



## Succinic Acid Production by *Actinobacillus succinogenes* in Chemostat and Biofilm Cultures

Hendrik G. Brink, Willie Nicol\*

University of Pretoria, Department of Chemical Engineering, University of Pretoria Main Campus, Corner Lynwood & Roper Street, Hatfield, Pretoria, 0002, South Africa  
 willie.nicol@gmail.com

Previously published results from a novel, homogeneously distributed shear, tubular fermenter used to study continuous chemostat (high shear) and biofilm (low shear) fermentations (Brink and Nicol, 2014a), were compared to results obtained in the same reactor at intermediate shear conditions as well as batch (biofilm) fermentations of *Actinobacillus succinogenes*.

It was found that the steady-state volumetric production rates increased by nearly an order of magnitude ( $1.8 \text{ g.L}^{-1}.\text{h}^{-1}$  vs  $15\text{--}17 \text{ g.L}^{-1}.\text{h}^{-1}$ ) as the shear was reduced from the chemostat ( $1.83 \text{ m.s}^{-1}$ ) to the lower shear biofilm conditions. The biofilm results indicated similar volumetric production rates for the different shear conditions, while the measured extracellular polymeric substances (EPS) in the biofilm exhibited a significant shear dependence; EPS fractions of  $0.50 \pm 0.05 \text{ g.g}^{-1}$  vs.  $0.16 \pm 0.02 \text{ g.g}^{-1}$  for the low and intermediate shear conditions respectively. The cell-based biofilm production rates were shown to be dependent on shear conditions at succinic acid titres less than the growth-maintenance boundary ( $10 \text{ g.L}^{-1}$ ), with a reduction in cellular production rate associated with an increase in shear. Under maintenance conditions the cell-based production rates appeared independent of the shear conditions in the fermenter, with the cell-based production rates decreasing with increasing succinic acid titres. The industrial implications are that for succinic acid titres in excess of  $10 \text{ g.L}^{-1}$ , the same mass of biomass under intermediate shear conditions should exhibit a greater volumetric production rate due to the higher fraction of cells as opposed to EPS.

The initial values for the batch cell-based succinic acid production rates corresponded to the steady-state values for biofilms grown under the same continuous conditions. However, during transient operation the production rates exceeded the steady-state values; a lag in the product inhibition response was observed. The transient production rates eventually ceased at succinic acid titres in excess of approximately  $60\text{--}72 \text{ g.L}^{-1}$ ; a residual glucose concentration of  $20 \text{ g.L}^{-1}$  was measured at the highest succinic acid titre ( $72 \text{ g.L}^{-1}$ ), indicating product related inhibition as opposed to substrate depletion. The transient results indicate that a larger average cell-based production rate can be obtained during transient operation when compared to the steady fermentation conditions due to a lag in the succinic acid inhibition during transient operation.

### 1. Introduction

Succinic acid (SA) has been identified as one of the US Department of Energy's "Top 10" biobased products for the replacement or supplementation of current petrochemically produced building block chemicals (Bozell and Petersen, 2010). The desirability of SA as integral platform chemical stems from the ease of fermentative production as well as the variety of chemicals to be produced (Werpy and Petersen, 2004), the chemicals include butanediol, tetrahydrofuran and gamma-butyrolactone (Kurzrock and Weuster-Botz, 2010) as well as the biodegradable polyester polybutylene succinate (Xu and Guo, 2010).

*A. succinogenes* is the most prominent wild strain used for the production of SA (Vlysidis et al., 2009; Brink and Nicol, 2014a). The popularity of this strain is due to its ability to metabolise most naturally occurring sugars (McKinlay et al., 2010; De Baros et al., 2013). Additionally the strain has shown a yield SA on glucose of 94%, SA titres close to the saturation point ( $> 95 \text{ g.L}^{-1}$ ) (Guettler et al., 1996) and volumetric production rates, during

continuous biofilm operation, in excess of  $10 \text{ g.L}^{-1}.\text{h}^{-1}$  (Brink and Nicol, 2014a; Maharaj et al., 2014). *A. succinogenes* is recognised as a biofilm forming microbe with unavoidable attachment to solid surfaces reported in all continuous *A. succinogenes* studies (Bradfield and Nicol, 2014; Brink and Nicol, 2014a; Maharaj et al., 2014; Urbance et al., 2004; Van Heerden and Nicol, 2013; Yan et al., 2014b), except for the external membrane separation study by Kim et al. (2009). The limited fermentation times in this study, however, indicated significant blockages of the membrane during fermentation as a result of biofilm formation. The use of an *A. succinogenes* biofilm fermenter can therefore be considered the only viable method of increasing the cellular biomass within the reactor in order increase the SA production rates. Currently only a limited number of continuous *A. succinogenes* biofilm studies have been published (Bradfield and Nicol, 2014; Brink and Nicol, 2014a; Maharaj et al., 2014; Urbance et al., 2004; Van Heerden and Nicol, 2013; Yan et al., 2014b). All these studies showed a significant improvement in volumetric production rates, with values higher than  $10 \text{ g.L}^{-1}.\text{h}^{-1}$  reported (Brink and Nicol, 2014a; Maharaj et al., 2014). The significant improvement in volumetric production rates were a result of the elevated biomass concentrations in the biofilm reactors, however, the increases did not necessarily correlate with the increased biomass concentrations (Urbance et al., 2004; Maharaj et al., 2014; Yan et al., 2014b), which were possibly a result of cellular death due to extended operational times and diffusional limitations within the biofilm, or significant fractions of EPS within the biofilm as a result of lower shear forces acting on the biofilms (Bradfield and Nicol, 2014; Brink and Nicol, 2014a; Maharaj et al., 2014; Urbance et al., 2004).

The theory explaining the underlying mechanisms that govern biofilm structures involve the balance between growth in the biofilm and detachment forces on the biofilm (Van Loosdrecht et al., 1995). The growth of biofilms depend on the diffusional gradients present within the biofilm (Wimpenny and Colasanti, 1997), high concentration gradients result in porous or filamentous biofilms, while low gradients result in more homogenously distributed biofilms. In contrast, the shear conditions acting on a biofilm removes the biofilm outer-layers and filamentous appendages, which results in smoother biofilm surfaces and therefore a more two-dimensional geometry. Higher shear also increases the turbulence and therefore mixing in the environment, which inherently leads to smaller external concentration gradients. The combination of smoother biofilm surfaces and smaller concentration gradients means that high shear tends to result in more homogenous biofilms (Paul et al., 2012; Van Loosdrecht et al., 1995).

The current study attempts to elucidate the influence of shear on the biofilm production characteristics by comparing previously published continuous results obtained at chemostat and low shear conditions (Brink and Nicol, 2014a) to new results obtained at intermediate shear conditions. The comparison is done with respect to the global (volumetric) and intrinsic (cell-based) production rates in both chemostat and biofilm reaction conditions. The biofilm composition is further estimated and compared with respect to the shear conditions. Finally the cellular production characteristics of biofilms grown at steady-state conditions and tested under batch conditions, are presented.

## 2. Materials and methods

### 2.1 Microorganism and growth medium

*A. succinogenes* 130Z (DSM 22257 or ATCC 55618) was acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ). Details on the growth conditions and growth media can be found in Brink and Nicol (2014a).

### 2.2 Media

The growth medium consisted of ( $\text{g.L}^{-1}$ ): YE: 6; clarified CSL (Bradfield and Nicol, 2014): 10; NaCl: 1.0,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ : 0.2,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ : 0.2, sodium acetate: 1.36,  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ : 0.16;  $\text{KH}_2\text{PO}_4$ : 3.2;  $\text{K}_2\text{HPO}_4$ : 1.6; D-glucose: 40; Antifoam A:  $1 \text{ mL.L}^{-1}$ . Details on the fermentation medium used can be found in Brink and Nicol (2014a). The same medium was used for batch fermentation, with the exception that initial glucose concentrations ( $C_{\text{Glc}0}$ ) of  $70\text{--}120 \text{ g.L}^{-1}$  was used (see Table 1).

### 2.3 Experimental setup and operation

For a complete description of the experimental setup refer to Brink and Nicol (2014a). The shear velocity used for the continuous/steady-state experiments were  $0.36 \text{ m.s}^{-1}$  ( $\text{Re} \approx 1600$  respectively, i.e. fully laminar flow). The time averaged rate of NaOH dosed, for pH control, was monitored continuously and used as an indication of steady-state in the system. To ensure steady-state in the reactor, the effluent was analysed twice with at least two volume turnovers between samples, when the rate of NaOH dosing fluctuated less than 5%.

The batch operation involved the growth of a biofilm under continuous operation at a shear velocity of  $0.09 \text{ m.s}^{-1}$  for approximately 2–3 days ( $\Delta t$ ) at a high dilution rate (D) ( $0.76\text{--}1.22 \text{ h}^{-1}$ ) under steady-state conditions. The

feed was subsequently ceased, i.e. batch mode was initiated in the reactor. The NaOH dosage was monitored and the results calibrated to the SA produced, measured in periodic samples from the reactor volume. Table 1 shows the continuous growth conditions of the respective batch runs.

*Table 1: Steady-state growth conditions of the respective batch runs*

Batch run	1	2	3	4
D (h <sup>-1</sup> )	1.22	0.97	0.76	1.07
C <sub>Glc0</sub> (g.L <sup>-1</sup> )	70	100	100	120
Δt (h)	46.33	66.88	41.00	45.51

## 2.4 Analytical Methods

The organic acid and glucose analysis was performed using an HPLC with an Aminex HPX-87H column. The biomass concentrations were quantified by calibrated absorbance measurements. The EPS and viable biomass separation was performed by base-hydrolysis of the EPS matrix using a pH 9 buffer. For details on the analysis methods and equipment used during steady-state operation, refer to the description in Brink and Nicol (2014a).

## 3. Results and discussion

### 3.1 Continuous operation

The results obtained from the continuous biofilm runs, as well as the previously published data (Brink and Nicol, 2014a), are shown in Figures 1–3. Figure 1 clearly shows a significant improvement in the volumetric productivity when comparing the chemostat ( $\Delta$ ) to the biofilm runs ( $\square$  and  $\diamond$ ). The maximum production rates increased by almost an order of magnitude (1.83 g.L<sup>-1.h<sup>-1</sup> vs. 15.2–17.1 g.L<sup>-1.h<sup>-1</sup>), which were most probably a result of the significant increase in the total biomass present in the biofilm (21.75 ± 6.12 g.L<sup>-1</sup> and 18.99 ± 5.69 g.L<sup>-1</sup> for the low and intermediate shear runs respectively) as opposed to the chemostat (1.92 ± 0.64 g.L<sup>-1</sup>). The total biomass concentrations for the biofilm runs were similar and therefore it appears intuitive that the volumetric production rates would correspond for the respective biofilm runs.</sup></sup>

This, however, contradicts the results from Figure 2 which shows the measured EPS fractions, as a function of the C<sub>SA</sub>, at each of the biofilm steady-state conditions. This figure shows the incredible differences in biofilm composition for the respective biofilm runs. The lower shear biofilm (0.09 m.s<sup>-1</sup>) had an EPS composition of 0.50 ± 0.05 g.g<sup>-1</sup>, while the higher shear biofilm (0.36 m.s<sup>-1</sup>) had an EPS fraction of 0.16 ± 0.02 g.g<sup>-1</sup>. Therefore the cellular concentration for the low shear biofilm was considerably lower than the intermediate shear case. This observation corresponds well to the mechanistic model by Van Loosdrecht et al. (1995) and Wimpenny & Colasanti (1997), which predicted a lower cellular fraction (higher EPS composition) at lower shear conditions and vice versa, as a result of shear forces acting on the biofilm surface as well as diffusional gradients within the biofilm. These results are further supported by a study on single-species *Lactobacillus rhamnosus* biofilms (Brink and Nicol, 2014b) in which it was observed that an increase in shear conditions elevated the estimated fraction of active cellular biomass in the biofilm. The increased shear removed inactive biomass such as extracellular polymeric substances (EPS). The change in composition was related to a significant change in metabolic product distributions, hypothesised to be due to different redox requirements of cellular biomass and EPS-biosynthesis.

The apparent contradictions in the results were analysed in Figure 3, which shows the experimentally determined cellular production rates (EPS excluded) for the biofilm operation (r<sub>SA\*</sub>), the previously published cellular production rates fit (Eq(1)) for the chemostat operation (Brink and Nicol, 2014a), as well as the published data fit (Eq(2)) for the low shear biofilm run (Brink and Nicol, 2014a).

$$r_{SA}^* = 1.5 \left( 1 - \exp(-6.0 \exp(-0.54 C_{SA})) \right) \quad (1)$$

$$r_{SA}^* = 4.5 \exp\left(-\frac{C_{SA}}{6.8}\right) \quad (2)$$

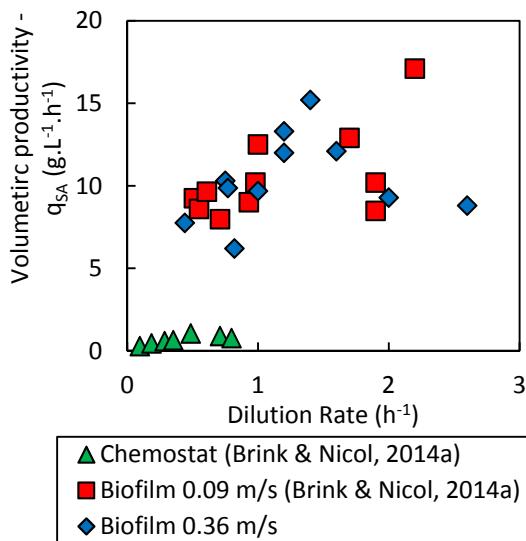


Figure 1: The volumetric production rates measured during continuous operation. The figure shows the significant improvement in volumetric production rates for the biofilm as compared to the chemostat modes.

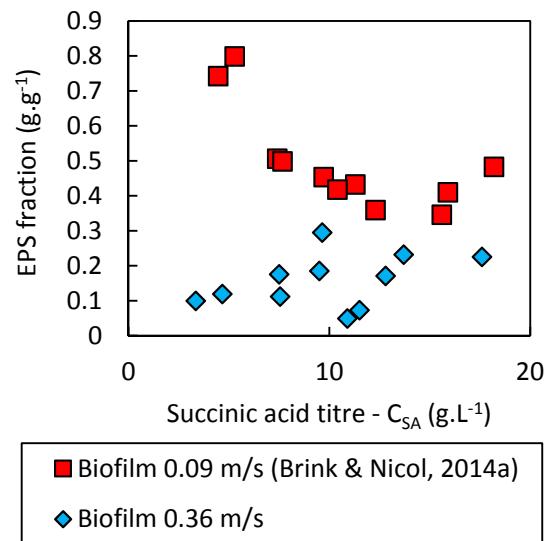


Figure 2: The EPS fractions measured for the respective biofilm runs. From the figure can be seen that the EPS fraction is significantly higher at lower shear conditions and vice versa.

Eq(1) demonstrates the significant product inhibition experienced by *A. succinogenes* during suspended cell operation, an observation shared by numerous authors during batch operation (Figure 1 published in Brink and Nicol (2014a)). At a  $C_{\text{SA}}$  of between  $8\text{--}12 \text{ g.L}^{-1}$  growth ceases almost entirely and the bacterium enters a stationary phase; in the chemostat the organism's productivity halts and the concentration does not exceed the growth-maintenance boundary. In contrast, the biofilm continues production of SA well in excess of this growth-maintenance boundary as a result of maintenance metabolism during which the cells produce SA merely for catabolic functions, while anabolic functions are down-regulated.

Eq(2) shows that the  $r_{\text{SA}}^*$  of the low shear biofilm ( $0.09 \text{ m.s}^{-1}$ ) decays exponentially as a function of the  $C_{\text{SA}}$ , indicating an inhibitory effect on the catabolic functions in the biofilm even after the growth has ceased. The extent of the inhibition is not known, as the  $C_{\text{SA}}$  were limited to  $<20 \text{ g.L}^{-1}$  and therefore extrapolation of the data is uncertain. For  $C_{\text{SA}} > 12 \text{ g.L}^{-1}$  the rate of  $r_{\text{SA}}^*$  reduction decrease, with the values remaining within a limited region possibly indicating an asymptotic minimum value. Interestingly the intermediate shear biofilm ( $0.36 \text{ m.s}^{-1}$ )  $r_{\text{SA}}^*$  exhibited a split from the low shear values at  $C_{\text{SA}} < 10 \text{ g.L}^{-1}$ , i.e. below the growth-maintenance boundary. The cause of the significantly lower  $r_{\text{SA}}^*$  values is uncertain, but it could possibly be related to some form of global population related regulation of the metabolism in the biofilm, indicating a shear dependence of the  $r_{\text{SA}}^*$  in the biofilm under growth conditions. For  $C_{\text{SA}} > 10 \text{ g.L}^{-1}$ , the intermediate and low shear biofilms appeared to follow the same trend, indicating that the maintenance metabolism is shear independent within the range of shear velocities tested. These results appear to explain, to some degree, the apparent incompatibility of Figures 1 and 2, i.e. differences in the maintenance and growth characteristics in the biofilm could account for the inconsistencies.

### 3.2 Batch operation

Figure 4 shows the  $r_{\text{SA}}^*$ , calculated from the SA calibrated NaOH dosing profiles, for the respective batch runs. This figure clearly shows that the initial conditions for the respective batch runs fell neatly on the low shear biofilm fit (Eq(2)), an expected observation considering that the biofilms were grown under steady-state conditions at  $0.09 \text{ m.s}^{-1}$  shear velocity. Initially there appeared to be a lag in the product inhibition; the drop in  $r_{\text{SA}}^*$  was much less pronounced than Eq(2). The  $r_{\text{SA}}^*$  remained nearly constant for  $C_{\text{SA}} < 40 \text{ g.L}^{-1}$ , while a nearly linear decrease was observed for  $C_{\text{SA}} > 40 \text{ g.L}^{-1}$ . The metabolic production appeared to cease at  $C_{\text{SA}}$  between  $60\text{--}72 \text{ g.L}^{-1}$  and a maximum  $C_{\text{SA}}$  of  $72.12 \text{ g.L}^{-1}$  was measured; a residual glucose concentration of  $19.8 \text{ g.L}^{-1}$  was measured at the maximum succinic acid titre which appears to support the observation that product inhibition as opposed to substrate depletion was the cause of the termination of  $r_{\text{SA}}^*$ . These results compared well to batch results obtained using the genetically modified *A. succinogenes* CCTCC M2012036 (Yan et al.,

2014a). The results indicate that a larger average  $r_{SA}^*$  can be obtained during batch operation as opposed to continuous biofilm operation due to a lag in the inhibition experienced by the organism in the biofilm.

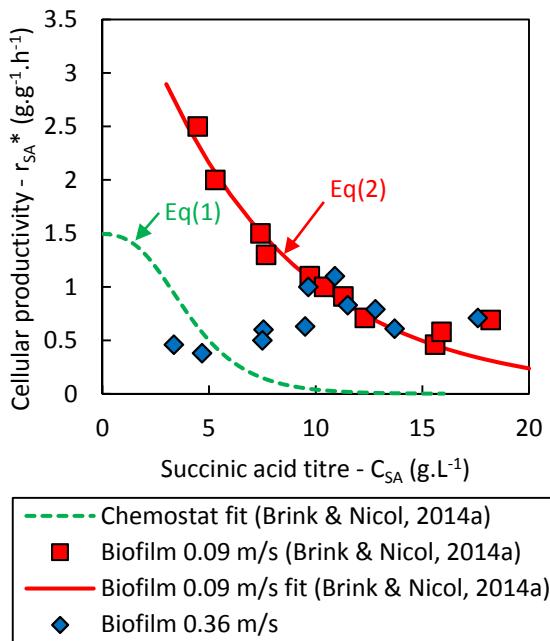


Figure 3: The biofilm cellular production rates ( $r_{SA}^*$ ) as well as the previously published (Brink and Nicol, 2014a) chemostat and low shear biofilm data fits (Eq(1) and Eq(2)).

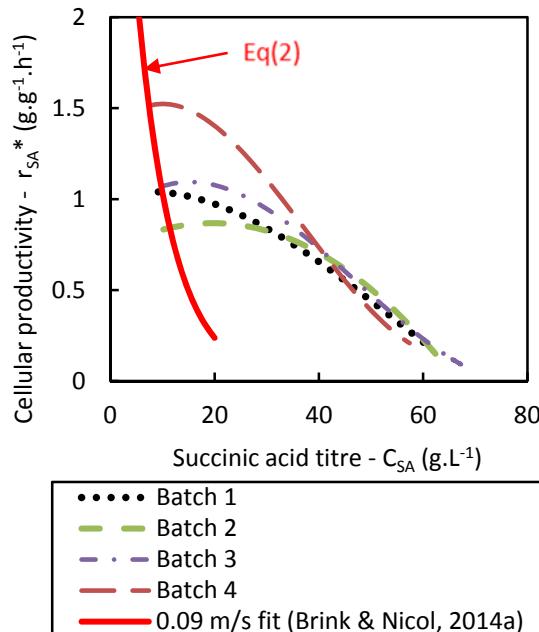


Figure 4: Batch profiles obtained from the calibration of the NaOH dosing profiles with the succinic acid concentrations measured from periodic samples.

#### 4. Conclusions

This study aimed to clarify the influence of shear on the production characteristics of *A. succinogenes* biofilms during continuous operation, while subsequently comparing the results to batch production characteristics. It was found that the volumetric production rates increased significantly when comparing the biofilm to chemostat operation ( $1.83 \text{ g.L}^{-1}.\text{h}^{-1}$  vs.  $15.2\text{--}17.1 \text{ g.L}^{-1}.\text{h}^{-1}$ ), corresponding to a significant increase in the total biomass concentration in the biofilm reactor. In contrast it was observed that the biofilm compositions differed significantly between the low shear and intermediate shear runs, with EPS fractions of  $0.50 \pm 0.05 \text{ g.g}^{-1}$  and  $0.16 \pm 0.02 \text{ g.g}^{-1}$  measured respectively. By analyzing the cellular production rates (excluding EPS) it was observed that a distinct split between the low shear and intermediate shear production rates were observed under growth conditions (low shear corresponded to high cellular production rates and vice versa), while under maintenance conditions the biofilm specific production rates appeared independent of shear. Finally the batch runs revealed a lag in product inhibition during transient biofilm operation, indicating that a higher average cellular production rate could be achieved under batch conditions as opposed to steady state operation.

The study's suggestion that under maintenance conditions *A. succinogenes* production rates are independent of shear, while the biofilm composition is not, has significant industrial implications because it implies that the same mass of biofilm in a higher shear reactor would have an overall greater volumetric productivity due to a smoother, more homogenous composition with a lower EPS fraction. Additionally the batch results indicate that a biofilm reactor with severe product inhibition might possibly be operated at a higher average production rate as a batch reactor as opposed to a continuous reactor, due to the delayed inhibition response.

#### References

- Bozell J.J., Petersen G.R., 2010, Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. *Green Chem.* 12, 539–554.
- Bradfield M.F.A., Nicol W., 2014, Continuous succinic acid production by *Actinobacillus succinogenes* in a biofilm reactor: Steady-state metabolic flux variation. *Biochem. Eng. J.* 85, 1–7.

- Brink H.G., Nicol W., 2014a, Succinic acid production with *Actinobacillus succinogenes*: rate and yield analysis of chemostat and biofilm cultures, *Microb. Cell Fact.* 13, 111.
- Brink H.G., Nicol W., 2014b, The influence of shear on the metabolite yield of *Lactobacillus rhamnosus* biofilms, *N. Biotechnol.* 31, 460–467.
- De Barros M., Freitas S., Padilha, G.S., Alegre R.M., 2013, Biotechnological production of succinic acid by *Actinobacillus succinogenes* using different substrate, *Chemical Engineering Transactions*, 32, 985-990, DOI: 10.3303/CET1332165
- Guettler M.V., Jain M.K., Rumler, D., 1996, Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. US5573931 A.
- Kim M. II, Kim N.J., Shang L., Chang Y.K., Lee S.Y., Chang H.N., 2009, Continuous production of succinic acid using an external membrane cell recycle system. *J. Microbiol. Biotechnol.* 19, 1369–1373.
- Kurzrock T., Weuster-Botz D., 2010, Recovery of succinic acid from fermentation broth. *Biotechnol. Lett.* 32, 331–9.
- Maharaj K., Bradfield M.F.A., Nicol W., 2014, Succinic acid-producing biofilms of *Actinobacillus succinogenes*: reproducibility, stability and productivity. *Appl. Microbiol. Biotechnol.* 98, 7379–7386.
- McKinlay J.B., Laivenieks M., Schindler B.D., McKinlay A.A., Siddaramappa S., Challacombe J.F., Lowry S.R., Clum A., Lapidus A.L., Burkhardt K.B., Harkins V., Vieille C., 2010, A genomic perspective on the potential of *Actinobacillus succinogenes* for industrial succinate production. *BMC Genomics* 11, 680.
- Paul E., Ochoa J.C., Pechaud Y., Liu Y., Liné A., 2012, Effect of shear stress and growth conditions on detachment and physical properties of biofilms. *Water Res.* 46, 5499–5508.
- Urbance S.E., Pometto A.L., DiSpirito A.A., Denli Y., 2004, Evaluation of succinic acid continuous and repeat-batch biofilm fermentation by *Actinobacillus succinogenes* using plastic composite support bioreactors. *Appl. Microbiol. Biotechnol.* 65, 664–670.
- Van Heerden C.D., Nicol W., 2013, Continuous succinic acid fermentation by *Actinobacillus succinogenes*. *Biochem. Eng. J.* 73, 5–11.
- Van Loosdrecht M.C.M., Eikelboom D., Gjatema A., Mulder A., Tijhuis L., Heijnen J.J., 1995, Biofilm structures. *Water Sci. Technol.* 32, 35–43.
- Vlysidis A., Bins M., Webb C., Theodoropoulos C., 2009, Utilisation of glycerol to platform chemicals within the biorefinery concept: A case for succíncico production, *Chemical Engineering Transactions*, 18, 537-542, DOI: 10.3303/CET0918087
- Werpy T., Petersen G.R., 2004, Top value added chemicals from biomass. Volume 1-results of screening for potential candidates from sugars and synthesis gas. National Renewable Energy Laboratory, Golden, USA.
- Wimpenny J.W.T., Colasanti R., 1997, A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. *FEMS Microbiol. Ecol.* 22, 1–16.
- Xu J., Guo B.-H., 2010, Poly(butylene succinate) and its copolymers: research, development and industrialization. *Biotechnol. J.* 5, 1149–63.
- Yan Q., Zheng P., Dong J.-J., Sun Z.-H., 2014a, A fibrous bed bioreactor to improve the productivity of succinic acid by *Actinobacillus succinogenes*. *J. Chem. Technol. Biotechnol.* 89, 1760–1766.
- Yan Q., Zheng P., Tao S.-T., Dong J.-J., 2014b, Fermentation process for continuous production of succinic acid in a fibrous bed bioreactor. *Biochem. Eng. J.* 91, 92–98.