Evaluation of productive biofilms for continuous lactic acid production

Laure Cuny[1]   Daniel Pfaff[1]   Jonas Luther[1]   Florian Ranzinger[1]   Peter Ödman[2]

Johannes Gescher[3]   Gisela Guthausen[1,4]   Harald Horn[1,5]   Andrea Hille-Reichel[1]

[1]Karlsruhe Institute of Technology, Engler-Bunte-Institut, Water Chemistry and Water Technology, Karlsruhe, Germany

[2]BASF SE, Ludwigshafen am Rhein, Germany

[3]Department of Applied Biology, Institute for Applied Biology, Karlsruhe Institute of Technology, Karlsruhe, Germany

[4]Karlsruhe Institute of Technology, Mechanical Process Engineering and Mechanics, Karlsruhe, Germany

[5]DVGW Research Laboratories for Water Chemistry and Water Technology, Karlsruhe, Germany

Correspondence

Andrea Hille-Reichel, Karlsruhe Institute of Technology, Engler-Bunte-Institut, Water Chemistry and Water Technology, 76131 Karlsruhe, Germany.
Email: andrea.hille reichel@kit.edu

Abstract

In white biotechnology research, the putative superiority of productive biofilms to conventional biotransformation processes based on planktonic cultures has been increasingly discussed in recent years. In the present study, we chose lactic acid production as a model application to evaluate biofilm potential. A pure culture of *Lactobacillus* bacteria was grown in a tubular biofilm reactor. The biofilm system was cultivated monoseptically in a continuous mode for more than 3 weeks. The higher cell densities that could be obtained in the continuous biofilm system compared with the planktonic culture led to a significantly increased space time yield. The productivity reached 80% of the maximum value 10 days after start up and no subsequent decline was observed, confirming the suitability of the system for long term fermentation. The analysis of biofilm performance revealed that productivity increases with the flow velocity. This is explained by the reduced retention time of the liquid phase in the reactor, and, thus, a minor pH drop caused by the released lactic acid. At low flow velocities, the pH drops to a value where growth and production are significantly inhibited. The biofilm was visualized by magnetic resonance imaging to analyze biofilm thickness. To deepen the understanding of the biofilm system, we used a simple model for cell growth and lactic acid production.

KEYWORDS

lactic acid production, long term fermentation, magnetic resonance imaging, productive biofilm, simulation, tubular biofilm reactor

1   INTRODUCTION

For decades, biofilms have been successfully used in bioremediation, mainly in the treatment of wastewater and off gas (Storhas, 2013; Tsoligkas et al., 2011). Recently, the interest in productive biofilms has greatly increased, since the application of biofilm technology has been extended to the production of bulk and fine chemicals (Gross, Schmid, & Buehler, 2012; Rosche, Li, Hauer, Schmid, & Buehler, 2009). Biotechnological production processes using bacteria are still mostly limited to batch and fed batch processes, in which planktonic cells grow suspended in a liquid medium and are disposed of at the end of each run (Halan, Buehler, & Schmid, 2012; Rosche et al., 2009). However, continuous reactor operation is generally expected to be economically advantageous due to the reduction of downtimes for reactor preparation and cleaning (Al Kaidy et al., 2015). The self immobilization and the resulting gentle cell retention of productive biofilm systems allow for a continuous mode of operation with little or no need for active cell separation (Karel, Libicki, & Robertson, 1985; Posten, 2018). Other well known advantages of biofilms are high resistance to toxic reactants and long term stability as required for continuous processing (Gross, Hauer, Otto, & Schmid, 2007; Li, Webb, Kjelleberg, & Rosche, 2006). Additionally, especially in anaerobic fermentation, a higher biomass density is expected in the reactor as compared with planktonic cells, resulting in an increase in volumetric productivity (Brink & Nicol, 2014;

Dagher, Ragout, Siñeriz, & Bruno Bárcena, 2010). However, mass transfer limitations in thicker biofilms, with respect to substrates as well as products, may pose a challenge, making the control of biofilm thickness indispensable (Muffler & Ulber, 2014). Biomass loss through sloughing events also has a negative impact on biofilm productivity (Brading, Jass, & Lappin Scott, 1995; Characklis & Marshall, 1990; Santek, Ivancić, Horvat, Novak, & Marić, 2006).

To evaluate the potential of biofilm based continuous production of value added compounds, the present study focuses on lactic acid as a model substance. Lactic acid is traditionally used in the food, pharmaceutical and textile industries (Sahm, Antranikian, Stahmann, & Takors, 2014). Since 2002 the demand has grown by a factor of 10, mainly due to the synthesis of polylactic acid (PLA), a biodegradable and biocompatible polymer (Ren, 2010). However, the high costs of lactic acid are the reason for its low competitiveness to petroleum derived plastics. The idea to reduce production costs by the use of productive biofilms came up over 20 years ago. So far, approaches are limited to the laboratory scale involving mostly carrier material like plastic composite supports (Demirci & Pometto, 1995; Ho, Pometto, & Hinz, 1997; Velázquez, Pometto Iii, Ho, & Demirci, 2001) or polyurethane foam (John, Nampoothiri, & Pandey, 2007; Rangaswamy & Ramakrishna, 2008). The use of membranes to increase the area to volume ratio and to facilitate cell attachment has also been investigated (Fan, Ebrahimi, Quitmann, & Czermak, 2015; Kwon, Yoo, Lee, Chang, & Chang, 2001). A rather new approach to achieve high productivities for lactic acid is the cultivation of microbial granules. However, the use of a mixed population derived from wastewater treatment makes the product unsuitable for food or cosmetic purposes (Kim et al., 2016).

In this study, we operated a horizontal tubular biofilm reactor (TBR). TBRs have been used for cultivation of different kinds of biofilms for decades (Harald Horn & Hempel, 1997; H. Horn, Reiff, & Morgenroth, 2003; Skoneczny & Tabiś, 2015; Wagner, Manz, Volke, Neu, & Horn, 2010), however, their application for productive biofilms is still very limited. Brink and Nicol (2014) investigated a heterofermentative *Lactobacillus* biofilm in a TBR with the focus on the influence of shear on general metabolic changes. The main advantages of TBRs as compared with other reactor types is their simple construction and their easy process set up, as no special carrier material or preliminary growth step is needed. Moreover, the absence of dead zones leads to a more reliable scale up procedure (Santek et al., 2006). The goal of this study was to characterize the performance of a pure culture biofilm of *Lactobacillus* bacteria for lactic acid production and to evaluate the applicability of a TBR. To deepen the understanding of the biofilm system, a simple model for cell growth and lactic acid production was developed.

2 MATERIALS AND METHODS

2.1 Materials

All chemicals used in this study were purchased either from Merck KGaA (Darmstadt, Germany) or from Carl Roth GmbH (Karlsruhe, Germany), unless stated otherwise.

The organism cultivated was *Lactobacillus delbrueckii*, a homofermentative lactic acid bacterium obtained from BASF SE (Ludwigshafen am Rhein, Germany).

2.2 Growth media and cultivation conditions

2.2.1 Preculture

A 100 ml flask with 45 ml MRS medium (De Man, Rogosa, & Sharpe, 1960) was inoculated with 5 ml frozen *L. delbrueckii* cell suspension. The culture was incubated on a magnetic stirrer under anaerobic conditions at 45 °C for 6 hr.

2.2.2 Batch production culture

A 2 L stirred tank bioreactor (Biostat® A; Sartorius Stedim Biotech GmbH, Göttingen, Germany) was inoculated with 50 ml preculture (BDM = 1.1 ± 0.2 g/L), cultivated as described above. The production medium had the following composition: 100 g/L glucose, 20 g/L yeast extract, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \bullet 7H_2O$, 100 g/L $CaCO_3$ as a buffering agent, 1 ml/L of both mineral salts and vitamin stock solution. The mineral salts stock solution consisted of: 5.80 g/L $ZnSO_4 \bullet 7H_2O$, 5.60 g/L $FeSO_4 \bullet 7H_2O$, 1.70 g/L $MnSO_4 \bullet H_2O$, 2.5 g/L $CuSO_4 \bullet 5H_2O$, 2.8 g/L $CoSO_4 \bullet 7H_2O$, and 40 g/L citric acid monohydrate. The vitamin stock solution contained: 0.05 g/L biotin, 1.0 g/L Ca pantothenate, 1.0 g/L nicotinic acid, 2.0 g/L myo inositol, 1.0 g/L thiamine HCl, 1.0 g/L pyridoxine HCl, 0.2 g/L para aminobenzoic acid, and 0.3 g/L riboflavin. The pH value was adjusted to 6.0 before inoculation and was kept around 5.0 by the buffering agent. The temperature was controlled at 45 °C.

2.2.3 Biofilm production culture

The biofilm culture was grown in a horizontal TBR made of glass (ID = 10 mm, *L* = 400 mm). Right before the inflow as well as right behind the outflow of the TBR, sampling sites were implemented in the system (Figure 1). As medium reservoirs and tempering units, two 2 L stirred tank bioreactors (Biostat® A; Sartorius Stedim Biotech GmbH, Göttingen, Germany) were installed in line with the TBR. The production medium had the same composition as in the batch culture apart from reducing the glucose concentration and replacing the buffering agent $CaCO_3$ with 0.1 g/L $CaCl_2 \bullet 2H_2O$ as a calcium source. After inoculation of the medium in reservoir 1, the biofilm was first grown in recycle mode. During this phase, the medium was pumped through the TBR in intermittent operation with 15 min pumping at a flow velocity of *v* = 1.06 mm/s alternating with 60 min rest. Glucose concentration was kept at approx. 1 g/L by regular addition of substrate. The pH was controlled automatically at 6.0 by NaOH addition. After a visible biofilm had developed after 48 hr, reactor operation was changed to continuous mode for further biofilm growth and lactic acid production. Therefore, reservoir 1 was replaced by reservoir 2, containing sterile medium with 5 g/L glucose and the biofilm was cultivated at a flow velocity of 0.3 mm/s without recycling the spent medium back to the reservoir. A bubble trap was
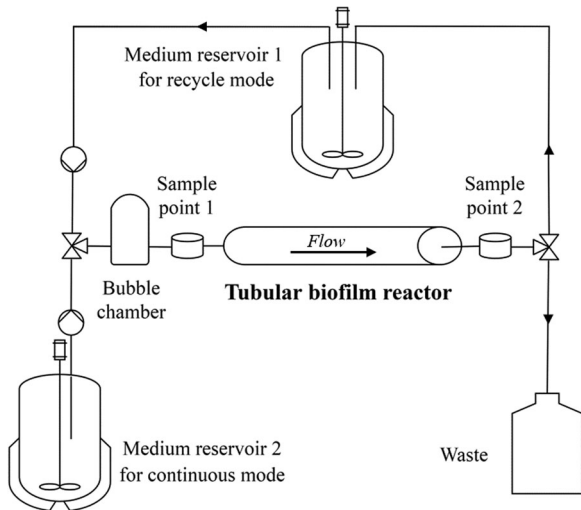
FIGURE 1 Experimental set up for the tubular biofilm reactor (ID = 10 mm, L = 400 mm)

used to avoid the introduction of gas into the TBR. To analyze the impact of flow velocity on biofilm productivity, short term experiments at flow velocities of 0.32, 1.06, and 2.12 mm/s were conducted almost daily. The experiment was terminated after approx. one month, when nearly constant productivity was achieved.

2.3 Determination of biofilm biomass

At the end of the experiment, the biomass growing on the inner walls of the TBR was determined as biomass wet weight (BWW) after drainage at an angle of 45° for 5 min. For the determination of biomass dry weight (BDW), the biofilm was scraped off the walls completely and the TBR was rinsed with normal saline. The suspension obtained was filtered and the filters (0.2 μm, nitrocellulose) were dried for 12 hr at 110 °C and weighed on a high precision balance. The water content was determined as the ratio of BDW to BWW.

2.4 Analytical methods

Planktonic cell growth was determined by measurement of optical density at a wavelength of 600 nm (OD_{600}; UV–Vis Spectrometer, Lambda XLS, Perkin Elmer, Waltham, MA) with cell free supernatant used as blank. Optical densities were related to biomass dry weight (BDW) concentrations by using a calibration curve.

Glucose and lactic acid concentrations were determined using high performance liquid chromatography (Agilent Technologies, Santa Clara, CA) combined with RI and UV detector (210 nm), respectively. Broth samples were centrifuged and the supernatant was diluted for analysis if necessary. An ion exclusion column (Rezex ROA organic acid H + (8%), 300 × 7.8 mm; Phenomenex, Torrance, CA) was used at a temperature of 55 °C with 2.5 mM H_2SO_4 as a mobile phase at a flow rate of 0.5 ml/min. The injection volume of the sample was 20 μl.

Biofilm visualization by magnetic resonance imaging (MRI; Herrling, Guthausen, Wagner, Lackner, & Horn, 2015; Wagner et al., 2010) and confocal laser scanning microscopy (CLSM; Neu & Lawrence, 2014; Neu et al., 2010) was performed after the experiment was terminated. To analyze the extent and distribution of biofilm growth over the cross sectional area of the TBR, three coplanar axial slices perpendicular to the horizontal axis of the TBR (position 1: 70 mm; position 2: 200 mm, position 3: 330 mm) were measured with MRI. Before imaging, the TBR was drained, re filled with normal saline and vertically positioned at the center of the MRI magnet. MRI was performed on a Bruker Avance 200 SWB spectrometer (Larmor frequency: 200 MHz, magnetic flux density: 4.7 T, vertical bore magnet with an ID of 150 mm, inner diameter of the ^{1}H bird cage: 20 mm; Bruker BioSpin GmbH, Rheinstetten, Germany), using a fast low angle shot sequence (Callaghan, 1993; Kimmich, 1997). The field of view was 20 mm × 20 mm, and an in plane spatial resolution of 156 μm was used. MRI parameters are provided in Table S1. Data was acquired within ParaVision 6.0.1. (Bruker BioSpin GmbH, Karlsruhe, Germany), and processed in Avizo (FEI Visualization Sciences Group, Merignac, France) and via self written scripts in Matlab (version R2012a; MatlabWorks Inc.; Natick, MA).

An undisturbed sample of biofilm at the inlet of the TBR was visualized on the microscale (0.08 μm/pixel) with CLSM on a Zeiss LSM700 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Nucleic acids and glycoconjugates were stained with SYTO60 (ThermoFisher Scientific Inc., Waltham, MA) and BanLec (Vector Laboratories Inc., Burlingame, CA), respectively, using a FITC and an A633 filter. Microscopy was done with a water immersible lens (magnification ×40, Objective W Plan Apochromat 40 × /1.0 DIC, Zeiss, Germany).

3 SIMULATION OF PRODUCT FORMATION IN THE TBR

3.1 Theory

The biofilm model used to represent the experimental data for cell mass (X), product (c_P) and substrate (c_S) concentrations have been developed based on the widely used equation of Monod (Monod, 1949), that includes a growth associated production. The maximum specific growth rate (μ_{max}) was determined by batch experiments whereas the Monod constant K_S of 0.01 g glucose/L was adopted from literature (Yabannavar & Wang, 1991). As the simulation results proved to be insensitive to the precise value of K_S in the range below 1 g/L (results not shown) and as the initial substrate concentrations in the experiments were by orders of magnitude higher, an experimental determination of K_S was considered unnecessary. So far, simulations for lactic acid production have only be reported for planktonic cells in the batch mode (Kumar Dutta, Mukherjee, & Chakraborty, 1996; Kwon et al., 2001; Yeh, Bajpai, & Iannotti, 1991). These simulations include a growth inhibition term taking into account the undissociated product (Kumar Dutta et al., 1996; Yeh

et al., 1991). As the product concentration in the present study is far below the inhibition concentration, this term can be neglected in the biofilm model. However, in contrast to batch experiments, the pH is not regulated inside the TBR. As the pH drop resulting from product formation has also an inhibitory effect on growth and production, a growth inhibition term was included, that depends on the concentration of released protons (c_H) and an inhibition constant (K_H). The prediction of pH change in the medium as a function of the formed acid was determined experimentally. According to Luedeking Piret (1959), a nongrowth related production term, depending on the actual cell density, was added for the rate of product formation, with a maximum specific production rate β_{max} determined by batch experiments. Biomass detachment was assumed to be a function of the growth velocity of the biofilm u_F (Horn & Hempel, 1997; Wanner & Reichert, 1996), with the proportionality constant referred to as detachment coefficient (k_D). Biomass and product yields, Y_{XS} and Y_{PS}, were determined in batch experiments or in the continuous study, respectively. The processes are summarized in Table 1.

3.2 | Model development

In this study, we used the AQUASIM 2.1 software (Reichert, 1994) to model the biotransformation of glucose to lactic acid within a productive biofilm. The confined biofilm reactor compartment offered by AQUASIM was used, which consists of a "bulk fluid," a "biofilm solid matrix," and a "biofilm pore water" zone (Wanner & Morgenroth, 2004). The bulk fluid is considered to be completely mixed whereas gradients perpendicular to the substratum for an arbitrary number of substances are calculated in the biofilm pore water. Biotic conversion reactions for example, Monod type kinetics are defined by the user, while the equations describing transport processes for example, Fick's law is implemented in AQUASIM. Due to the high flow rates normally applied, TBRs are typically simulated as one completely mixed reactor compartment (Horn & Hempel, 1997), especially when operated in recycle mode. To consider the axial changes in concentrations characteristic for plug flow at lower flow rates, we modeled the TBR as a series of three interconnected biofilm segments, named S1 to S3 (Figure 2). Thus, the 1D model transforms into a pseudo 2D model. This approach is recommended (Rittmann et al., 2018; Wanner & Morgenroth, 2004) but has not yet been applied to real data. The specific surface area of each of the three segments was equal to 0.025 m², reflecting the actual inner
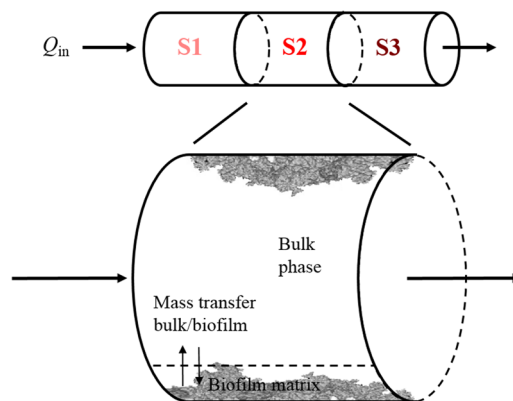

FIGURE 2 Schematic representation of the biofilm reactor in the model. The segments S1, S2, and S3 refer to a length of 133 mm [Color figure can be viewed at wileyonlinelibrary.com]

surface area of the TBR of 0.075 m² in total. The actual biofilm surface area is a function of the distance from the substratum. As initial values, all concentrations in the biofilm and bulk liquid were assumed equal to the corresponding influent concentrations as the impact of the very thin initial biofilm layer (10 μm, see Table 2) is considered negligible. Mass transfer of substrate, product, and protons into and out of the biofilm, as well as their mass transport within the biofilm matrix, were described by diffusion. As mass transport is considered only in the liquid phase of the biofilm ($1 - \varepsilon_x = 0.86$, Table 2), the corresponding diffusion coefficients in water were used in the model. The kinetic model parameters were calculated by minimizing the sum of the squares of the weighted deviations (χ^2) among measurements and calculated model results. Table 2 summarizes the important parameters of the simulation.

4 | RESULTS AND DISCUSSION

4.1 | Preliminary experiment: Batch lactic acid production with planktonic cells

To analyze lactic acid production with planktonic cells and to determine the growth parameters, batch experiments ($n = 2$) were performed. Growth, substrate consumption and lactic acid production data for one representative experiment as well as the deduced growth parameters are given in Figure 3a,b. Cell growth ceased before substrate depletion, resulting in a low BDW concentration of

TABLE 1 Process matrix for the simulation of lactic acid production

	Components				
	Substrate	Product	Proton	Biomass	
Process	**S**	**P**	**H**	**X**	**Process rate r_i**
Cell growth and growth-associated production	$-\frac{1}{Y_{XS}}$	$\frac{Y_{PS}}{Y_{XS}}$	$\frac{Y_{PS}}{Y_{XS}}$	1	$\mu_{max} \cdot \frac{c_S}{K_S + c_S} \cdot \frac{K_H}{K_H + c_H} \cdot X$
Non growth-associated production	$-\frac{1}{Y_{XS}}$	1	1	-	$\beta_{max} \cdot \frac{c_S}{K_S + c_S} \cdot X$
Biomass detachment	-	-	-	−1	$k_D \cdot u_F$

TABLE 2 Kinetic parameters for the simulation of lactic acid production

Parameter	Symbol	Value	Unit	Source
Rates				
Maximum growth rate	μ_{max}	0.63	1/h	This study, batch experiment
Maximum nongrowth associated production rate	β_{max}	0.10	1/h	This study, batch experiment
Constants				
Monod constant for substrate	K_S	0.01	g/L	Yabannavar and Wang (1991)
Inhibition constant for proton	K_H	$1.0 \cdot 10^{6}$	g/L	Fitted
Yield coefficients				
Biomass yield	Y_{XS}	0.05	g/g	This study, batch experiment
Product yield	Y_{PS}	0.94	g/g	This study, continuous mode
Diffusion coefficients				
Substrate	D_S	0.835	cm^2/d	Ribeiro et al. (2006)
Product	D_P	1.120	cm^2/d	Ribeiro et al. (2005)
Proton	D_H	8.808	cm^2/d	Calculated
Detachment coefficient	k_D	0.83	-	Fitted
Dry biomass of solid fraction in biofilm volume	ρ_X	205	kg/m^3	This study, continuous mode
Volume fraction of biomass	ε_X	0.14	-	This study, continuous mode
Initial biofilm thickness	$L_{F,0}$	10	µm	Fitted
Concentrations in influent				
Substrate	$c_{S,in}$	5	g/L	
Proton	$c_{H,in}$	10^{6}	g/L	
Flow rate	v	0.32	mm/s	

only 1.5 g/L. As the pH value is controlled in the batch culture, this indicates a possible product inhibition at approx. 30 g/L of lactic acid, as has already been observed before (Goncalves, Xavier, Almeida, & Carrondo, 1991; Kumar Dutta et al., 1996). Lactic acid production continued at a reduced rate even when net growth ceased after 42 hr, showing that the production is both growth and nongrowth associated. Despite high specific productivity, the maximum volumetric productivity reached only 1.2 g·L^{-1}·hr^{-1} due to the low biomass concentration. This is in accordance with recent studies, where volumetric productivities with different bacterial strains and fermentation conditions varied from 0.65 to 3.20 g·L^{-1}·hr^{-1} (Gao, Wong, Ng, & Ho, 2012; Liu et al., 2010; Meng, Xue, Yu, Gao, & Ma, 2012; Velázquez et al., 2001). The low biomass but high product yields ($Y_{XS} = 0.06 \pm 0.01$ g/g; $Y_{PS} = 0.87 \pm 0.01$ g/g) are characteristic for anaerobically cultivated microorganisms and represent the desired features for efficient product formation. Our results identify
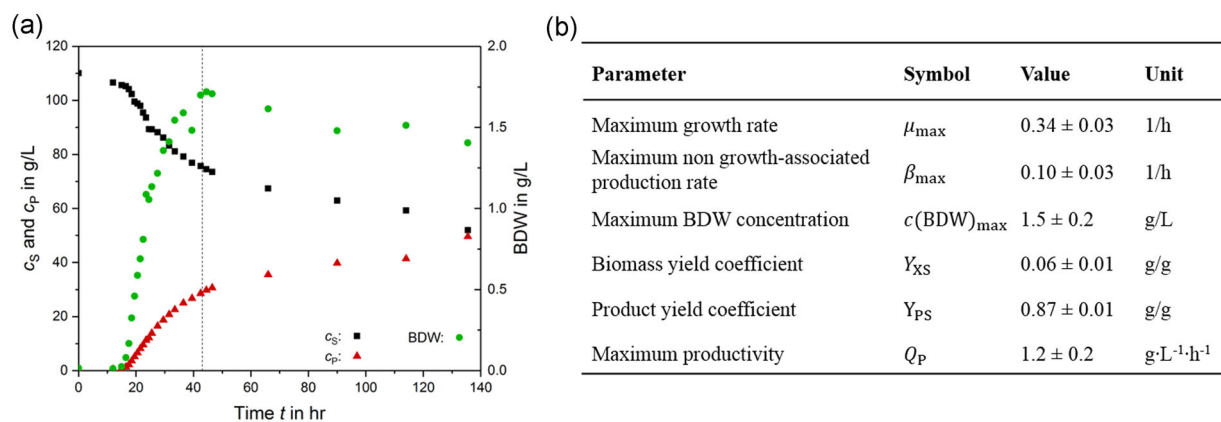

FIGURE 3 (a) Growth (given as CDW), substrate consumption and lactic acid production data (c_S and c_P) for one representative batch culture using planktonic cells. The dotted line separates growth and nongrowth associated lactic acid production. (b) Growth and lactic acid production parameters as deduced from batch experiments ($n = 2$) [Color figure can be viewed at wileyonlinelibrary.com]

Figure 3(b) table:

Parameter	Symbol	Value	Unit
Maximum growth rate	μ_{max}	0.34 ± 0.03	1/h
Maximum non growth-associated production rate	β_{max}	0.10 ± 0.03	1/h
Maximum BDW concentration	$c(BDW)_{max}$	1.5 ± 0.2	g/L
Biomass yield coefficient	Y_{XS}	0.06 ± 0.01	g/g
Product yield coefficient	Y_{PS}	0.87 ± 0.01	g/g
Maximum productivity	Q_P	1.2 ± 0.2	g·L^{-1}·h^{-1}

the major known bottlenecks in conventional lactic acid production, namely low biomass concentration and high residual substrate concentration due to product inhibition as well as short fermentation times for growth associated production, and substantiate the need for alternative production processes.

4.2 | Continuous lactic acid production in the TBR

4.2.1 | Biofilm visualization and analysis

Biofilm growth started at the bottom of the TBR over its entire length, indicating that gravity promoted initial cell attachment. From there, biofilm grew upwards until after 4 days of cultivation the entire cross sectional area of the TBR was covered (data not shown). At the end of the cultivation period, the TBR was analyzed by MRI (Figures 4a,b). The drainage during sample preparation led to the detachment of 22% of the total biofilm biomass as determined by weighing. To consider the lost biomass within the simulation, this amount was uniformly added to the measured values of biofilm thickness $L_{F\,meas}$, as deduced from the MR images. These corrected values are subsequently referred to as $L_{F\,corr}$. Consequently, the analysis of MR images revealed a final mean biofilm thickness $L_{F\,corr}$ of approx. 1,350 μm before drainage. However, $L_{F\,corr}$ is subject to large variations over the reactor length. Whereas the mean thickness averages 2,300 μm at the beginning of the glass TBR, it only amounts to approximately 850 μm in the two thirds downstream. The decrease of $L_{F\,corr}$ might reflect a possible growth inhibition that will be discussed below.

The biofilm grown at the inlet of the TBR was visualized by CLSM (Figure 5). Subsequent studies showed that the structure of the biofilm is independent of the position along the TBR (data not shown). Staining of the glycoconjugates revealed only a small amount of extracellular polymeric substances (EPS) consisting mostly of cell wall bound glycopolymers of the glycocalyx (Staudt, Horn, Hempel, & Neu, 2003). The inner cohesion of the biofilm might be additionally
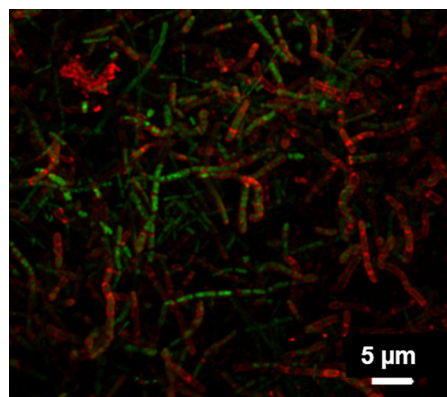
FIGURE 5 Confocal laser scanning microscopy image of 27 days old biofilm. Green: nucleic acid stain (SYTO60); red: glycoconjugates stain (BanLec) [Color figure can be viewed at wileyonlinelibrary.com]

assured through cell cell adhesion by pili. A low EPS production is advantageous in biotechnological production, because the product yield is not significantly reduced as compared with planktonic cultivation.

Analysis of the biofilm by BWW and BDW determination (Section 2.3) revealed a high water content of 96.5%, and a biofilm dry density of 28.5 g/L, defined as BDW per biofilm volume. Due to the negligible amount of EPS, the high biofilm density is equivalent to a high cell density, which should have a positive effect on the productivity of the process (Dagher et al., 2010; Halan et al., 2012; John et al., 2007).

4.2.2 | Biofilm productivity

The productivity of the biofilm Q_P over the course of the cultivation and the corresponding pH values measured at the outlet of the TBR are depicted in Figure 6a,b. Values were obtained for three different flow velocities. In each case, lactic acid productivity reached a stable value after a short start up phase and could be maintained for the
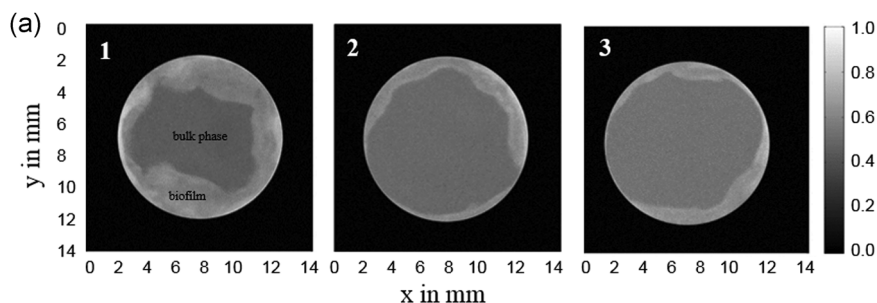
Position	1	2	3
Mean $L_{F\,corr}$ in μm	2300	850	850
Minimum $L_{F\,corr}$ in μm	200	0	50
Maximum $L_{F\,corr}$ in μm	3750	1750	1750

FIGURE 4 (a) Magnetic resonance images of 27 days old biofilm at the beginning (position 1: 70 mm), the middle (position 2: 200 mm) and the end (position 3: 330 mm) of the biofilm reactor. (b) Mean, minimum, and maximum values for the corrected biofilm thickness $L_{F\,corr}$
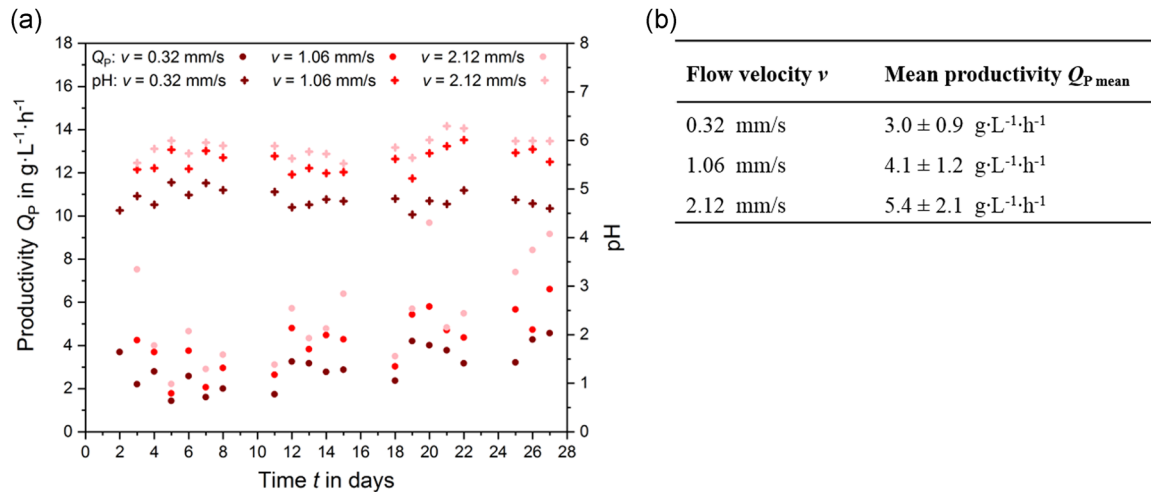
FIGURE 6 Biofilm productivity Q_P and corresponding pH value in the bulk liquid phase measured at the outlet of the reactor over the course of the cultivation for three different flow velocities v. Mean productivities $Q_{P\,mean}$ for each flow velocity v are indicated [Color figure can be viewed at wileyonlinelibrary.com]

rest of the cultivation period of approx. 3 weeks, proving the suitability of the system for long term cultivation. The variations in Q_P over time can be explained by occasional sloughing events of the biofilm, leading to certain variations in biofilm thickness. It was clearly shown that the mean productivity $Q_{P\,mean}$ increased with the flow velocity. As will be later on revealed by the simulation results (Section 4.2.3), the main impact of the flow velocity is the magnitude of pH change along the reactor length. At the lowest flow velocity (0.32 mm/s), the lactic acid production leads to a strong pH drop to a value of 4.5 at the outlet of the TBR. At the highest velocity (2.12 mm/s) in contrast, the pH can be maintained close to the optimum pH value of 6.0 throughout all three reactor segments. As additional batch experiments with planktonic cells showed, there is a significant correlation between pH value and productivity: the lactic acid production rate achieved at a pH of 6.0 was diminished by $25 \pm 15\%$ at a pH value of 5 and even by $67 \pm 5\%$ at a value of 4.5 as compared with the maximum productivity achieved. At a pH of 4.0, production was completely prevented (data not shown). Furthermore, deviation of the pH from its optimum value has a negative impact on the growth rate itself and cell growth completely ceases below a pH value of 4 (Stenroos, Linko, & Linko, 1982). Since lactic acid production leads to a strong pH gradient along the TBR, pH inhibition, thus, also explains the decrease in biofilm thickness over the reactor length as observed by MRI (Section 4.2.1).

The maximum productivity, achieved at a flow velocity of 2.12 mm/s, reached a value of 7–10 g·L^{-1}·hr^{-1} with a high product yield of 0.94 ± 0.06 g/g. In comparison to the batch culture with planktonic cells as used in industrial production, the maximum productivity in the biofilm system could hence be increased by a factor of 6–8. As this productivity increase is ascribed to the high cell density and not to the continuous operation itself, alternative continuous processes without some sort of cell retention are considered uncompetitive, especially since product concentrations have to be kept below the inhibitory level. The production rates for

pure cultures, achieved recently in different operational systems using biofilms on carrier material, vary between 4.2 and 8.95 g·L^{-1}·hr^{-1} (Cotton, Pometto, & Gvozdenovic Jeremic, 2001; Rangaswamy & Ramakrishna, 2008; Velázquez et al., 2001). However, a direct comparison of the different processes is hardly possible, as the volumetric productivity varies highly with the reactor design, the surface to volume ratio (SA/V) being a major factor determining reactor efficiency. The flux is typically used as a parameter describing biofilm turnover performance. It is defined as product transport out of the biofilm per biofilm surface area and amounts to 324 g·m^{-2}·d^{-1} in this experiment. Therefore, adapting the SA/V and overcoming the pH limitation hold potential for further increasing the volumetric productivity in this application.

4.2.3 | Simulation results

The measured and predicted consumption rates of glucose Q_S, the production rates of lactic acid Q_P and the pH values in the bulk liquid are depicted over the course of the cultivation in Figure 7a. All data refer to the lowest flow velocity of 0.32 mm/s. The increase of Q_S and Q_P, and the decrease of the pH value result from the process of biofilm growth. To avoid the complexity of the model, the occasional, inevitable sloughing events are not taken into account, and the variations in Q_S and Q_P are, thus, approximated by a steady increase. Apart from the start up phase of approx. 2 days, the simulation can satisfactorily represent the general trend of the data. Figure 7b shows the comparison between measured (given as $L_{F\,corr}$) and predicted data for the biofilm thickness L_F. As experimental data are only available at the end of the experiment, the development of biofilm thickness over time is not verified. However, as the modeled substrate and product concentrations (Figure 7a) depend on the biomass present, the model is coherent in itself. The model predicts the decreasing thickness over the reactor length due to the increasing
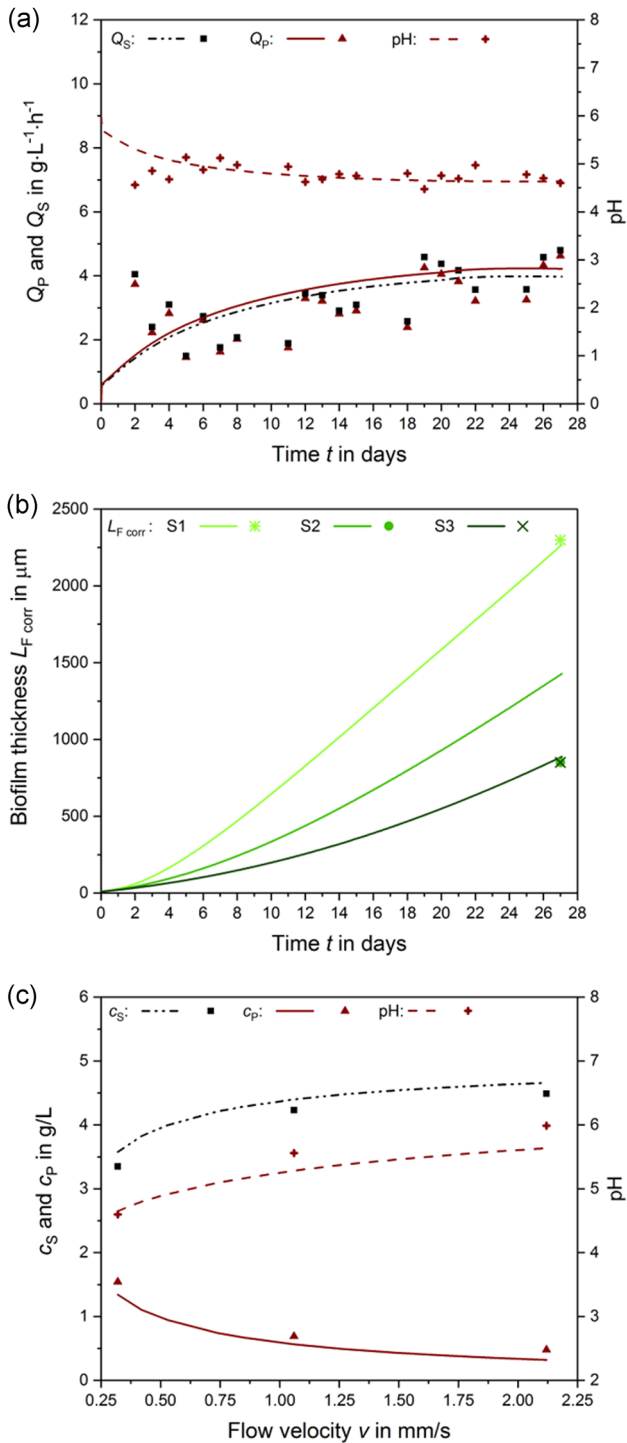
FIGURE 7 Comparison of experimentally determined and predicted parameters in the bulk liquid phase over the course of cultivation ($v = 0.32$ mm/s): (a) Glucose consumption and lactic acid production rates Q_S and Q_P, as well as pH value. (b) Biofilm thickness $L_{F\,corr}$ in the three interconnected segments S1 to S3. (c) Substrate and lactic acid concentrations (c_S and c_P) and pH value as a function of flow velocity at Day 27 [Color figure can be viewed at wileyonlinelibrary.com]

pH inhibition. However, as sloughing is not considered, the model assumes a constant decrease, and therefore overestimates L_F in the second segment of the TBR, which was determined to be similar to the one of the third segment (cf. Figure 4). Figure 7c

shows the influence of the flow velocity on the concentrations c_S and c_P, and the resulting pH values obtained in a short term experiment on Day 27. The figure confirms the previous statement (Figure 6) that the major impact of the flow velocity is the magnitude of pH change along the reactor length: The smaller the flow velocity, the higher the lactic acid concentration and, thus, pH drop and the more significant the pH inhibition. The experimental values can be satisfactorily represented by the model, which further confirms its validity.

After comparison against the experimental data, the model was used to predict data that are difficult to obtain experimentally. The changes of Q_S and Q_P, which could only be determined as integral values at the outlet of the TBR, were calculated over the reactor length and are depicted in Figure 8a. It can be seen that in each segment of the TBR, the substrate consumption rate is only slightly inferior to the production rate, as a result of the aforementioned high product yield. In the first segment, Q_P increases with the cultivation time due to the growth of biofilm, and thus increase of biomass. However, the increase decelerates after approx. 4 days of cultivation and from Day 23 on, Q_P even starts dropping. It seems that when a certain biofilm thickness is reached, mass transport limitations of lactic acid out of the biofilm lead to pH inhibition within the film, which causes the productivity increase to slow down. The simulation clearly shows that already from Day 23 on, additional mass transport limitations of glucose occur, resulting in a substrate depletion towards the substratum (data not shown, cf. c_S in Figure 8b). The amount of productive biomass is, thus, reduced, causing the observed productivity decline. In the second and third segment, Q_P follows cell growth, indicating that in the thinner biofilm, mass transport limiting conditions are minor.

Substrate and product concentrations c_S and c_P, and pH values inside the biofilm at the end of the cultivation period (Day 27) are shown for S1 (Figure 8b) and S3 (Figure 8c) at the lowest and the highest flow velocity (0.32 and 2.12 mm/s). In the first segment of the TBR, the biofilm thickness of 2,300 μm leads to limiting conditions close to the substratum for both flow velocities. Whereas substrate is depleted at a biofilm depth of approx. 1,800 μm (within the first 500 μm biofilm above the substratum), the pH decreases to a value of 4.0 already at a depth of approx. 1,000 μm (1,300 μm above the substratum). As lactic acid production is prevented at pH < 4.0, almost two thirds of the biofilm volume in this segment do not contribute to production, leading to reduced specific productivity as compared with planktonic cells. In segment S3, with a mean biofilm thickness of 850 μm, glucose concentration is not limiting the turnover processes and the pH drop is limited to values > 4.0 throughout the whole biofilm depth. However, the lactic acid concentration at the substratum of 2.2 – 2.5 g/L creates pH values between 4.2 and 4.4, a range where pH inhibition already occurs and lactic acid production is significantly decelerated. Production is thus strongly inhibited by mass transport limitations, where the insufficient product removal is by far more significant than the substrate supply. Increasing the flow velocity from 0.32 to 2.12 mm/s results in a minor pH drop in the bulk phase and increases the mass transfer of product out of the biofilm. This has an impact on the lactic acid
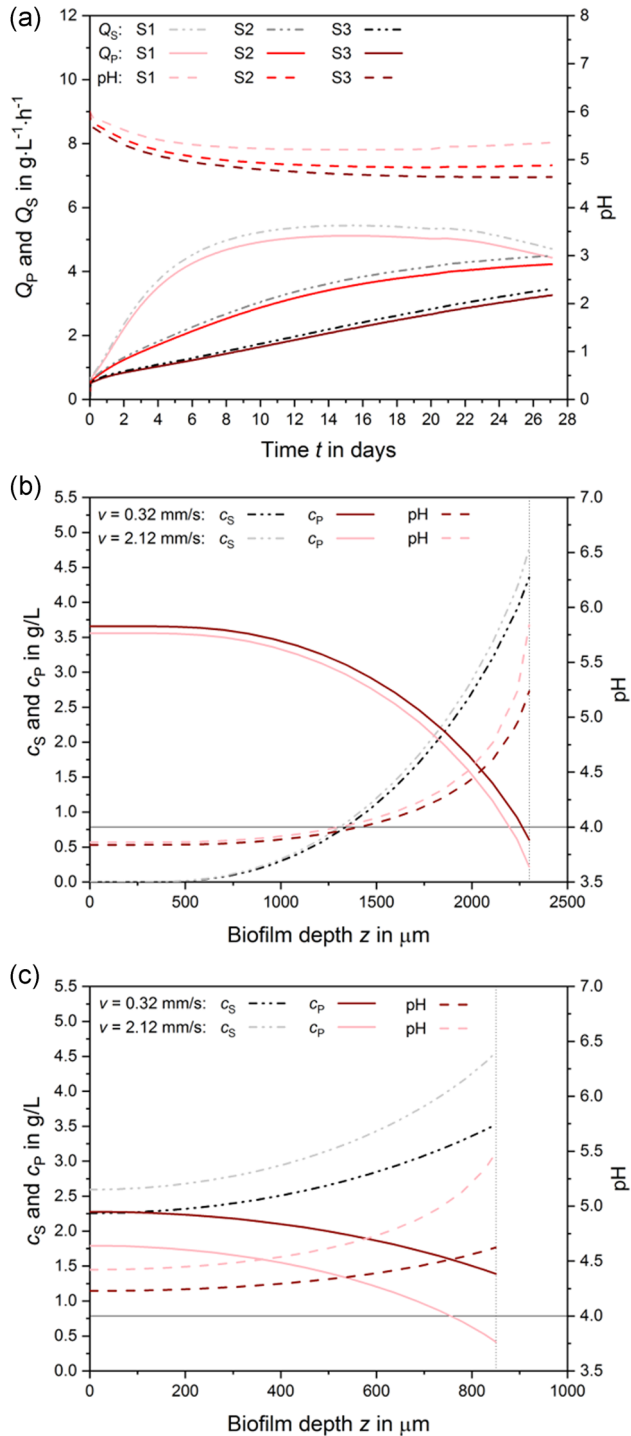
FIGURE 8 (a) Predicted values of Q_S, Q_P, and pH over the course of the cultivation along the reactor length (segments S1 to S3). Comparison of c_S, c_P, and pH inside the biofilm at Day 27 for a flow velocity of 0.32 and 2.12 mm/s in segment S1 (b) and S3 (c). Dotted lines represent the biofilm thickness $L_{F\,corr}$ in S1 and S3 as measured by magnetic resonance imaging. The pH value of 4.0 is highlighted by a straight line [Color figure can be viewed at wileyonlinelibrary.com]

magnitude of the encountered limitations is decreased, which confirms the previous statement that the productivity increase is due to an improved mass transport. To prevent a significant productivity decline, biofilm thickness should therefore be limited to 500 μm in the present system. The simulation shows the importance of considering the axial gradients in a plug flow reactor. The gradual increase of product concentration and, thus, growth inhibition has a significant impact on biofilm thickness and biofilm productivity over the reactor length, which cannot be represented by a model assuming a completely mixed reactor as is still the current state of the art (Horn et al., 2003; Rittmann et al., 2018).

Figure 8 illustrates that the inhibition of lactic acid productivity is mainly due to excessive biofilm thickness and the resulting mass transport limitations, causing substrate depletion and product accumulation and hence pH drop. Biofilm thickness, however, can be controlled, for example, by modifying the flow velocity: Since higher shear forces result in biofilm erosion, an "optimum" biofilm thickness can be adjusted (Costerton, Lewandowski, Caldwell, Korber, & Lappin Scott, 1995). In this regard, the uniform flow conditions in a TBR are advantageous as compared with other cultivation processes (e.g. fixed bed reactor). The study thus confirms the control of biofilm thickness as a key factor in productive biofilm processes. An additional pH regulation over the reactor length could also contribute to a significant increase of the achieved turnover rates.

5 CONCLUSIONS

Our results lead to the following conclusions:

(i) A strain of *L. delbrueckii*, although initially selected for high lactic acid production in planktonic state, can be cultivated in a biofilm. Biofilm formation and cultivation was achieved without preliminary immobilization steps in a glass TBR, a very simple system and allowing good insight.

(ii) The cell density in the biofilm system was increased by a factor of 19 as compared with the planktonic culture. This resulted in an increased volumetric productivity by a factor of 6–8 when working at the very low substrate and product concentrations. Control of biofilm thickness to overcome the pH inhibition resulting from lactic acid production and optimization of the dimensions (e.g. increasing the specific surface area) and design of the cultivation system present a high potential for further productivity increase.

(iii) Lactic acid production reached 80% of the maximum value after 10 days and could be maintained for a period of more than 2 weeks, proving the suitability of the biofilm system for long term cultivation.

(iv) A simple model for cell growth and lactic acid production within a biofilm was developed for the first time, which deepens the understanding of the biofilm system and can facilitate future optimization.

concentrations and the pH values inside the biofilm, especially at low depths. The importance of this effect increases axially along the reactor length so that the influence of the velocity is further pronounced towards the outlet of the TBR. Consequently, the

REFERENCES

Al-Kaidy, H., Duwe, A., Huster, M., Muffler, K., Schlegel, C., Sieker, T., ... Ulber, R. (2015). Biotechnology and bioprocess engineering From the first Ullmann's article to recent trends. *ChemBioEng Reviews*, *2*(3), 175 184. https://doi.org/10.1002/cben.201500008

Brading, M. G., Jass, J., & Lappin-Scott, H. M. (1995). Dynamics of bacterial biofilm formation. In H. M. Lappin-Scott, & J. W. Costerton (Eds.), *Microbial Biofilms* (pp. 46 63). Cambridge: Cambridge University Press.

Brink, H. G., & Nicol, W. (2014). The influence of shear on the metabolite yield of *Lactobacillus rhamnosus* biofilms. *New Biotechnology*, *31*(5), 460 467.

Callaghan, P. T. (1993). *Principles of nuclear magnetic resonance microscopy*. Clarendon Press.

Characklis, W. G., & Marshall, K. C. (1990). *Biofilms*. Wiley.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annual Reviews in Microbiology*, *49*(1), 711 745.

Cotton, J., Pometto, A., & Gvozdenovic-Jeremic, J. (2001). Continuous lactic acid fermentation using a plastic composite support biofilm reactor. *Applied Microbiology and Biotechnology*, *57*(5-6), 626 630. https://doi.org/10.1007/s002530100820

Dagher, S. F., Ragout, A. L., Siñeriz, F., & Bruno-Bárcena, J. M. (2010). Cell immobilization for production of lactic acid: Biofilms do it naturally. *Advances in Applied Microbiology*, *71*, 113 148.

De Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, *23*(1), 130 135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

Demirci, A., & Pometto, A. L., III (1995). Repeated-batch fermentation in biofilm reactors with plastic-composite supports for lactic acid production. *Applied Microbiology and Biotechnology*, *43*(4), 585 589. https://doi.org/10.1007/BF00164758

Fan, R., Ebrahimi, M., Quitmann, H., & Czermak, P. (2015). Lactic acid production in a membrane bioreactor system with thermophilic Bacillus coagulans:Fouling analysis of the used ceramic membranes. *Separation Science and Technology*, *50*(14), 2177 2189. https://doi.org/10.1080/01496395.2015.1031401.

Gao, T., Wong, Y., Ng, C., & Ho, K. (2012). L-lactic acid production by *Bacillus subtilis* MUR1. *Bioresource Technology*, *121*, 105 110. https://doi.org/10.1016/j.biortech.2012.06.108

Goncalves, L., Xavier, A., Almeida, J., & Carrondo, M. (1991). Concomitant substrate and product inhibition kinetics in lactic acid production. *Enzyme and Microbial Technology*, *13*(4), 314 319.

Gross, R., Hauer, B., Otto, K., & Schmid, A. (2007). Microbial biofilms: New catalysts for maximizing productivity of long-term biotransformations. *Biotechnology and Bioengineering*, *98*(6), 1123 1134.

Gross, R., Schmid, A., & Buehler, K. (2012). Catalytic biofilms: A powerful concept for future bioprocesses. In G. Lear, & G. D. Lewis (Eds.), *Microbial biofilm: Current research and applications* (pp. 193 222). Norfolk, UK: Caister Academic Press.

Halan, B., Buehler, K., & Schmid, A. (2012). Biofilms as living catalysts in continuous chemical syntheses. *Trends in Biotechnology*, *30*(9), 453 465.

Herrling, M. P., Guthausen, G., Wagner, M., Lackner, S., & Horn, H. (2015). Determining the flow regime in a biofilm carrier by means of magnetic resonance imaging. *Biotechnology and Bioengineering*, *112*(5), 1023 1032. https://doi.org/10.1002/bit.25510

Ho, K., Pometto, A. L., & Hinz, P. N. (1997). Optimization of L-(+)-lactic acid production by ring and disc plastic composite supports through repeated-batch biofilm fermentation. *Applied and Environmental Microbiology*, *63*(7), 2533 2542.

Horn, H., & Hempel, D. C. (1997). Growth and decay in an auto-/heterotrophic biofilm. *Water Research*, *31*(9), 2243 2252. https://doi.org/10.1016/S0043-1354(97)00081-X

Horn, H., Reiff, H., & Morgenroth, E. (2003). Simulation of growth and detachment in biofilm systems under defined hydrodynamic conditions. *Biotechnology and Bioengineering*, *81*(5), 607 617. https://doi.org/10.1002/bit.10503

John, R. P., Nampoothiri, K. M., & Pandey, A. (2007). Polyurethane foam as an inert carrier for the production of L(+)-lactic acid by *Lactobacillus casei* under solid-state fermentation. *Letters in Applied Microbiology*, *44*(6), 582 587. https://doi.org/10.1111/j.1472-765X.2007.02137.x

Karel, S. F., Libicki, S. B., & Robertson, C. R. (1985). The immobilization of whole cells: Engineering principles. *Chemical Engineering Science*, *40*(8), 1321 1354. https://doi.org/10.1016/0009-2509(85)80074-9

Kim, D. H., Lee, M. K., Hwang, Y., Im, W. T., Yun, Y. M., Park, C., & Kim, M. S. (2016). Microbial granulation for lactic acid production. *Biotechnology and Bioengineering*, *113*(1), 101 111. https://doi.org/10.1002/bit.25540

Kimmich, R. (1997). *NMR: Tomography, diffusometry, relaxometry*. Berlin, Heidelberg: Springer.

Kumar Dutta, S., Mukherjee, A., & Chakraborty, P. (1996). Effect of product inhibition on lactic acid fermentation: Simulation and modelling. *Applied Microbiology and Biotechnology*, *46*(4), 410 413. https://doi.org/10.1007/bf00166238

Kwon, S., Yoo, I.-K., Lee, W. G., Chang, H. N., & Chang, Y. K. (2001). High-rate continuous production of lactic acid by *Lactobacillus rhamnosus* in a two-stage membrane cell-recycle bioreactor. *Biotechnology and Bioengineering*, *73*(1), 25 34.

Li, X. Z., Webb, J. S., Kjelleberg, S., & Rosche, B. (2006). Enhanced benzaldehyde tolerance in Zymomonas mobilis biofilms and the potential of biofilm applications in fine-chemical production. *Applied and Environmental Microbiology*, *72*(2), 1639 1644. https://doi.org/10.1128/aem.72.2.1639-1644.2006

Liu, B., Maohua, Y., Qi, B., Chen, X., Su, Z., & Yinhua, W. (2010). Optimizing L-(+)-lactic acid production by thermophile *Lactobacillus plantarum* As.1.3 using alternative nitrogen sources with response surface method. *Biochemical Engineering Journal*, *52*(2/3), 212 219.

Luedeking, R., & Piret, E. L. (1959). A kinetic study of the lactic acid fermentation. Batch process at controlled pH. *Journal of Biochemical and Microbiological Technology and Engineering*, *1*(4), 393 412. https://doi.org/10.1002/jbmte.390010406

Meng, Y., Xue, Y., Yu, B., Gao, C., & Ma, Y. (2012). Efficient production of L-lactic acid with high optical purity by alkaliphilic *Bacillus* sp. WL-S20. *Bioresource Technology*, *116*, 334 339. https://doi.org/10.1016/j.biortech.2012.03.103

Monod, J. (1949). The growth of bacterial cultures. *Annual Review of Microbiology*, *3*, 371 394. https://doi.org/10.1146/annurev.mi.03.100149.002103

Muffler, K., & Ulber, R. (2014). *Productive biofilms*. Berlin: Springer International Publishing.

Neu, T. R., & Lawrence, J. R. (2014). Investigation of microbial biofilm structure by laser scanning microscopy. *Advances in Biochemical Engineering/ Biotechnology*, *146*, 1 51. https://doi.org/10.1007/10 2014 272

Neu, T. R., Manz, B., Volke, F., Dynes, J. J., Hitchcock, A. P., & Lawrence, J. R. (2010). Advanced imaging techniques for assessment of structure, composition and function in biofilm systems. *FEMS Microbiology Ecology*, *72*(1), 1 21. https://doi.org/10.1111/j.1574-6941.2010.00837.x

Posten, C. (2018). *Integrated bioprocess engineering*. Walter der Gruyter GmbH.

Rangaswamy, V., & Ramakrishna, S. (2008). Lactic acid production by *Lactobacillus delbrueckii* in a dual reactor system using packed bed biofilm reactor. *Letters in Applied Microbiology*, *46*(6), 661 666.

Reichert, P. (1994). AQUASIM   A tool for simulation and data analysis of aquatic systems. *Water Science and Technology*, *30*(2), 21 30. https://doi.org/10.2166/wst.1994.0025

Ren, J. (2010). *Biodegradable poly(lactic acid): Synthesis, modification, processing and applications*. Beijing; Heidelberg: Tsinghua University Press; Springer.

Ribeiro, A. C. F., Lobo, V. M. M., Leaist, D. G., Natividade, J. J. S., Veríssimo, L. P., Barros, M. C. F., & Cabral, A. M. T. D. P. V. (2005). Binary diffusion coefficients for aqueous solutions of lactic acid. *Journal of Solution Chemistry*, *34*(9), 1009 1016. https://doi.org/10.1007/s10953-005-6987-3

Ribeiro, A. C. F., Ortona, O., Simões, S. M. N., Santos, C. I. A. V., Prazeres, P. M. R. A., Valente, A. J. M., & Burrows, H. D. (2006). Binary mutual diffusion coefficients of aqueous solutions of sucrose, lactose, glucose, and fructose in the temperature range from (298.15 to 328.15) K. *Journal of Chemical & Engineering Data*, *51*(5), 1836 1840. https://doi.org/10.1021/je0602061

Rittmann, B. E., Boltz, J. P., Brockmann, D., Daigger, G. T., Morgenroth, E., Sørensen, K. H., & Vanrolleghem, P. A. (2018). A framework for good biofilm reactor modeling practice (GBRMP). *Water Science and Technology*, *77*(5), 1149 1164. https://doi.org/10.2166/wst.2018.021

Rosche, B., Li, X. Z., Hauer, B., Schmid, A., & Buehler, K. (2009). Microbial biofilms: A concept for industrial catalysis? *Trends in Biotechnology*, *27*(11), 636 643.

Sahm, H., Antranikian, G., Stahmann, K. P., & Takors, R. (2014). *Industrielle mikrobiologie*. Berlin, Heidelberg: Springer.

Santek, B., Ivancić, M., Horvat, P., Novak, S., & Marić, V. (2006). Horizontal tubular bioreactors in biotechnology. *Chemical and Biochemical Engineering Quarterly*, *20*(4.

Skoneczny, S., & Tabiś, B. (2015). The method for steady states determination in tubular biofilm reactors. *Chemical Engineering Science*, *137*, 178 187. https://doi.org/10.1016/j.ces.2015.06.024

Staudt, C., Horn, H., Hempel, D. C., & Neu, T. R. (2003). Screening of lectins for staining lectin-specific glycoconjugates in the EPS of biofilms. In P. Lens, A. P. Moran, T. Mahony, & P. Stoodley (Eds.), *Biofilms in medicine, industry and environmental biotechnology* (pp. 308 326). London, United Kingdom: IWA Publishing.

Stenroos, S.-L., Linko, Y.-Y., & Linko, P. (1982). Production of L-lactic acid with immobilized *Lactobacillus delbrueckii*. *Biotechnology Letters*, *4*(3), 159 164. https://doi.org/10.1007/BF00144317

Storhas, W. (2013). *Bioverfahrensentwicklung*. Wiley-VCH.

Tsoligkas, A. N., Winn, M., Bowen, J., Overton, T. W., Simmons, M. J. H., & Goss, R. J. M. (2011). Engineering biofilms for biocatalysis. *ChemBio-Chem*, *12*(9), 1391 1395.

Velázquez, A., Pometto Iii, A. L., Ho, K.-L. G., & Demirci, A. (2001). Evaluation of plastic-composite supports in repeated fed-batch biofilm lactic acid fermentation by *Lactobacillus casei*. *Applied Micro-biology and Biotechnology*, *55*(4), 434 441.

Wagner, M., Manz, B., Volke, F., Neu, T. R., & Horn, H. (2010). Online assessment of biofilm development, sloughing and forced detachment in tube reactor by means of magnetic resonance microscopy. *Biotechnology and Bioengineering*, *107*(1), 172 181. https://doi.org/10.1002/bit.22784

Wanner, O., & Morgenroth, E. (2004). Biofilm modeling with AQUASIM. *Water Science and Technology*, *49*(11-12), 137 144.

Wanner, O., & Reichert, P. (1996). Mathematical modeling of mixed-culture biofilms. *Biotechnology and Bioengineering*, *49*(2), 172 184. https://doi.org/10.1002/(SICI)1097-0290(19960120)49:2<172::AID-BIT6>3.0.CO;2-N

Yabannavar, V. M., & Wang, D. I. (1991). Analysis of mass transfer for immobilized cells in an extractive lactic acid fermentation. *Biotechnology and Bioengineering*, *37*(6), 544 550. https://doi.org/10.1002/bit.260370608

Yeh, P. L.-H., Bajpai, R. K., & Iannotti, E. L. (1991). An improved kinetic model for lactic acid fermentation. *Journal of Fermentation and Bioengineering*, *71*(1), 75 77. https://doi.org/10.1016/0922-338X(91)90309-5

Repository KITopen