 1. The respective proportions of substrates used for biosynthesis and maintenance and the proportions of products associated with biosynthesis and maintenance can be deduced from Eqs. 1 and 5. These are $k_1(x(x_0)$, and $k_2 \int x\ \mathrm{d}t$, for biosynthesis and cell maintenance, respectively.

Peptone consumption commenced approximately 25 h after inoculation. As peptones were the sole source of nitrogen, their utilization during growth was mostly directed toward biosynthesis and this is shown in Fig. 2. Utilization of peptones for cell maintenance was significant after 50 h and approximated a 15% of the total amount of initial peptone concentration (Fig. 2). Lactate consumption was in parallel with peptones consumption and similarly lactate utilization for cell maintenance became significant after about 60 h (Fig. 3). According to Fig. 3, the amount of lactate utilized for cell maintenance did not exceed 20% of the amount provided with the medium and this is indicative of lactate utilization mainly for biosynthesis.

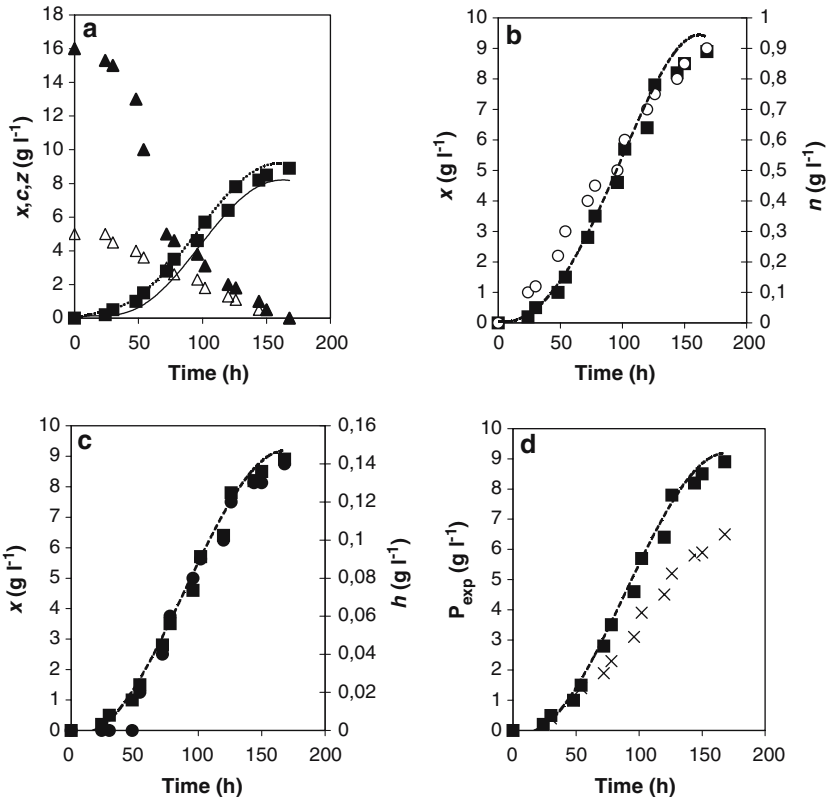

Fig. 1 **a** Biomass (*x*, *filled square*), peptones (*z*, *filled triangle*) and lactate (*c*, *open square*) concentrations time-courses. The dashed line represents the model-derived biomass based on the kinetics of peptone assimilation. The *solid line* represents the model-derived biomass based on the kinetics of lactate assimilation. **b** Time-course of ammonia release (*n*, *open circle*) and experimental (*x*, *filled square*) and model-derived (*dashed line*) biomass based on the kinetics of ammonia formation. **c** Proton concentration (*h*, *filled circle*) time-course and experimental (*x*, *filled square*) and model-derived (*dashed line*) biomass based on the kinetics of proton transfer. **d** Time-course of total mass of produced carbon dioxide (P_{exp}, *x*) and experimental (*x*, *filled square*) and model-derived (*dashed line*) biomass based on the kinetics of carbon dioxide production

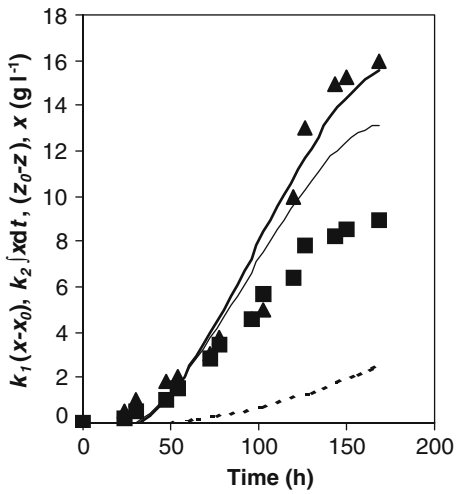

Fig. 2 Model-derived amounts of peptones assimilated for biosynthesis [*solid line*, $k_1(x–x_0)$], cell maintenance [*dashed line*, $k_2 \int x\, dt$] and the sum of both (+). Also shown, experimental values for peptone (*z*, *filled triangle*) and biomass (*x*, *filled square*) concentrations

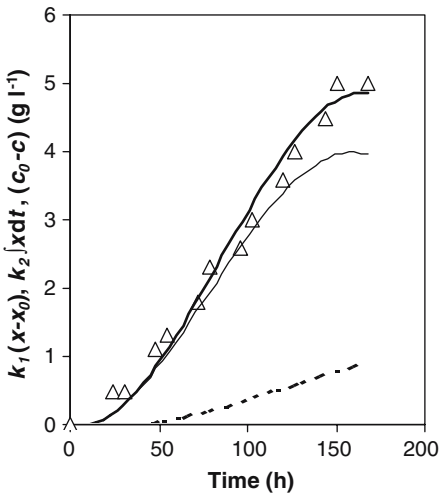

Fig. 3 Model-derived amounts of lactate assimilated for biosynthesis [*solid line*, $k_1(x–x_0)$], cell maintenance [*dashed line*, $k_2 \int x\, dt$] and the sum of both (thick solid line). Experimentally derived values for lactate concentrations (*c*, *open triangle*) are also shown

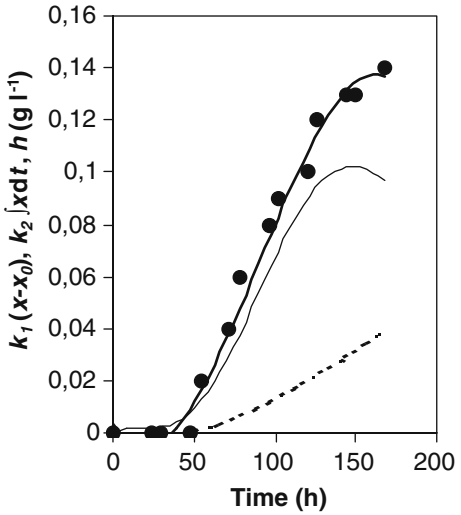

Fig. 4 Model-derived proportions of protons transferred for biosynthesis [*solid line*, $k_1(x–x_0)$], cell maintenance [*dashed line*, $k_2 \int x\, dt$] and the sum of both (*thick solid line*). Experimentally derived amounts of proton concentrations (*h*, *filled circle*) are also shown

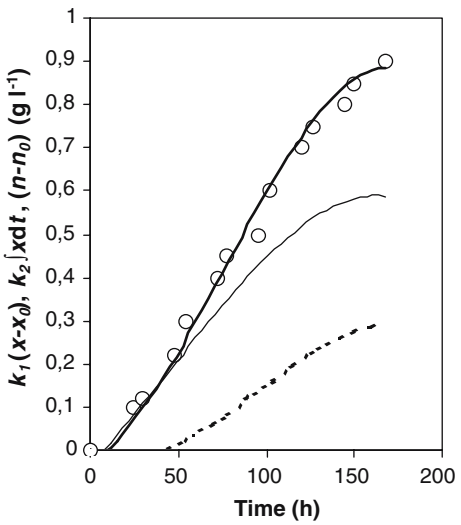

Fig. 5 Model-derived amounts of ammonia resulting from biosynthesis [*solid line*, $k_1(x–x_0)$], cell maintenance [*dashed line*, $k_2 \int x\, dt$] and the sum of both (*thick solid line*). Experimentally derived values of ammonia concentrations (*n*, *filled circle*) are also shown

As shown in Fig. 1, ammonia production, and therefore peptone consumption, was commenced at 24 h of fermentation, while a pH increase was noted at the same time. According to Fig. 4, the contribution of a maintenance mechanism in proton transfer started after approximately 50 h from inoculation. Lactate utilization for cell maintenance was significant at that time and lactate metabolism contributed to proton transfer. The contribution of the growth-associated

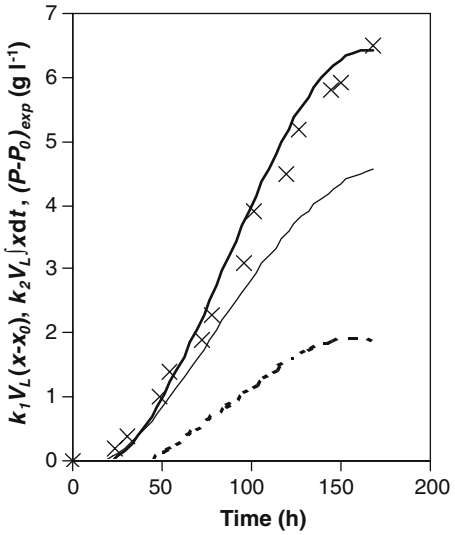

Fig. 6 Model-derived amounts of carbon dioxide resulting from biosynthesis [*solid line*, $k_1V_L(x–x_0)$], cell maintenance [*dashed line*, $k_2V_L \int x dt$] and the sum of both (*thick solid line*). Experimentally derived values of carbon dioxide concentrations (P_{exp}, *x*) are also shown

Table 1 Multiple linear regression on Eqs. 1 and 5 yielded the values of coefficients k_1 and k_2

Medium component	k_1 (h^{-1})	K_2 (h^{-1})
Peptones	0.80	1.55×10^{-3}
Lactate	0.91	3.91×10^{-3}
Protons	11.80×10^{-3}	6.00×10^{-5}
Ammonia	0.08	2.10×10^{-4}
Carbon dioxide	1.31	9.21×10^{-3}

mechanism to the total proton exchange was estimated to be 75% while the contribution of the non-growth associated mechanism increased during the growth phase and reached a maximum of 25%. As expected, the main proportion of ammonia was produced through a growth-associated mechanism, while a non-growth associated mechanism contributed to ammonia production from 50 h in fermentation (Fig. 5).

Carbon dioxide production was evident during germination of spores (12 h). Since peptones consumption was almost zero at that phase, CO_2 production resulted from amino acid assimilation. The total mass yield for CO_2 from biosynthesis and cell maintenance is shown in Fig. 6. The contribution of a maintenance mechanism was evident at 40 h (Fig. 6). CO_2 production was growth-associated and remained such even at the end of fermentation at 168 h when growth rate was very low.

The unstructured model presented is based on a partial association of substrate assimilation and product formation with growth. Experimentally derived

values for substrates peptones and lactate and products ammonia, carbon dioxide and protons were compared with model-derived values and their proportions corresponding to growth-associated parts, used for—or resulted from—biosynthesis, and non-growth associated parts, used for, or resulted from, maintenance. In all cases examined, the partially growth associated model showed good agreement with the experimental data and allows accurate determination of the amounts of substrates or products related to biosynthesis and cell maintenance.

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