

Experimental Investigation and Reaction Kinetics Modeling of Biomass Formation, Substrate Consumption and Product Formation During Start-up of Fixed-Bed Cultures with Immobilized *Lactococcus lactis* ssp. *lactis*

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Abstract

Lactic acid bacteria (LAB) like *Lactococcus lactis* ssp. *lactis* are used in food industry as well as for production of bacteriocins and optically pure lactic acid. Fixed-bed processes are highly productive but mathematical description is often complex. The aim of this work was to establish a mathematical model for process design. Biomass formation, lactose consumption and lactate production during start-up of fixed-bed cultures with immobilized *L. lactis* was investigated experimentally and described by a reaction kinetics model. Appropriate modeling and simulation of fixed-bed processes require biomass data. Therefore, a low-volume multiple fixed-bed reactor system (Multiferm) was used to investigate biomass formation of a *L. lactis* strain during the start-up phase of fixed-bed cultivation. The generation of data in parallel experiments was fast and easily compared to larger single reactor systems. Biomass data obtained from both fractions, retained and free suspended biomass, was used for modeling and simulation, together with data for lactose and lactate. The underlying Luedeking-Piret-like model structure was developed based on the results from suspension cultivations with the same strain. The fixed-bed system was described as perfusion culture with cell retention. For this, merely four additional parameters had to be defined to extend the suspension model to fixed-bed cultures. Experimental trends and steady states of both biomass fractions besides substrate and product could be described very well. Thus, this model could be used for process layout during process development.

Keywords: Fixed-bed reactor; Immobilization; *Lactococcus lactis*; LAB; Luedeking-Piret; Modeling; Start-up

Abbreviations and Symbols: D: Dilution rate (h^{-1}); DMB: Dry matter of biomass ($\text{g}\cdot\text{L}^{-1}$); F: Flow rate ($\text{L}\cdot\text{h}^{-1}$); F_{in} : Inlet flow rate ($\text{L}\cdot\text{h}^{-1}$); F_{out} : Outlet flow rate ($\text{L}\cdot\text{h}^{-1}$); g: Gravitation constant ($9.81 \text{ m}\cdot\text{s}^{-2}$); j: Running index; k_{lys} : Lysis rate (h^{-1}); K_{S} : Substrate saturation constant ($\text{g}\cdot\text{L}^{-1}$); LAB: Lactic acid bacteria; m_{r} : Maintenance rate of retained cells (h^{-1}); n: Number of data points/values; OD: Optical density (-); OD_{600} : Optical density determined at 600 nm (-); P: Product concentration ($\text{g}\cdot\text{L}^{-1}$); PLA: Polylactic Acid; P_{out} : Product concentration at the outlet ($\text{g}\cdot\text{L}^{-1}$); R: Fraction of retained biomass in perfusion fermentation (-); R^2 : Correlation coefficient (-); RSS: Residual sum of squares; S: Substrate concentration ($\text{g}\cdot\text{L}^{-1}$); S_{in} : Substrate concentration at the inlet ($\text{g}\cdot\text{L}^{-1}$); S_{out} : Substrate concentration at the outlet ($\text{g}\cdot\text{L}^{-1}$); TCC: Total cell concentration ($\text{cells}\cdot\text{mL}^{-1}$); TSS: Total sum of squares; V: Volume (L); WLS: Weighted least square sum; w_{xf} : weighting factor for biomass (=4); X_{f} : Free suspended biomass concentration ($\text{g}\cdot\text{L}^{-1}$); $X_{\text{f,out}}$: Free suspended biomass concentration at the outlet ($\text{g}\cdot\text{L}^{-1}$); X_{r} : Retained biomass concentration ($\text{g}\cdot\text{L}^{-1}$); Y_{j} : Concentration of either biomass, substrate or product ($\text{g}\cdot\text{L}^{-1}$); \hat{Y}_{j} : Corresponding simulated value to Y_{j} ($\text{g}\cdot\text{L}^{-1}$); \bar{Y}_{j} : Arithmetic mean of all Y_{j} either biomass, substrate or product ($\text{g}\cdot\text{L}^{-1}$); $Y_{\text{X/S}}$: Biomass yield coefficient from substrate ($\text{g}\cdot\text{g}^{-1}$); α : Growth associated product formation rate ($\text{g}\cdot\text{g}^{-1}$); β : Non-growth associated product formation rate (h^{-1}); γ : Constant for unspecific substrate loss ($\text{L}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$); μ : Specific growth rate (h^{-1}); μ_{max} : Maximum specific growth rate (h^{-1}); η_{p} : Effectiveness factor production (-).

Introduction

Lactic acid bacteria (LAB) are commonly used in the food industry e.g., for fermenting dairy products or vegetables in order to serve as a preserving agent or to change organoleptic properties of the product [1]. Moreover, some of them evidently act as probiotics [2-5]. High-value compounds produced from LAB can be bacteriocins like nisin, which have an antimicrobial effect and are already added to tooth paste, for instance [6-11]. Another product is microbially produced lactic acid or

lactate, which can be provided in an enantiopure manner either as D- or L-lactate [12-14]. Thus, microbial lactate is interesting as a reactant in the PLA (polylactic acid) industry, where the properties of the resulting polymer is strongly dependent on the defined enantiomeric mixture used for polymerization [15].

The Global Industry Analysts Inc. estimated the world production of lactic acid to be 258,000 tons in 2009 and projected it to increase up to 367,000 tons in 2017. Combined with a price of around 1.5 US\$/kg for 88 % purity, lactic acid has the potential to become a high-volume chemical intermediate [16]. However, microbial production processes are not yet feasible to compete with chemically produced lactic acid. One possibility to make these processes more profitable is the use of cheap raw materials [17] and to increase productivity. For the latter, fixed-bed processes, where cells are immobilized within macroporous carriers, are a promising alternative to common processes with suspended cells [18-20]. Immobilization of cells on inert porous carriers is an easy way to retain biomass as described in literature for many applications [20-26]. Due to cell retention it is possible to run fixed-bed reactors in a perfusion mode at a steady state with dilution rates higher than the maximum specific growth rate of the used strain.

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By this, a very high volume specific productivity with respect to lactate can be reached and maintained for long periods of time. We recently reported on fixed-bed cultures of *L. lactis*, where the volume-specific lactic acid production rate was up to 30 times higher as in batch culture, and continuous cultivation could be maintained for approx. 50 days and a successful scale-up to 1 L-fixed-bed-column with perfusion rates of up to approx. 35 L per day [27]. Fixed-bed systems can be scaled up to industrial scale by changing the flow pattern from radial to axial perfusion [28].

Nevertheless, the number of industrial fixed-bed processes is quite small. This is due to some extent to the lack of process development tools for fixed-bed processes. A further complicating factor is that fixed-bed processes cannot be designed as easy as processes with homogeneously suspended cells. Especially reaction kinetics modeling of biomass formation, substrate consumption and product formation during start-up of fixed bed cultures with immobilized cells has hardly been addressed so far [27]. A description of this initial transient by an appropriate model could be used for prediction of the steady state of fixed-bed processes regarding time and concentrations. Furthermore, influences like changing substrate concentration, dilution rate and initial cell concentration could be simulated. Thereby, the number of experiments could be reduced. The latter one is important during process development to reduce costs because experiments are extraordinarily expensive in case of fixed-bed processes due to long running periods and high dilution rates.

One problem for description and thus modeling of fixed-bed processes using an immobilization matrix is the tedious assessment of retained biomass, which is essential to investigate the time course of biomass formation during start-up and steady state. Sampling is difficult, because cells settle within carriers and only free suspended biomass could be withdrawn from the system. Even if this problem would be addressed somehow, this sample would not be representative for the whole system since fixed-bed reactors are non-homogeneous systems. Attachment of cells within the immobilization matrix can dramatically hinder sampling, especially when solid, inert carriers are used. In homogeneities such as gradients over perfusion height further contribute to this problem. Hence, total retained biomass can only be determined accurately, if the fixed-bed reactor is dismantled and the whole biomass is separated from the carriers and analyzed. As this implies the end of cultivation, a large number of fixed-bed runs would be required to determine the time course of biomass formation within the fixed-bed.

For this purpose, the multi-fixed-bed bioreactor “Multiferm” provides an ideal downscaled system for the development of fixed-bed processes with immobilized microorganisms or cells. It consists of twelve single fixed-bed units that can be operated independently [29]. As experiments can be run in parallel, it offers the possibility to determine the progress of biomass formation by analyzing some of the fixed-bed units at different time points. These data were used here to investigate the development of biomass during transient initial phase until a steady state was reached and link these to data on substrate consumption and product formation.

Furthermore, a reaction kinetics model for the fixed-bed culture was set-up based on a model for suspension culture. Modeling approaches from literature showed that the growth behavior of *L. lactis* can be described appropriately by Monod growth kinetics [30]. Concerning homofermentative lactic acid production from LAB in suspended fermentations, the Luedeking-Piret model is the most predominantly used relation in the literature since its first postulation

[31]. This model employs two empirical constants α and β that connect the production of lactic acid to growth and biomass, respectively. Most LAB, including *L. lactis*, show a behavior leading to values of α which are approximately one order of magnitude larger than those for β indicating a strong link of product formation to growth rather than maintenance metabolism [32].

Luedeking and Pirets relation was a starting point for many researchers when developing unstructured models for lactic acid fermentations with homofermenters. Jørgensen and Nikolajsen [33] developed a set of differential equations to predict fermentations of *L. lactis* in both, batch and continuous cultivations. Substrate consumption is related to growth via a yield coefficient $Y_{X/S}$ and in addition, a term for microbial maintenance m reflects a specific substrate consumption, which is governed by other cellular processes than growth and proportionally dependent on the current biomass concentration. The Luedeking-Piret expression is then modified by the introduction of a negative constant γ expressing reduced product formation in the presence of high sugar levels, which was originally claimed to improve the model in continuous mode.

However, despite its widely distributed application, the Luedeking-Piret equation remains challenging to apply for other cultivation modes than batch operation due to the strong dependence of the growth and non-growth associated constants on the culturing condition. Thus, Zacharof and Lovitt [34] suggested the use of yield coefficients for both, substrate and production rates and furthermore, introduced a term for product inhibition into the specific growth rate. This model also considers microbial maintenance. With adequate extensions of the balance equations, this model was successfully tested in batch, continuous and membrane bioreactor systems.

In order to identify a suitable model for different fermentation modes with *L. lactis* ssp. *lactis* in this work, both approaches were used as starting point for model set-up. Moreover, since none of the said models is reportedly used to describe immobilized LAB fermentation, further assumptions were required. Since in this work *L. lactis* was cultivated in a fixed-bed on macroporous carriers, where biomass is retained to some extent within the inner structures of the carriers and between them, suspended and retained biomass had to be considered separately. For this, some authors have employed different interpretations of the dimensionless, internal effectiveness factor η , which ranges from 0 to 1 and was originally postulated by Thiele to describe reaction rates affected by diffusion limitations within carriers. Up to date, this factor has successfully been used for immobilized ethanol fermentations with yeasts wherein η was used in different works to describe the efficiency of the immobilized biocatalyst versus free biocatalyst with respect to biomass formation [35], substrate consumption [36] and ethanol production [35,37].

Materials and Methods

Bacterial strain

In this study the type strain *Lactococcus lactis* ssp. *lactis* (DSM-No. 20481), in the following denoted as *L. lactis*, was used. It is a homofermentative strain producing exclusively L-lactate under mesophilic growth conditions (30°C, pH 7). As it requires a broad spectrum of nutrients, the complex M17 broth (Difco™, BD) was used, which was supplemented with lactose to a final concentration of 5 g·L⁻¹. The medium and the lactose solution were autoclaved separately to avoid the decrease of lactose concentration due to Maillard’s reaction.

Since *L. lactis* is anaerobic and aerotolerant, there was no aeration installed. Thus, also low agitation rates were sufficient to homogenize

the broth. Mentioned conditions regarding medium, temperature, pH and aeration were used for all experiments.

Cultivation

Inoculum preparation: A new preculture was prepared in baffled shaking flasks (250 mL nominal volume) from cryostock for every experiment in the Multiferm. For this purpose, a cryovial containing 10^9 cells in 1.5 mL freezing medium (M17 broth with 10 % (v/v) glycerol) was thawed and transferred to 50 mL of pre-warmed M17 broth to which lactose was added ($5 \text{ g}\cdot\text{L}^{-1}$ final concentration). After overnight incubation for approx. 16 h at 30°C and 160 rpm in an orbital shaker, 5 mL were used to inoculate a second preculture with an inoculation volume of 10 % (v/v) in shaking flasks. Biomass was determined after 6 h by counting, and twelve times 10^9 cells were centrifuged (10 min, $3\cdot 10^3\text{-g}$). The cell pellet was resuspended in fresh lactose supplemented M17 broth to achieve a final concentration of $6.66\cdot 10^7$ cells per mL, which equals 10^9 cells per 15 mL fixed-bed.

Reactor system used for fixed-bed cultivations: Fermentations of immobilized bacteria were carried out in the multiple fixed-bed reactor system Multiferm (medorex e. K, Nörten-Hardenberg, Germany). This system comprises twelve single fixed-bed units with a working volume of 10 ml fixed-bed (carriers) plus 15 mL of fluid volume each. All units are installed within a conditioning vessel and can be operated independently. For fermentation trials, each unit was filled with 8 g of borosilicate carriers (diameter: 4 mm, VitraPOR®, ROBU® Glasfilter-Geräte GmbH, Hattert, Germany). The pH was not controlled during continuous cultivation of immobilized cells because preliminary experiments showed that the pH drops within the units to a level at the outlet, which is not limiting (in average pH of 6 at $D=\mu_{max}$). Temperature was controlled at 30°C with a Pt100 sensor and a heating rod, which was located in the water-filled conditioning vessel of the Multiferm. Heating was supported by placing the whole reactor system on a heating plate with magnetic stirrer. Pumps were installed to obtain bottom-to-top perfusion for better degassing of the units. The applied dilution rate was 0.5 h^{-1} corresponding to the fixed-bed volume of 10 mL, which equals the maximum specific growth rate μ_{max} of the used strain in batch cultivation in shaking flasks. All samples except for retained biomass were withdrawn at the outlet (harvest flow) of each fixed-bed unit. This reactor system was established and precisely described in a previous study using a *Lactobacillus* strain [29].

During continuous fixed-bed cultivation, samples were taken every 48 h. Medium at the outlet was drawn for analysis of lactose and lactate. Moreover, three fixed-bed units were disconnected and dismantled to determine free suspended and immobilized biomass.

Inoculation of the fixed-bed units for continuous cultivation: The autoclaved system, with all twelve units installed and each filled with the carriers, was treated as described in the following:

The water in the conditioning vessel was heated up to 30°C . A volume of 15 mL lactose supplemented M17 broth containing 10^9 cells was transferred to every unit. Cells settled within the fixed-bed during 16 h of incubation. The next morning the exhausted medium was replaced carefully with syringes *via* the Luer lock connections of each unit. Afterwards perfusion was started at above mentioned dilution rate.

Bioreactor set-up for continuous chemostat cultivation of suspended cells: Fermentations in continuous chemostat mode were realized using the multifermmentation system DASGIP® (ependorf AG, Germany). The system allows installing of up to four independently

controlled reaction vessels with a maximum working volume of 1 L into a platform where each glass reactor is fixed by a heating collar which was used to maintain a temperature of 30°C throughout the fermentations. The Rushton-type stirrer was set at 300 rpm and the pH regulation was managed by the systems own software DASware® control at 7.0 ± 0.3 *via* peristaltic pumps and calibrated pH electrodes with 5 M NaOH aq. Medium at room temperature was fed through external, calibrated peristaltic pumps at the bottom of the fermentation broth and the exit flow was installed at the height of the broth surface at a higher flow rate than the inlet flow to ensure a constant volume over time. Before fermentations were started, the pH regulation system was rinsed with 70 % EtOH aq according to the systems integrated clean in place (CIP) protocol and three fermentation units were filled with 350 mL medium without lactose and autoclaved for 15 min at 121°C . Subsequently, the fermentation units were placed in the platform and when the medium reached 30°C , 20 mL 10 % (w/w) lactose aq was added to a final concentration of $5 \text{ g}\cdot\text{L}^{-1}$ with the help of Luer lock syringes. For inoculation, a total of $4\cdot 10^{10}$ cells from a preparatory culture were prepared in 30 mL fresh medium and inoculated with a syringe.

Each fermenter was connected to an own medium reservoir. Due to the large amount of required medium, reservoirs had to be exchanged twice per fermenter, which was done by reconnecting the respective Luer lock connections under a 70% EtOH aq saturated paper towel. Samples of 2 mL were drawn to collect data about biomass, lactate and lactose. Continuous fermentations were performed three times independently and in parallel and the resulting data is expressed as means \pm SD. The dilution rates were chosen as 0.2, 0.4, 0.6, 0.8 and 0.9 times the maximum specific growth rate determined from the batch experiments in the Vario 500 system, which was 0.49 h^{-1} .

Analytcs

Biomass: Biomass was analyzed as total cell concentration (TCC) in $\text{cells}\cdot\text{mL}^{-1}$ *via* optical density (OD). TCC was determined using a Neubauer improved counting chamber with a depth of 0.02 mm. To obtain an appropriate cell density, fermentation broth was diluted with 0.9 % (w/v) NaCl solution, if necessary.

OD measurement was carried out in semi-micro cuvettes (Sarstedt, Nümbrecht, Germany) at 600 nm with a spectrophotometer (V-1200, VWR International, Darmstadt, Germany). Again, samples were diluted with 0.9 % (w/v) NaCl solution to have values in the linear range below 0.6. In order to distinguish the influence of the broth itself on the measurement, samples were centrifuged (10 min, $3\cdot 000\text{-g}$) and cell-free broth was re-measured. Consequently, OD data in this paper is exclusively the difference of these values.

For determination of immobilized biomass, carriers were rinsed carefully and transferred to 30 mL of 0.9 % (w/v) NaCl solution. Then, cells were detached *via* ultrasonic sound (2 min, Branson bath 221, Branson, Dietzenbach, Germany).

The dry content of bacterial mass (DMB, dry matter of biomass) was assessed by drying and weighing of washed fermentation samples according to the following procedure. Samples of 10 mL fermentation broth were centrifuged for 10 min at $3\cdot 10^3\text{-g}$ in tared glass vials. After discarding the supernatant, the pellet was resuspended in 2 mL 0.9 % (w/w) NaCl aq and centrifuged again under the same conditions. Subsequently, the vial was spilled and placed in an oven at $+80^\circ\text{C}$ for complete water removal. After at least two days, the biomass was determined by balancing the sample immediately after removal from the oven and DMB was expressed as a concentration in $\text{g}\cdot\text{L}^{-1}$.

Parallel determination of TCC, OD and DMB during batch cultivations yielded the following correlations:

$$TCC = OD_{600} \cdot 7.36 \cdot 10^8 \quad (\text{cells} \cdot \text{mL}^{-1}) \quad (1)$$

$$DMB = OD_{600} \cdot 0.32 + 0.10 \quad (\text{g} \cdot \text{L}^{-1}) \quad (2)$$

Lactate and lactose concentration: Filter-sterilized samples (0.22 μm pore size) were measured with HPLC. Two systems were used, both having a cation exchange column. The first device used 2.5 mM H_2SO_4 , which flows through the pre-warmed column (40°C, 0.3 mL \cdot min $^{-1}$). A refractive index detector analyzed all components. The second device used 5 mM sulfuric acid as eluent at a flow rate and has 0.6 mL \cdot min $^{-1}$. Lactose was again measured by a refractive index detector and additionally, a UV-detector was used to assess lactate (Kontron Instruments, Kontron HPLC 332 UV detector, RID-6A Shimadzu infrared detector, BioRad Aminex HPX-87H column at 50°C).

Methods used with respect to modeling

Parameter estimation: Model-specific parameters were determined by fitting of the model curve to the experimental data. This was realized by minimizing a weighted least sum of squares function by using the MATLAB subroutine *fmin* search. Beforehand, differential equations of the model were solved by the *ode45* algorithm with variable step width from MATLAB using initial guesses. Since biomass concentrations are four times lower in average, DMB values were weighted by a factor of 4 to have the same influence as lactose and lactate levels on the parameter estimation, which was realized by minimizing a weighted least sum of squares function comparing simulated to experimental data according to the following equation:

$$WLS = \sum_{j=1}^n (\hat{X}_{f,j} - X_{f,j})^2 \cdot w_{xf} + \sum_{j=1}^n (\hat{S}_j - S_j)^2 + \sum_{j=1}^n (\hat{P}_j - P_j)^2 \quad (3)$$

With

WLS=weighted least square

n=number of values

j=running index

$X_{f,j}$, S_j , P_j =concentration of either free suspended biomass, substrate or product

$\hat{X}_{f,j}$, \hat{S}_j , \hat{P}_j =corresponding simulated value

w_{xf} =weighting factor for free suspended biomass (=4)

Note that for cultivations with immobilized biomass the weighting factor for free suspended biomass fraction equals 1 in order to hit the behavior during fixed-bed cultivation. Additionally, another term for retained biomass containing $X_{r,j}$ was included.

Evaluation of simulations: The simulation was evaluated by calculating the coefficient of determination R^2 as

$$R^2 = 1 - (\text{RSS}/\text{TSS}) \quad (4)$$

where the residual sum of squares (RSS) was normalized to the total sum of squares (TSS) according to the following two equations:

$$\text{RSS} = \sum_{j=1}^n (\hat{Y}_j - Y_j)^2 \quad (5)$$

with

n number of values

J running index

Y_j concentration of either biomass, substrate or product

\hat{Y}_j corresponding simulated value

\bar{Y}_j arithmetic mean of all measured values of either biomass, substrate or product

Results and Discussion

Time course of biomass, substrate and product during fixed-bed cultivation

The aim of these studies was to investigate how bacteria proliferate during transient initial phase of fixed-bed cultivation until a steady state point of operation is reached, where all concentrations of substrate, product and biomass in the outlet are constant. The chronology of pictures in Figure 1 shows how biomass changes over the cultivation period of eight days (192 h). In the beginning, there is only few biomass within the system. At the end, an enormous formation of biomass is visible. Additionally, it can be seen that biomass grew from bottom to top. The underlying mechanism was not investigated further but compared with recent studies it seems to happen due to gravitational effects instead of any limitation at this fixed-bed size and dilution rate. For scale-up, limitation is addressed by changing the flow from axial to radial perfusion.

L. lactis did not form a stable biofilm under the applied conditions and cells are rather retained by deep-filtration effects. Furthermore, cells sedimented on the bottom of the fixed-bed unit where the medium enters the reactor from the inner tube in bottom-to-top perfusion. Therefore, the biomass was divided into two fractions, one being free suspended cells and the other summarizing sedimented cells and cells within the carriers. The latter fraction is referred as retained biomass. Retained biomass is related to the whole liquid volume of 15 mL within a reaction unit.

The time course of free suspended and retained cells as well as of substrate (lactose) and product (lactate) concentrations are plotted in Figure 2. The standard deviation is very small, underlining the high reproducibility of the multiple fixed-bed runs. The suspended DMB remained stable at concentrations between 0.16 g \cdot L $^{-1}$ and 0.47 g \cdot L $^{-1}$. In contrast, retained biomass increased linearly from initially 0.3 g \cdot L $^{-1}$ to 11.20 g \cdot L $^{-1}$ on day six to stay constant until day eight.

The lactose concentration decreased from the initial value of 5 g \cdot L $^{-1}$ to nearly zero (below detection limit) for all subsequent sampling points. Simultaneously, the lactate concentration increased up to a maximum of 16.28 g \cdot L $^{-1}$ on the second day and remained constant for days six and eight at 12.55 g \cdot L $^{-1}$. It is obvious that the strain was able to metabolize complex components of the M17 broth since the lactate values were three to five times elevated versus the initial lactose value. The volume specific lactate productivities resulted in values of 4.69 g \cdot L $^{-1}$ \cdot h $^{-1}$ (day six) and 4.72 g \cdot L $^{-1}$ \cdot h $^{-1}$ (day eight). These values are in very good agreement with data from experiments in larger fixed-beds (fixed-bed volume 100 mL: 4.7 g \cdot L $^{-1}$ \cdot h $^{-1}$, fixed-bed volume 1 L: 4.8 g \cdot L $^{-1}$ \cdot h $^{-1}$, data not shown). The biomass specific productivity was 0.42 g \cdot g $^{-1}$ \cdot h $^{-1}$, whilst the yield was calculated to be 1.89 g \cdot g $^{-1}$. This value is greater than 1, again for the above mentioned reason of complex medium component utilization. This data set was used for modeling as described in the next section.

Model development for biomass, substrate and product

To consider both biomass fractions (free and retained), the fixed-bed system was described as a perfusion system consisting of a cell suspension coupled with a cell retention unit, where not all free cells

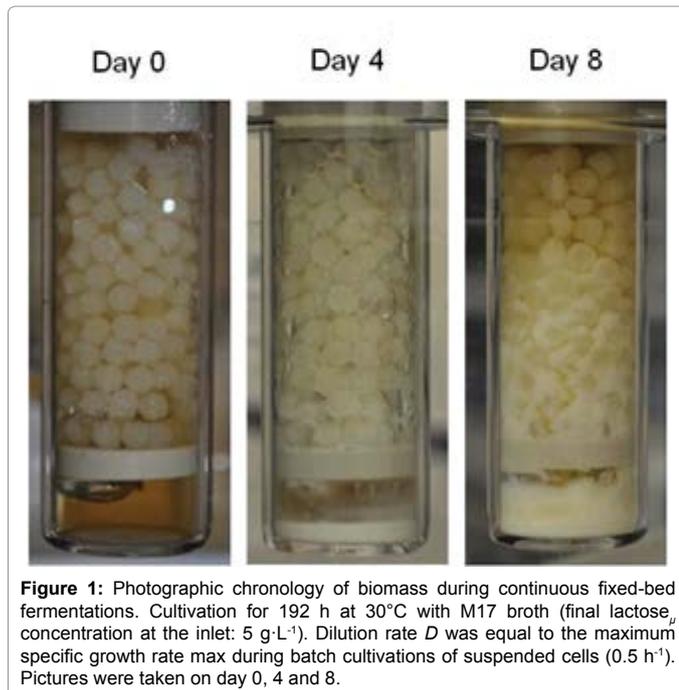


Figure 1: Photographic chronology of biomass during continuous fixed-bed fermentations. Cultivation for 192 h at 30°C with M17 broth (final lactose_{in} concentration at the inlet: 5 g·L⁻¹). Dilution rate *D* was equal to the maximum specific growth rate max during batch cultivations of suspended cells (0.5 h⁻¹). Pictures were taken on day 0, 4 and 8.

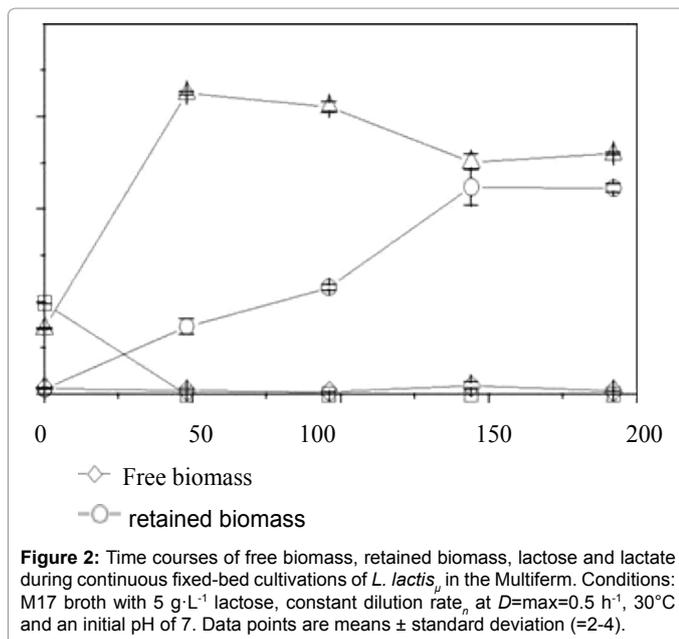


Figure 2: Time courses of free biomass, retained biomass, lactose and lactate during continuous fixed-bed cultivations of *L. lactis* in the Multiferm. Conditions: M17 broth with 5 g·L⁻¹ lactose, constant dilution rate_{in} at $D = \mu_{max} = 0.5 \text{ h}^{-1}$, 30°C and an initial pH of 7. Data points are means \pm standard deviation ($n=2-4$).

leave the system but stay within the system (fixed-bed reactor) and contribute to the fraction of retained cells (Figure 3).

Therefore, a perfusion constant *R* had to be introduced to the differential equations for biomass (see below). The retained biomass reaches a steady state after constant increase. Hence, this fraction is not regarded as growing rather than being produced *via* retaining of free cells. Furthermore, the retained fraction is allowed to “lyse” in order to reach and stay at a maximum value, which was realized by introducing a lysis constant k_{lys} . Growth of free suspended biomass was described by Monod growth kinetics [30].

$$\mu = \mu_{max} \cdot \frac{S}{S + K_s} \quad (7)$$

μ =specific growth rate

μ_{max} =maximum specific growth rate

S=running index

K_s =Monod-constant

Own studies revealed that at substrate concentrations below 5 g·L⁻¹ lactose no term for product inhibition has to be included to improve the model notably. Substrate consumption of free suspended biomass was described by using a yield coefficient $Y_{x/s}$ because it turned out that maintenance is negligible for suspended cells. Product formation can be modeled with a Luedeking-Piret equation [31] containing expressions for growth correlated formation (α -term), non-growth correlated formation by plain biomass (β -term) and unspecific substrate consumption (γ -term) [33] that does not contribute to the product.

Substrate consumption by retained cells was considered by a maintenance coefficient m_r in the differential equation for substrate. An additional β -term for retained biomass introduced in the differential equation for product, which is adjusted with an effectiveness factor η_p [35-37] that stands for the relative difference between non-growth associated production of lactate from free and retained cells.

Finally, the model to describe fixed-bed cultivations consists of the following set of differential equations:

$$\frac{dX_f}{dt} = X_f \cdot \mu - D \cdot (1 - R) \cdot X_f \quad (8)$$

$$\frac{dX_r}{dt} = D \cdot R \cdot X_f - k_{lys} \cdot X_r \quad (9)$$

$$\frac{dS}{dt} = -\frac{X_f \cdot \mu}{Y_{x/s}} - m_r \cdot X_r + D \cdot (S_{in} - S) \quad (10)$$

$$\frac{dP}{dt} = \alpha \cdot X_f \cdot \mu + \beta \cdot X_r - \gamma \cdot X_f \cdot S + \beta \cdot X_r \cdot \eta_p - D \cdot P \quad (11)$$

X_f free suspended biomass

X_r retained biomass

P product concentration

D dilution rate

Note that compared to the reaction kinetic model of a chemostat cultivation of suspended *L. lactis* cells, there is only one additional equation for retained biomass and overall, only four new parameters (R , k_{lys} , m_r and η_p) were introduced.

Those model parameters in eq. 7-10 related to growth, substrate consumption and product formation of free suspended biomass (μ_{max} , K_s , $Y_{x/s}$, α , β , γ) were estimated from chemostat cultures. The results of the parameter estimation are listed in Table 1. Model selection and parameter estimation are discussed in detail in Ref. [38].

These parameters were used as a starting point for estimation of the parameters specific for the retained biomass (R , k_{lys} , m_r and η_p). Table 2 summarizes the results of the estimated four fixed-bed specific parameters.

A perfusion constant *R* of 0.37 reveals that only 63 % of free suspended cells leave the system. The fitted lysis rate k_{lys} means that 0.3 % of retained cells lyse per hour. The calculated effectiveness factor η_p is

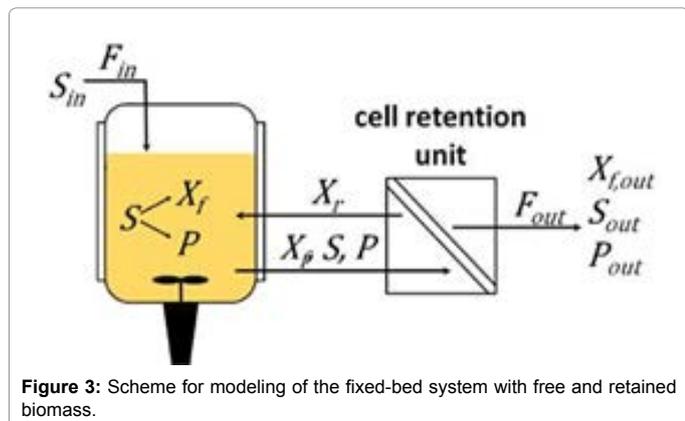


Figure 3: Scheme for modeling of the fixed-bed system with free and retained biomass.

μ_{max}	K_s	$Y_{x/s}$	α	β	γ
(h ⁻¹)	(g·L ⁻¹)	(g·g ⁻¹)	(g·g ⁻¹)	(h ⁻¹)	(L·g ⁻¹ ·h ⁻¹)
0.48	0.24	0.18	10.22	0.12	0.53

Table 1: Parameter values from curve fitting of batch fermentation data of suspended *L. lactis* (M17 broth, 30°C, 5 g·L⁻¹ lactose). Model and parameters were evaluated in continuous fermentation.

R	k_{lys}	m_r	η_p
(-)	(h ⁻¹)	(h ⁻¹)	(-)
0.37	2.92×10^{-3}	0.21	6.16

Table 2: Parameter values from curve fitting of fixed-bed fermentation data of *L. lactis* (M17 broth, 30°C, 5 g·L⁻¹ lactose).

equal to a six-fold increase of non-growth correlated lactate production from retained cells versus free cells.

Simulation versus experimental data

Figure 4 shows simulations of time courses for free and retained biomass as well as levels of lactose and lactate versus time compared to the corresponding experimental data set. In general, simulations of free and suspended biomass (both expressed as DMB) show a good description of the time courses for both fractions. The simulation of free biomass reaches a maximum in the beginning after 10 h of fermentation time of 1.08 g·L⁻¹. Afterwards, the steady state value of 0.15 g·L⁻¹ was reached after 120 h. The simulation of retained biomass reaches the steady state earlier than the experimental data indicates and only to 76 % of the experimental value. The simulated time course of lactose concentration predicts a decrease within the first 10 h of fermentation from initially 5 g·L⁻¹ to 0.36 g·L⁻¹ what represents very well what was observed in the experiment, where lactose level also dropped under the detection limit of 0.5 g·L⁻¹. The simulated lactate trend shows a mild decrease in the first 1.5 h of fermentation due to dilution effects. Then it increases logarithmically up to 14.73 g·L⁻¹ at 120 h process time. This value is very close to the steady state of experimental data.

Tables 3-6 summarizes the coefficients of determination for all simulated trends. The time courses of retained cells and for the product concentration can be described adequately by the simulation as indicated by values of 0.724 and 0.796, respectively. An excellent description by simulation was received with the underlying model for the substrate trend. The calculated R² value for simulated free suspended biomass led to a negative value. This is due the low concentrations of free suspended biomass and thus, the relative deviations between simulated and experimental data are large.

	X_r	$X_{r,ret}$	S	P
	(g·L ⁻¹)	(g·L ⁻¹)	(g·L ⁻¹)	(g·L ⁻¹)
R ²	-2.076	0.724	0.979	0.796

Table 3: Coefficients of determination R² for the simulations of free biomass, retained biomass, lactose and lactate as shown in Figure 4.

μ_{max}	K_s	$Y_{x/s}$	α	β	γ
(h ⁻¹)	(g·L ⁻¹)	(g·g ⁻¹)	(g·g ⁻¹)	(h ⁻¹)	(L·g ⁻¹ ·h ⁻¹)
0.48	0.24	0.18	10.22	0.12	0.53

Table 4: Parameter values from curve fitting of batch fermentation data of suspended *L. lactis* (M17 broth, 30°C, 5 g·L⁻¹ lactose). Model and parameters were evaluated in continuous fermentation.

R	k_{lys}	m_r	η_p
(-)	(h ⁻¹)	(h ⁻¹)	(-)
0.37	2.92×10^{-3}	0.21	6.16

Table 5: Parameter values from curve fitting of fixed-bed fermentation data of *L. lactis* (M17 broth, 30°C, 5 g·L⁻¹ lactose).

	X_r	$X_{r,ret}$	S	P
	(g·L ⁻¹)	(g·L ⁻¹)	(g·L ⁻¹)	(g·L ⁻¹)
R ²	-2.076	0.724	0.979	0.796

Table 6: Coefficients of determination R² for the simulations of free biomass, retained biomass, lactose and lactate as shown in Figure 4.

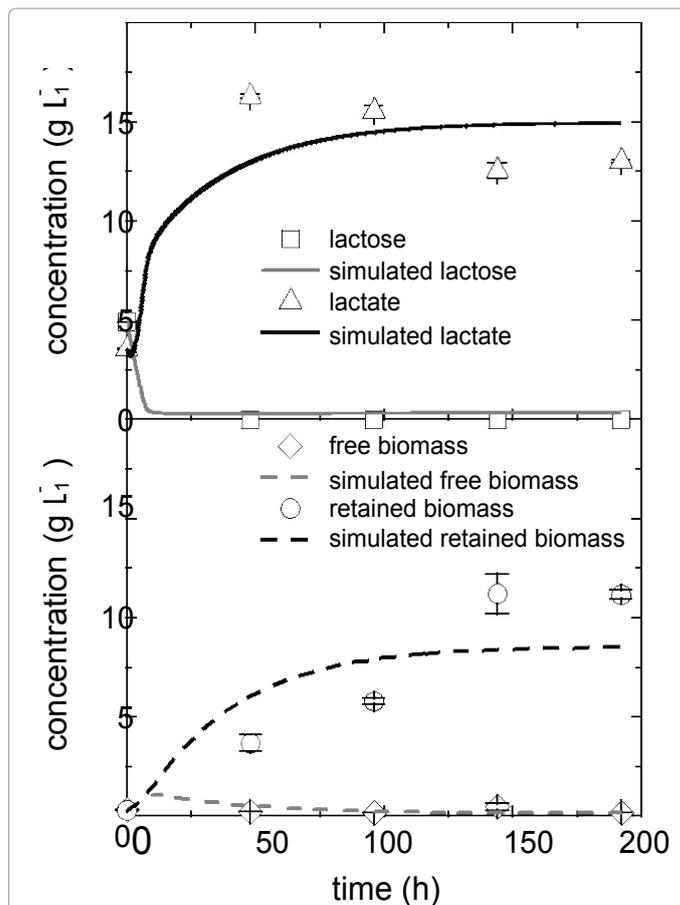


Figure 4: Simulation of free and retained biomass, lactose and lactate vs. experimental data.

Conclusion

In this study the down-scaled multiple fixed-bed fermenter system Multiferm was used to investigate biomass formation during cultivations of a *L. lactis* ssp. *lactis* strain. Especially the initial transient state was of interest in order to use the data for modeling and simulation. Retained and free suspended biomass was determined successfully by dismantling three units every 48 h beginning on day zero up to day eight resulting in five sample points and 15 values in total. Since the Multiferm has a compact design with twelve single fixed-bed units in one vessel, the system had to be prepared only twice to get all necessary values. Therefore, the usage of this reactor system accelerated the experimental effort drastically.

The straightforward modeling approach based on suspension cultivations in chemostat mode resulted in a model, which could be extended to adequately describe the fixed-bed process by introducing only four new parameters. The main idea of modeling the free suspended biomass as a perfusion-like system, where only a part leaves the reactor and the other contributes to non-growing but accumulating retained biomass, led to an adequate description of the fixed-bed process. Further studies will focus on scale-up of the fixed-bed system and optimization of process parameters, e.g., perfusion rates.

Conflict of Interest

The authors confirm that they do not have any conflicts of interest.

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