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Valorization of spent sulphite liquor for succinic acid production via continuous fermentation system

Dimitrios Ladakis[a], Katerina Michailidi[a], Anestis Vlysidis[b], Apostolis Koutinas[b,*], Ioannis K. Kookos[a,*]

[a] Department of Chemical Engineering, University of Patras, 26504 Patras, Rio, Greece
[b] Department of Food Science and Human Nutrition, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece

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ABSTRACT

Spent sulphite liquor has been evaluated in continuous cultures for succinic acid production using *Actinobacillus succinogenes* and *Basfia succiniciproducens*. Continuous cultures were initially carried out at constant dilution rate (0.04 h^{-1}) and varying commercial xylose concentrations (23–55 g/L) or constant xylose concentration (40 g/L) and varying dilution rates (0.02–0.25 h^{-1}) showing that dilution rates of 0.02–0.15 h^{-1} led to satisfactory succinic acid production by both strains. In continuous cultures using nanofiltrated spent sulphite liquor, the highest yields were achieved at dilution rate of 0.02 h^{-1} (0.48 g/g for *A. succinogenes* and 0.55 g/g for *B. succiniciproducens*), while the highest productivities were obtained at dilution rates of 0.04 h^{-1} for *A. succinogenes* cultures (0.67 g/L/h) and 0.1 h^{-1} for *B. succiniciproducens* cultures (1.6 g/L/h). Metabolic flux analysis demonstrated higher biomass concentrations in *A. succinogenes* cultures carried out in both xylose and nanofiltrated spent sulphite liquor, while *B. succiniproducens* cultures were more robust regarding succinic acid production efficiency in either xylose or nanofiltrated spent sulphite liquor.

1. Introduction

The development of innovative technologies for establishing biorefineries based on renewable feedstocks as a viable substitute for the existing petroleum refinery based on fossil resources is of paramount importance. A crucial parameter in the commercialization of a biorefinery, which usually deals with microbial fermentation, is its economic competitiveness with the petrochemical one. Taking this into consideration, it is vital to optimize, apart from the performance of the microorganism employed, key operational parameters influencing the bioprocesses in terms of final product concentration, productivity and yield.

Spent sulphite liquor (SSL) is the liquid waste stream produced by the acidic sulphite wood pulping process. This process separates the main components of wood using aqueous solution of $SO_2/MHSO_3/MSO_3$ (M stands for Na, Ca, Mg or NH_3) at 135–145 °C under acidic conditions [1]. Sulphonation, hydrolysis, and condensation occur during pulping of wood leading to the solubilisation of lignin in the aqueous solution. The main product is cellulose fibers, while spent sulphite liquor is obtained as a waste stream. One t of pulp requires 2.5 t of wood. SSL contains C5 and C6 sugars, organic acids, furfural, 5-hydroxy-methyl-furfural, phenolic compounds, wood extractives, lignosulphonates and dissolved solids [2]. The sugar fraction of weak SSL varies between 3% and 4% depending on the wood species used. In the case of softwood, the sugars present are mainly hexoses, while when hardwood is pulped, more than 50% of the sugars are pentoses and mostly xylose [3].

In 2015, the world production of bleached sulphite pulp amounted to 2×10^6 t [4]. The sulphite pulping process generates approximately 8–9 m^3 of weak SSL per t of pulp produced [5]. The weak SSL is condensed in multiple-effect evaporators and the resulting liquor is called thick SSL. The valorization of SSL could create sustainable biorefinery concepts. SSL has been used as a fermentation substrate for the production of ethanol [3,5], poly(3-hydroxybutyrate) [6] and succinic acid [7–9].

Succinic acid has potential as platform chemical and is listed by the US Department of Energy among the top ten chemical building blocks that could be produced from renewable resources [10]. Succinic acid in combination with other chemicals, such as diamines and diols, can serve as building blocks for the production of polyesters, polyamides and polyester amides. Among them, poly(butylene succinate) and its copolymers can be used in

* Corresponding authors.
E-mail addresses: akoutinas@aua.gr (A. Koutinas), i.kookos@chemeng.upatras.gr (I.K. Kookos).

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various applications such as plastic bags, packaging and mulch film as well as other disposable articles [11]. The production of bio-based succinic acid is currently placed at a Technology Readiness Level of 8 with BioAmber, Reverdia, Myriant and Succinity being major companies focusing on its production [12]. The bio-based succinic acid has a market price of ca. 2.94 $/kg with around 38,000 t of annual production capacity [12]. Although bio-based succinic acid production was projected to reach 600,000 t by 2020, this is a highly optimistic scenario as it is based on a production cost of 1 $/kg [12]. The carbon sources used in industrial fermentations for succinic acid production are mainly glucose or starch hydrolysates derived from corn or wheat and glycerol. The industrial production of succinic acid from crude renewable resources is necessary in order to develop sustainable processes following biorefinery and bioeconomy principles.

Among wild-type succinic acid producers, *Actinobacillus succinogenes* stands out as one of the most promising strains with reported succinic acid concentrations up to 145.2 g/L [13] and volumetric productivities higher than 10 g/L/h [14]. *Basfia succiniciproducens* is a promising wild-type succinic acid producing strain with limited literature-cited studies focusing on this strain [15]. Due to its industrial potential, attributed to wide substrate utilization, genetic tractability and facultative anaerobic metabolism [16], *B. succiniciproducens* has been modified via metabolic engineering [17] resulting in succinic acid yields of 0.71 g/g. The production of succinic acid has been investigated using various lignocellulosic resources [16,18]. Alexandri et al. [7] fractionated SSL via nanofiltration and solvent extraction for the recovery of lignosulphonates and phenolic-rich extract, while the remaining solution was used for the cultivation of *B. succiniciproducens* that led to the production of 39 g/L succinic acid. Pateraki et al. [9] demonstrated the effective utilisation of permeates from nanofiltrated SSL by *B. succiniciproducens* resulting in succinic acid concentration of 33.8 g/L and yield of 0.58 g/g.

This study presents the optimization of continuous cultures using both *A. succinogenes and B. succiniciproducens* for the production of succinic acid with SSL as carbon source. The fermentation parameters were initially identified using a synthetic medium containing commercial xylose as this was the major sugar contained in SSL. Continuous fermentations were subsequently carried out using nanofiltrated SSL as carbon source. Metabolic flux analysis (MFA) was employed in order to estimate the biomass concentration and carbon source to biomass conversion yield achieved in continuous cultures because wall growth prevents the determination of dry cell weight.

2. Materials and methods

2.1. Microorganisms and pre-culture medium

The bacterial strains employed for succinic acid production were *Actinobacillus succinogenes* 130Z (DSM 22257) and *Basfia succiniciproducens* JF 4016 (DSM 22022), which were purchased from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. Bacterial stock cultures were maintained at −80 °C in cryovials using 50% (v/v) glycerol solution. The medium used for inoculum preparation constituted of 30 g/L Tryptic Soya Broth (TSB). The medium was initially sterilized at 121 °C for 20 min. Precultures were then placed in an incubator at 37 ºC for 12 h with agitation at 170 rpm.

2.2. Spent sulphite liquor and pretreatment protocol

The SSL utilized in this study was generated by the acidic sulphite pulping process of *Eucalyptus globulus* and was supplied by the company Sniace S.A. (Torrelavega, Spain). The composition of SSL was 176.5 ± 4.85 g/L total sugars (128.1 ± 0.6 g/L xylose, 21.5 ± 2.5 g/L galactose, 19.3 ± 0.4 g/L glucose, 7.4 ± 1.3 g/L mannose and 0.2 ± 0.05 g/L arabinose), 458.8 ± 2.7 g/L lignosulphonates, 12.4 ± 0.8 g/L phenolics, pH value of 2.7 and 64 ± 0.2% dry matter as was described by Alexandri et al. [7]. Although the concentration of total sugars in the thick SSL was 176.5 g/L, it was not possible to use it after simple dilution as fermentation medium due to the presence of inhibitors, such as lignosulphonates and phenolic compounds. For this reason, nanofiltration of SSL was carried out in order to reduce the concentration of lignosulphonates and phenolic compounds in the permeate. Alexandri et al. [7,8] and Pateraki et al. [9] have carried out several fed-batch fermentations using pretreated SSL as fermentation medium leading to the conclusion that pretreatment of SSL via ultrafiltration or nanofiltration is essential in order to maximize succinic acid production efficiency. In this study, the SSL was diluted 3.5 times in order to maintain a reasonably high total sugar concentration in the permeate.

Pretreatment of SSL was conducted by nanofiltration using a vibratory shear-enhanced processing filtration unit (V-SEP, New Logic Research, Emeryville, CA). The nanofiltrated SSL was provided by the company AVECOM NV (Belgium). The membranes used in the V-SEP filter had molecular weight cut-offs (MWCO) of 800 Da (polyethersulfone, NF-PES-10) and surface area of 1.5 m^2. Filtration using the V-SEP equipment was carried out with 3.5 times diluted SSL. The total filtration volume used was 560 L of diluted SSL. Flushing with hot water during operation was employed in order to increase the membrane flux. Periodical caustic cleaning recovered entirely the initial membrane flux during operation. The temperature and pressure used during filtration were 70 °C and 400 psi. The filtration flux was modified during operation to achieve an acceptable flux during filtration in the range of 40.7–10 L m^{-2} h^{-1}. The concentrations of sugars, lignosulphonates and phenolic compounds in the permeate were 44.7 g/L, 5.7 g/L and 0.2 g/L respectively. This nanofiltrated SSL was used in continuous fermentations in this study. The retention of lignosulphonates, sugars and phenolics in the retentate stream during filtration were 97%, 16% and 96%.

2.3. Continuous cultures for succinic acid production

Continuous fermentations with the bacterial strains *A. succinogenes* and *B. succiniciproducens* were carried out in a bench-top bioreactor (Labfors 4, Infors HT) with working volume of 1 L at constant temperature of 37 °C, agitation of 100 rpm in the case of *A. succinogenes* or 250 rpm in the case of *B. succiniciproducens*, and 10% (v/v) inoculum. The pH was maintained at 6.7 using 5 M NaOH. CO_2 was supplied continuously at 0.1 vvm. The vessel and the fermentation medium were sterilized at 121 °C for 20 min. Every continuous culture started in batch mode. After 24 h in batch mode, the continuous operation was initiated. The fermentation medium used at the beginning of the batch mode operation contained 22.5 g/L xylose and 5 g/L yeast extract, a mineral solution and 5 g/L of $MgCO_3$. The composition of the mineral solution used was 1.16 g/L $NaH_2PO_4{\cdot}H_2O$, 0.31 g/L Na_2HPO_4, 1 g/L NaCl, 0.2 g/L $MgCl_2{\cdot}6H_2O$, 0.2 g/L $CaCl_2{\cdot}2H_2O$.

The continuous cultures were initially carried out with *A. succinogenes* and *B. succiniciproducens* at constant dilution rate of 0.04 h^{-1} and a synthetic medium that contained commercial xylose as carbon source. The dilution rate is defined as the volumetric flow rate of the feeding supplied to the bioreactor divided by the working volume of the culture. Six different feeding media were used with six xylose concentrations (23, 35, 40, 47, 50 and 55 g/L) supplemented with yeast extract concentration of 7.5 g/L and the same mineral medium as the one mentioned above. The continuous fer-

Table 1
Dilution rate (D), HRT, HRT cycles and steady state total duration of continuous fermentations conducted with *A. succinogenes* and *B. succiniciproducens* using either commercial xylose (40 g/L) or SSL as carbon source in the feeding medium.

Carbon source	*A. succinogenes*			*B. succiniciproducens*		
	D (h^{-1})	HRT (h)	HRT cycles	D (h^{-1})	HRT (h)	HRT cycles
Xylose	0.02	50	9	0.02	50	9
	0.03	33	9	0.04	25	9
	0.04	25	10	0.08	12.5	12
	0.06	16.6	15	0.10	10	17
	0.10	10	20	0.15	6.25	17
	0.15	6.6	20			
	0.20	5	20			
	0.25	4	20			
Total duration (h)	1857				1101	
SSL	0.02	50	9	0.02	50	9
	0.04	25	9	0.04	25	9
	0.06	16.6	15	0.10	10	15
Total duration (h)	924				825	

mentations lasted for 1545 h. All feeding media were supplied for approximately 250 h or 10 hydraulic retention times (HRT).

Based on the results of the previous fermentation, subsequent continuous fermentations using *A. succinogenes* and *B. succiniciproducens* were conducted using constant commercial xylose concentration (40 g/L) in the feeding solution and 7.5 g/L yeast extract and the same mineral medium as the one mentioned above. The dilution rates, the HRT, the HRT cycles and the total duration of the fermentations for both microbial strains are presented in Table 1.

The final set of continuous fermentations was carried out by both strains using nanofiltrated SSL as fermentation medium. The operational parameters of these cultures were indicated by the continuous fermentations carried out with commercial xylose. The continuous fermentations were initiated using a feeding medium consisted of commercial xylose (40 g/L), yeast extract (7.5 g/L) and the same mineral medium as the one mentioned above in order to reach steady state operation at a dilution rate of 0.02 h^{-1}. In the second stage of each fermentation carried out with SSL, the feeding medium contained nanofiltrated SSL with a total sugar concentration of 40 g/L and 7.5 g/L yeast extract as nitrogen source and the same mineral medium as the one mentioned above. The SSL feeding medium was used until steady state was achieved at the lowest dilution rate followed by step wise increase of dilution rate. The dilution rates, HRT, HRT cycles and the total duration of the continuous fermentations carried out with both microbial strains are presented in Table 1.

2.4. Analytical methods

The concentration of sugars and carboxylic acids (succinic, lactic, acetic and formic acids) was measured by a Shimadzu HPLC system using a Rezex ROA-Organic acid H^+ column and a Shimadzu RI detector. The temperature of the column was 70 °C and the mobile phase was a 10 mM H_2SO_4 aqueous solution with 0.6 mL min^{-1} flow rate.

The phenolic content (% OH) and lignosulphonate content of SSL were determined spectrophotometrically using a double-beam UV–vis spectropthotometer (Jasco V-530), according to UNE EN 16109:2012 protocol.

2.5. Metabolic flux analysis

The objective of MFA was to estimate the formation of biomass and the relationship between biomass yield and succinic acid yield at different dilution rates during continuous cultures of *B. succiniciproducens* and *A. succinogenes*. Biomass formation could not be accurately quantified due to wall growth.

The metabolic network of *B. succiniciproducens* was constructed based on the one proposed by Becker et al. [17] and was extended to include the reactions involved in the consumption of xylose or the sugars contained in SSL. The network contains glycolysis, pentose phosphate pathway, tricarboxylic acid (TCA) cycle, pyruvate metabolism, fermentation pathways, biomass formation and energy metabolism. The metabolic network of *B. succiniciproducens* was modified in order to obtain the metabolic pathway of *A. succinogenes*. *A. succinogenes* lacks a complete TCA cycle resulting in only one pathway for succinate production. Specifically, it lacks the enzymes citrate synthase and isocitrate dehydrogenase and therefore it is unable to synthesize a-ketoglutarate [19]. Moreover, a-ketoglutarate cannot be produced from succinate because of the unidirectionality of the reaction. *A. succinogenes* produces formate and acetate, but it does not produce lactate [19].

The flux analysis was based on the maximization of biomass formation for a given production of succinic acid and by-products. Assuming steady state the problem can be written as in Eq. (1):

$$\sum_{r \in R} S_{m,r} f_r = \begin{cases} 0, & \text{if } i \text{ is an intracellular metabolite} \\ f_m, & \text{if } i \text{ is an extracellular metabolite} \end{cases}, \quad m \in M \quad (1)$$

where r is the r-*th* reaction in the set R (= 1, 2, . . ., NR) of the reactions considered in the network and m is the m-*th* metabolite in the set M (= 1, 2, . . ., NM) that participated in the network. Set R is divided in two disjoint sets: the set R_{Irr} which consists of all irreversible reactions and set R_{Rev}, which consists of all reversible reactions. The following constraints are defined over these sets:

$$0 \le f_r \le F, \quad r \in R_{Irr} \quad (2)$$

$$-F \le f_r \le F, \quad r \in R_{Rev} \quad (3)$$

where F>0 is a sufficiently large number. As it was stated before, the objective function aims at maximizing the biomass produced as follows (Eq. (4)):

$$\max_{f_r, f_m} f_{biomass} \quad (4)$$

In order to estimate the biomass produced during the experiments, the experimentally measured extracellular fluxes for the main products (i.e. succinic, formic, lactic and acetic acids) are imposed on the model (Eq. (5)):

$$f_m = f_m^{\text{experimental}}, \quad m \in \{SUCCINIC, FORMIC, LACTIC, ACETIC\} \quad (5)$$

The model developed for both microbial strains was implemented into MATLAB and solved using the linear programming solver available in MATLAB.

3. Results and discussion

Xylose constitutes the predominant sugar in spent sulphite liquor. For this reason, the initial continuous fermentations were conducted utilizing only commercial xylose in the feeding medium as the sole carbon source for both microorganisms. The aim of these experiments was to optimize the xylose concentration in the feeding medium and the dilution rate that maximize the production of succinic acid by *A. succinogenes* and *B. succiniciproducens*. The optimum parameter values were subsequently used in the continuous fermentation carried out with nanofiltrated SSL as carbon source.

Both bacterial strains create biofilms on the wall of the bioreactor during continuous fermentations. Although the free volume at the headspace of the bioreactor was low, wall growth was observed. Due to biofilm formation, the dry cell weight was not measured during all continuous fermentations as liquid samples were not

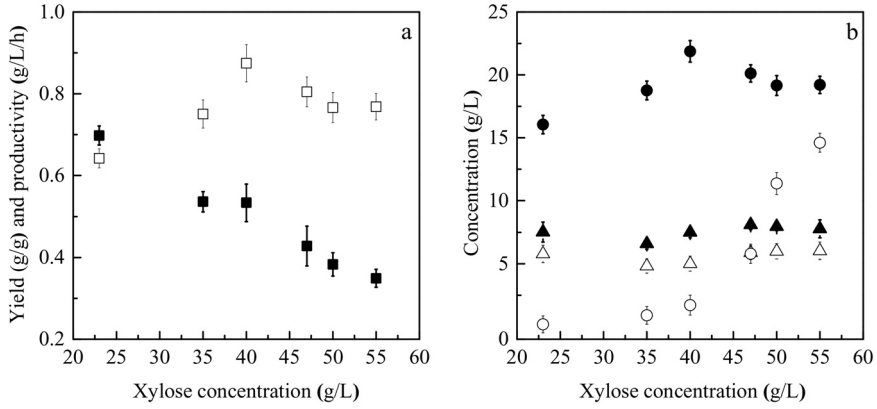

Fig. 1. Xylose to succinic acid conversion yield (■), productivity of succinic acid (□) and concentrations of succinic acid (●), acetic acid (▲), formic acid (△), and unconsumed xylose (○) obtained during continuous cultivation of *A. succinogenes* using constant dilution rate (0.04 h^{-1}) and feeding media with six different xylose concentrations (23, 35, 40, 47, 50 and 55 g/L). All experimental points represent average values (more than 10 experimental results) calculated at each steady-state for each feeding medium.

representative of the bacterial growth taking place inside the bioreactor. It has been reported in several studies that *A. succinogenes* creates biofilm under prolonged continuous operations [14,20,21]. Brink and Nicol [20] reported biofilm formation by *A. succinogenes* at continuous cultures with low shear conditions. van Heerden and Nicol [21] reported biofilm formation when *A. succinogenes* was cultivated in an external recycle bioreactor resulting in the prevention of kinetic analysis of suspended cell fermentation. Wall growth has been also observed in continuous cultures of *B. succiniciproducens* using crude glycerol as carbon source [22]. To the best of our knowledge, there are no published studies on continuous fermentations of *B. succiniciproducens* cultivated on xylose. In this study, it has been observed that biofilm formation also occurs in the case of *B. succiniciproducens*, which was more evident than the one observed in the case of *A. succinogenes*. Due to this observation, higher agitation was applied in the case of *B. succiniciproducens* experiments in order to reduce wall growth. This was based mainly on empirical observation that facilitated steady state operation at prolonged fermentation duration.

At high dilution rates, aggregation of bacterial cells often created operational problems at the outlet of the bioreactor. The aforementioned phenomenon was more intense in the case of *B. succiniciproducens* than *A. succinogenes* resulting sometimes in lack of synchronization between the inlet and outlet flow rate when dilution rates higher than 0.15 h^{-1} were applied. In order to avoid this operational problem, the maximum dilution rate used in continuous cultures of *B. succiniciproducens* was 0.15 h^{-1}, while in the case of *A. succinogenes* the highest dilution rate used was 0.25 h^{-1} (Table 1).

3.1. Continuous cultures using varying xylose concentrations and constant dilution rate

The first continuous fermentation was performed at constant dilution rate (0.04 h^{-1}) in order to investigate the optimum xylose concentration in the feeding medium using the bacterial strain *A. succinogenes*. Steady state conditions were achieved at all xylose concentrations. To ensure this, the duration of feeding of each medium was at least 10 HRT.

Fig. 1 presents the concentrations of organic acids and total sugars as well as the total sugar to succinic acid conversion yield and the productivity achieved during the first continuous fermentation. The effect of the different xylose concentrations in the feeding media used were evaluated considering the yields, productivities, consumed xylose concentration and metabolic product concentrations achieved. The yield was calculated as the ratio of succinic acid concentration to the xylose concentration used in each feeding medium. The maximum yield of 0.7 g/g was achieved when 23 g/L of xylose concentration was used in the feeding medium (Fig. 1a). However, at this xylose concentration the observed productivity was the lowest one achieved (0.64 g/L/h). The highest productivity (0.87 g/L/h) was achieved when 40 g/L of xylose concentration was used in the feeding medium. The yield observed at 40 g/L xylose concentration was higher than the yields obtained at xylose concentrations of 47, 50 and 55 g/L in the feeding medium and similar to the one achieved at 35 g/L xylose concentration. The highest succinic acid concentration (22 g/L) was also achieved at the xylose concentration of 40 g/L, while the lowest succinic acid concentration (16 g/L) was observed at the lowest xylose concentration (23 g/L). The succinic acid concentration and productivity increased steadily from 23 g/L to 40 g/L xylose concentration in the feeding medium. At xylose concentrations higher than 40 g/L, the succinic acid concentration and yield were reduced. Significant accumulation of xylose was observed in the fermentation broth at xylose concentrations higher than 40 g/L. When xylose concentration in the feeding medium was up to 40 g/L, the concentration of unconsumed xylose in the effluent was higher than 1.7 g/L (Fig. 1b).

By-product formation varied in the range of 6.5–8.1 g/L in the case of acetic acid and 4.8–6.0 g/L in the case of formic acid within the xylose concentration range used (Fig. 1b). The lowest by-product concentrations were observed at xylose concentrations of 35 g/L and 40 g/L. The succinic acid to by-product ratio (SA:FA:AA, on a g basis) achieved (1:0.24:0.34) is quite similar with the ratio (1:0.23:0.33) reported by Pateraki et al. [9] in fed-batch fermentations using *A. succinogenes* cultivated on commercial mixed sugars using the same ratio present in SSL.

The results presented above indicate that at the specific dilution rate (0.04 h^{-1}) employed in this fermentation, the xylose concentration of 40 g/L leads to the highest productivity and succinic acid concentration as well as the unconsumed xylose concentration was at very low levels (1.7 g/L) in the fermentation effluent. A similar experiment was carried out in the case of *B. succiniciproducens* showing similar results to *A. succinogenes* (results not presented). Consequently, the feeding containing 40 g/L xylose was chosen as the most appropriate for both bacterial strains for the subsequent continuous fermentations.

3.2. Continuous fermentations on synthetic xylose using varying dilution rates

Subsequent continuous fermentations with both *A. succinogenes* and *B. succiniciproducens* were carried out at varying dilution rates

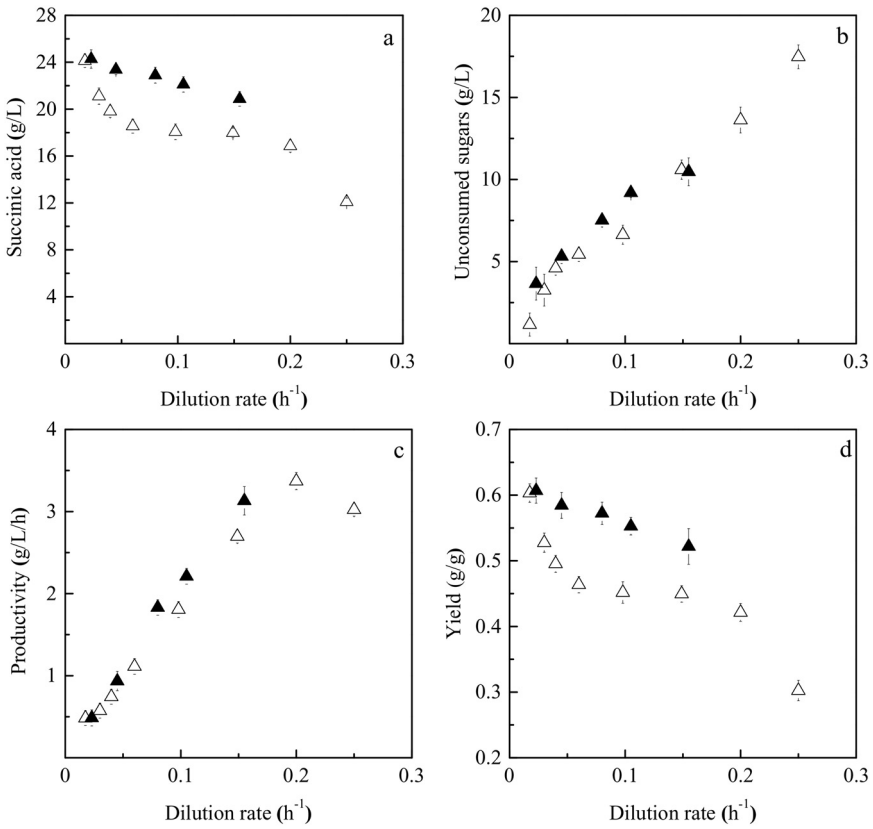

Fig. 2. Succinic acid concentration, productivity, yield and unconsumed sugars achieved during continuous cultures of *A. succinogenes* (unfilled symbols) and *B. succiniciproducens* (filled symbols) carried out on xylose-based synthetic fermentation media at various dilution rates. Data represent the average values (more than 10 experimental results) of the steady-states at each dilution rate.

using synthetic medium based on commercial xylose as carbon source. Eight dilutions rates were used in the case of *A. succinogenes* and five dilution rates were used in the case of *B. succiniciproducens* (Fig. 2). Satisfactory conversion of xylose into succinic acid was observed at dilution rates in the range of 0.02–0.15 h^{-1} for *A. succinogenes* and 0.02–0.1 h^{-1} for *B. succiniciproducens*. The highest succinic acid concentration (ca. 24 g/L) and yield (ca. 0.6 g/g) were achieved at dilution rate of 0.02 h^{-1} for both strains (Fig. 2a and d). In the case of *A. succinogenes*, succinic acid concentration and yield were gradually decreased to 18 g/L and 0.45 g/g, respectively, when the dilution rate was increased to 0.15 h^{-1}. In the case of *B. succiniciproducens* cultures carried out at dilution rates up to 0.15 h^{-1}, the succinic acid concentration and yield were gradually reduced to around 20.5 g/L and 0.51 g/g, respectively. The highest productivities were observed at 0.2 h^{-1} in the case of *A. succinogenes* (3.37 g/L/h) and 0.15 h^{-1} in the case of *B. succiniciproducens* (3.27 g/L/h) (Fig. 2c). Increasing dilution rates led to increasing productivity (Fig. 2c) and unconsumed xylose concentration (Fig. 2b). The remaining xylose concentration in the fermentation broth reached 17.5 g/L at 0.25 h^{-1} in *A. succinogenes* cultures and 10.4 g/L at 0.15 h^{-1} in *B. succiniciproducens* cultures. Xylose consumption rate followed increasing trends with increasing dilution rates for both *A. succinogenes* cultures (0.8–6.6 g/L/h) and *B. succiniciproducens* cultures (0.7–4.4 g/L/h).

Table 2 presents the fermentation efficiency achieved in continuous cultures conducted by either *A. succinogenes* or *B. succiniciproducens* cultivated on synthetic xylose, glucose or glycerol using different bioreactor types. Bradfield et al. [23] carried out continuous cultures of immobilized *A. succinogenes* cells in a custom biofilm bioreactor using synthetic xylose as carbon source leading to succinic acid concentration of 26.4 g/L, productivity of 2.64 g/L/h, yield of 0.77 g/g and sugar conversion of 60% at dilution rate of 0.1 h^{-1}. Bradfield and Nicol [24] carried out continuous cultures of *A. succinogenes* on synthetic xylose using a custom biofilm reactor leading to succinic acid concentrations of 10.9–29.4 g/L, yields of 0.55–0.68 g/g and productivities of 1.5–3.4 g/L/h at dilution rates of 0.05, 0.1 and 0.3 h^{-1}. The concentrations and yields achieved in this study with both *A. succinogenes* and *B. succinciproducens* are lower than the respective values reported by Bradfield et al. [23] and Bradfield and Nicol [24]. The productivities achieved in this study lay at the highest values reported by Bradfield and Nicol [24]. Most literature-cited publications on continuous cultures have been carried out using synthetic glucose and *A. succinogenes* in order to produce succinic acid. In these studies, higher succinic acid concentrations (up to 55.3 g/L), yields (0.91 g/g) and productivities (6.35 g/L/h) than xylose-based continuous cultures have been reported. This is the first study reporting the succinic acid production efficiency of *B. succiniciproducens* continuous cultures on xylose.

Fig. 3a presents the production of acetic acid (AA) and formic acid (FA) by both strains and the production of lactic acid (LA) by *B. succiniciproducens*. In the case of *A. succinogenes*, the production of acetic acid and formic acid varied in the range of 5.6–7.5 g/L and 3.8–5.1 g/L, respectively (Fig. 3a). The production of acetic acid (around 6.8 ± 0.16 g/L) and formic acid (around 4.6 ± 0.27 g/L) was almost stable at dilution rates from 0.03 h^{-1} to 0.2 h^{-1}. Both by-product concentrations declined at the highest dilution rate of 0.25 h^{-1}. In the case of *B. succiniciproducens*, the ranges of acetic acid, formic acid and lactic acid concentrations varied at 5.5–6.3 g/L, 3.8–4.4 g/L and 0.6–1.7 g/L, respectively (Fig. 3a). The highest concentration of total by-products was observed at dilution rate of 0.06 h^{-1} for *A. succinogenes* (11.8 g/L) and at dilution rate of 0.1 h^{-1} for *B. succiniciproducens* (11.9 g/L).

Table 2
Succinic acid production efficiency achieved by continuous cultures using *A. succinogenes* or *B. succiniciproducens*.

Strain	Carbon source	Bioreactor system	Dilution rate (h^{-1})	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Ref
Continuous cultures using xylose-based fermentation media							
A. succinogenes	Xylose	Bioreactor	0.02–0.25	12–24.1	0.30–0.60	0.48–3.37	This study
B. succiniciproducens	Xylose	Bioreactor	0.02–0.15	20.4–24.3	0.51–0.60	0.48–3.27	This study
A. succinogenes	SSL	Bioreactor	0.02, 0.04 & 0.06	10.6–19.2	0.26–0.48	0.38–0.68	This study
B. succiniciproducens	SSL	Bioreactor	0.02, 0.04 & 0.1	16.0–22	0.40–0.55	0.4–1.6	This study
A. succinogenes	Xylose	Custom, biofilm reactor with a novel agitator fitting[1]	0.05 & 0.1	32.5 & 26.4	0.72 & 0.77	1.54 & 2.64	[23]
	Corn stover hydrolysate		0.04 & 0.05	39.6 & 33.6	0.78 & 0.69	1.77	
A. succinogenes	Xylose	Custom, biofilm reactor	0.05, 0.1 & 0.3	10.9–29.4	0.55–0.68	1.5–3.4	[24]
Continuous cultures using glucose- or glycerol-rich fermentation media							
A. succinogenes	Glucose	External-recycle, biofilm reactor	0.05–0.5	48.5	0.91	–	[25]
A. succinogenes	Glucose	Fibrous-bed bioreactor	0.05	55.3	0.8	2.77	[26]
A. succinogenes	Glucose	Biofilm reactor with Poraver® beads	0.11	29.5	0.9	3.24	[14]
A. succinogenes	Glucose	External recycle bioreactor	0.56	12	0.69	6.35	[21]
B. succiniciproducens	Crude glycerol	Suspended cells	0.018	5.21	1.02	0.094	[22]
Mannheimia succiniciproducens	Wood hydrolysate	Suspended cells	0.4	7.98	0.55	3.19	[18]
Batch or fed-batch cultures using xylose-rich fermentation media							
A. succinogenes	Ultrafiltrated SSL with 3 kDa membrane	Fed-batch	–	27.4	0.52	0.39	[9]
B. succiniciproducens	Nanofiltrated SSL with 500 Da membrane		–	33.8	0.58	0.48	
B. succiniciproducens	SSL treated via nanofiltration and ethyl acetate extraction	Fed-batch	–	39	0.54	0.31	[7]
B. succiniciproducens	Corn stover hydrolysate	Batch	–	30	0.69	0.43	[16]
A. succinogenes	Corn stover hydrolysate	Batch	–	42.8	0.74	0.3	[27]
A. succinogenes	Sugarcane bagasse hemicellulose hydrolysate	Batch	–	22.5	0.43	1.01	[28]
B. succiniciproducens	*Arundo donax*[2] hydrolysate	Batch	–	17	0.75[3]	0.35	[29]
A. succinogenes	Corn stover hydrolysate[4]	Batch	–	56.4	0.73	1.08	[30]
A. succinogenes	Biotin-supplemented spent yeast cell hydrolysate with corn fiber hydrolysate	Batch	–	47	0.68	0.63	[31]

[1] central porous polypropylene tube perforated with a multitude of holes into which porous polypropylene or silicone arms were affixed.
[2] a non-food dedicated energy crop – it contains higher glucose (25–37 g/L) content than xylose (13–22 g/L).
[3] mol mol^{-1}.
[4] it contains higher glucose (180.12 g/L) content than xylose (92.2 g/L) and arabinose (44.61 g/L).

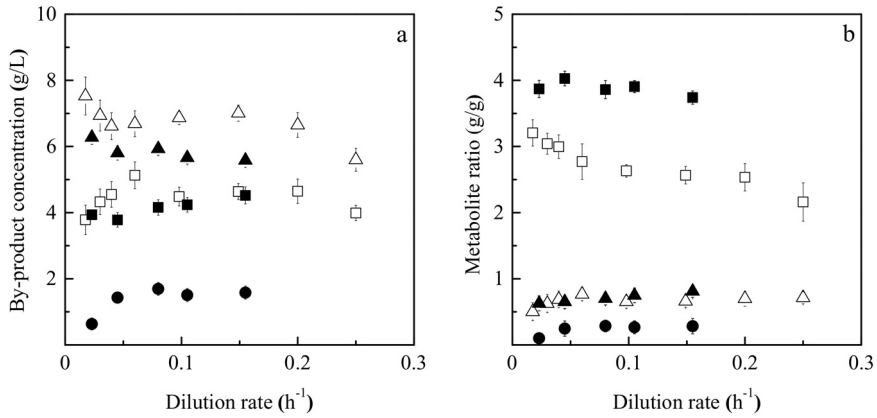

Fig. 3. (a) Production of acetic acid (▲,△), formic acid (■,□) and lactic acid (●) as well as (b) ratios of SA:AA (■,□), FA:AA (▲,△), LA/AA (●) achieved during continuous cultures of *A. succinogenes* (unfilled symbols) and *B. succiniciproducens* (filled symbols) using synthetic xylose as carbon source at different dilution rates. Data represent the average (more than 10 experimental results) of the steady-states at each dilution rate.

Fig. 3b presents the ratios of SA:AA, FA:AA and LA:AA for both strains cultivated on xylose at different dilution rates. The SA:AA ratio was higher in the case of *B. succiniciproducens* (3.7–4.0 g/g) than *A. succinogenes* (2.2–3.2 g/g), whereas the FA:AA ratio varied almost within the same range (*i.e.* 0.6–0.8 for *B. succiniciproducens* and 0.5–0.8 g/g for *A. succinogenes*). The LA:AA ratio varies at a narrow range of 0.1 – 0.3 g/g for *B. succiniciproducens*. The lowest succinic acid to by-product ratio (SA:FA:AA:LA, g/g) was observed at the lowest dilution rate (0.02 h^{-1}) used for both strains with respective values of 1.00:0.15:0.3 for *A. succinogenes* and 1.00:0.16:0.25:0.02 for *B. succiniciproducens*. Pateraki et al. [9] reported SA:FA:AA:LA (g/g) ratios of 1.00:0.23:0.33 for *A. succinogenes* and 1.00:0.15:0.16:0.14 for *B. succiniciproducens* achieved via fed-batch cultures using a synthetic medium containing the sugar ratio of SSL.

3.3. Continuous fermentations using SSL

Based on the results presented in Fig. 1, the optimal fermentation efficiency was observed at xylose concentration of 40 g/L. The same total sugar concentration was used in continuous cultures using SSL as carbon source because xylose constitutes around 73% of total sugars in SSL.

Continuous cultures of *A. succinogenes* were carried out with the dilution rates of 0.02, 0.04 and 0.06 h^{-1}, while continuous cultures with *B. succiniciproducens* were carried out with dilution rates of 0.02, 0.04 and 0.1 h^{-1} (Table 1). Fig. 4 presents the average values of succinic acid concentration, unconsumed sugars, productivity and yield obtained at each dilution rate for both bacterial strains. The highest succinic acid concentration (19 g/L) and yield (0.48 g/g) in the case of *A. succinogenes* were achieved at dilution rate of 0.02 h^{-1} (Fig. 4a and d), while the highest productivity (0.68 g/L/h) was achieved at dilution rate of 0.04 h^{-1} (Fig. 4c). In the case of *B. succiniciproducens,* the highest succinic acid concentration (22 g/L) and yield (0.55 g/g) were achieved at dilution rate of 0.02 h^{-1}. The highest productivity (1.6 g/L/h) achieved by *B. succiniciproducens* (Fig. 4c) was accomplished at the highest dilution rate used (0.1 h^{-1}). Continuous cultures of *B. succiniciproducens* led to higher succinic acid concentration, yield and productivity than *A. succinogenes* at all dilution rates used. The accumulation of total sugars in the fermentation broth (Fig. 4b) increased with increasing dilution rate for both strains. In *A. succinogenes* cultures the unconsumed sugar concentration increased from 4.0 g/L at dilution rate of 0.02 h^{-1} to 12.1 g/L at dilution rate of 0.06 h^{-1}. The respective unconsumed total sugar concentration in *B. succiniciproducens* cultures were 6.6 g/L at dilution rate of 0.02 h^{-1} and 14.2 g/L at dilution rate of 0.1 h^{-1}.

The highest succinic acid concentration, yield and productivity achieved in continuous cultures using synthetic xylose-based fermentation media were higher than the respective values observed when SSL-based media were used for both strains (Table 2). Bradfield et al. [23] carried out continuous immobilized cultures of *A. succinogenes* on xylose-rich corn stover hydrolysate at dilution rates of 0.04 and 0.05 h^{-1} using a custom biofilm reactor with a novel agitator fitting leading to succinic acid concentrations, yields and productivities of up to 39.6 g/L, 0.78 g/g and 1.77 g/L/h respectively. Kim et al. [18] reported similar yield (0.55 g/g), lower succinic acid concentration (7.98 g/L) and higher productivity (3.19 g/L/h), as compared to respective values achieved in this study by *B. succiniciproducens* cultivated on nanofiltrated SSL, when continuous cultures of *Mannheimia succiniciproducens* MBEL55E were carried out on glucose- and xylose-rich wood hydrolysate.

Table 2 also presents the succinic acid production efficiency achieved in batch or fed-batch cultures on various xylose-based hydrolysates. Pateraki et al. [9] conducted fed-batch cultures on ultrafiltrated or nanofiltrated SSL leading to succinic acid concentration up to 27.8 g/L in the case of *A. succininogenes* and 33.8 g/L in the case of *B. succiniciproducens*. Alexandri et al. [7] conducted fed-batch cultures of *B. succiniciproducens* on nanofiltrated SSL followed by solvent extraction for the separation of phenolic compounds leading to succinic acid concentrations of 39 g/L. Table 2 shows that the highest succinic acid concentration (42.8 g/L) and yield (0.74 g/g) achieved with a crude hydrolysate in which xylose was the predominant sugar has been reported by Salvachúa et al. [27].

Fig. 5a presents the production of acetic acid, formic acid and lactic acid observed by both continuous cultures using SSL as fermentation medium. The acetic acid concentrations produced by *A. succinogenes* were 4.7 g/L, 6.6 g/L and 6.7 g/L at dilution rates 0.02, 0.04 and 0.06 h^{-1}, respectively. When *A. succinogenes* was cultivated on commercial xylose based medium (Fig. 3a), the same acetic acid concentrations were obtained at dilution rates of 0.04 h^{-1} and 0.06 h^{-1}, whereas higher acetic acid concentration (7.5 g/L) was produced at dilution rate of 0.02 h^{-1}. The formic acid concentrations produced by *A. succinogenes* when cultivated on SSL were 3.1 g/L, 3.9 g/L and 6.5 g/L at the dilution rates 0.02, 0.04 and 0.06 h^{-1}, respectively (Fig. 5a). In the case of continuous cultures of *A. succinogenes* on commercial xylose based medium (Fig. 3a), slightly higher formic acid concentrations were produced at dilution rates of 0.02 h^{-1} (3.8 g/L) and 0.04 h^{-1} (4.5 g/L), while slightly lower formic acid concentration was observed at 0.06 h^{-1} (5.1 g/L). The formic acid concentration during *A. succinogenes* continuous

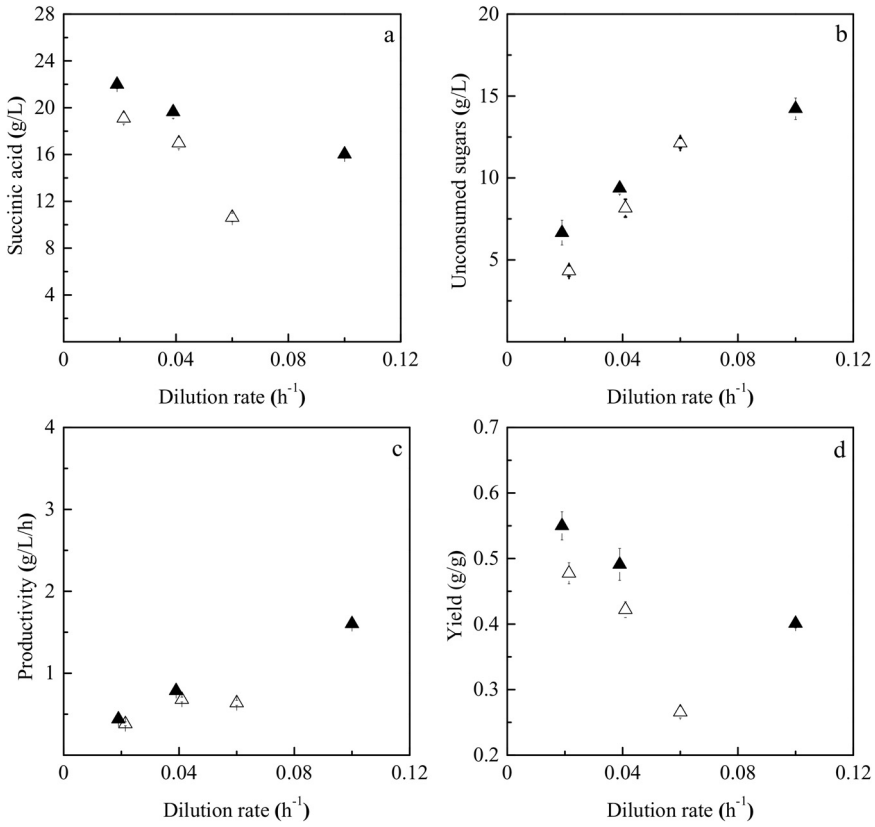

Fig. 4. Succinic acid concentration, productivity, yield and unconsumed sugars achieved during continuous cultures of *A. succinogenes* (unfilled symbols) and *B. succiniciproducens* (filled symbols) carried out on nanofiltrated SSL at various dilution rates. Data represent the average values (more than 10 experimental results) of the steady-states at each dilution rate.

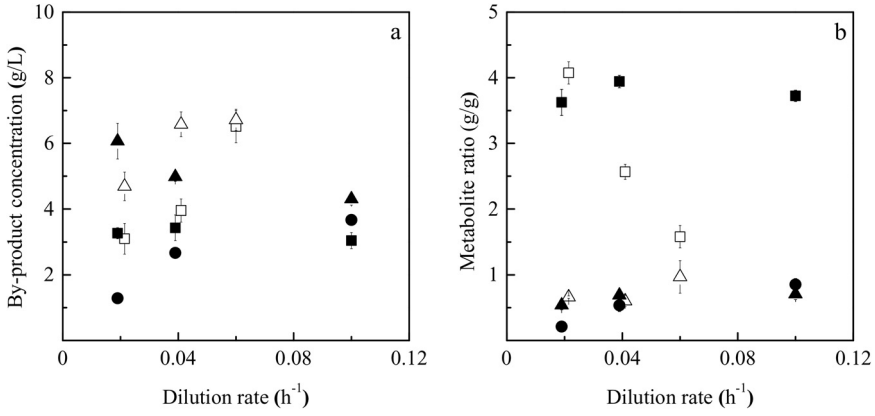

Fig. 5. (a) Production of acetic acid (▲,△), formic acid (■,□) and lactic acid (●) as well as (b) ratios of SA:AA (■,□), FA:AA (▲,△), LA/AA (●) achieved during continuous cultures of *A. succinogenes* (unfilled symbols) and *B. succiniciproducens* (filled symbols) using nanofiltrated SSL at different dilution rates. Data represent the average (more than 10 experimental results) of the steady-states at each dilution rate.

cultures on SSL increased with increasing dilution rate, which was also observed in the case of xylose-based continuous cultures at the same range of dilution rates (0.02–0.06 h^{-1}). It should be mentioned that at the highest dilution rate used (Fig. 5a), the formic acid concentration was similar to the acetic acid concentration. The highest concentration of total by-products for *A. succinogenes* (13.2 g/L) was observed at dilution rate of 0.06 h^{-1}, which is higher than the highest total by-product concentration observed in xylose-based *A. succinogenes* continuous cultures (11.8 g/L) at dilution rate 0.15 h^{-1}.

In the case of *B. succiniciproducens* continuous cultures on SSL, the acetic acid concentration (6.1, 5.0 and 4.3 g/L) was reduced with increasing dilution rate (0.02, 0.04 and 0.1 h^{-1}), while lactic acid concentration (1.3, 2.7 and 3.7 g/L) was increased with increasing dilution rate (Fig. 5a). When *B. succiniciproducens* was cultivated on commercial xylose (Fig. 3a), the acetic acid concentration remained almost stable (around 6 g/L) at dilution rates of 0.02, 0.04, 0.08 and 0.1 h^{-1} (Fig. 3a). The lactic acid concentrations observed in SSL-based cultures were higher than the respective concentrations observed in xylose-based cultures (Fig. 3a). The formic acid production by *B. succiniciproducens* in the SSL-based continuous cultures (Fig. 5a) varied at a narrow range (3.0–3.4 g/L), which is lower than the formic acid concentrations observed in xylose-based cultures at the same dilution rates. The highest concentration of

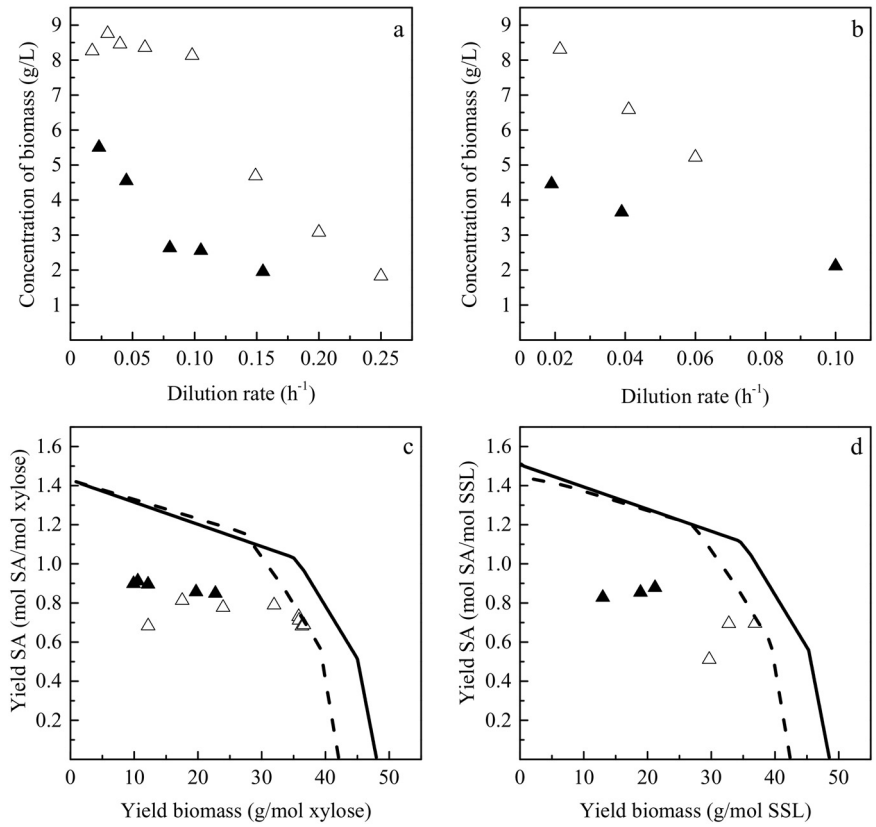

Fig. 6. Biomass concentration at different dilution rates achieved on xylose (a) and nanofiltrated SSL (b) by *A. succinogenes* (unfilled symbols) and *B. succinogenes* (filled symbols). Succinic acid yield per mol consumed xylose as related to biomass yield per mol consumed xylose (c) and succinic acid yield per mol consumed sugars contained in SSL as related to biomass yield per mol consumed sugars contained in SSL (d) achieved at different dilution rates in continuous cultures of *A. succinogenes* (unfilled symbols) and *B. succiniciproducens* (filled symbols). Each point is the average value estimated for each dilution rate presented for each continuous culture in Table 1. The continuous (*B. succiniciproducens*) and dashed (*A. succinogenes*) lines designate the maximum succinate yield for a given biomass yield when 1 mol of xylose or sugars in SSL are consumed. The yields were expressed considering the consumed sugars in each dilution rate. The units of yields used in Fig. 6c and d are the same as the ones used in Fig. 5 included in the study of Becker et al. [17] for comparison purposes.

total by-products for *B. succiniciproducens* (11.1 g/L) was observed at dilution rate of 0.04 h^{-1}, which is similar to the respective concentration (11.9 g/L) observed in xylose-based *B. succiniciproducens* cultures at 0.1 h^{-1}.

The reduction of succinic acid concentration with increasing dilution rates in continuous cultures of *A. succinogenes* coincides with the increasing concentrations of acetic acid and formic acid. In the case of *B. succiniciproducens*, the reduction of succinic acid concentration at increasing dilution rates coincides with stable formic acid concentration, reducing acetic acid concentration and increasing lactic acid concentration. Becker et al. [17] has shown that the dominant cofactor of the total redox flux is NADH. Succinic acid production utilises the reductive branch of TCA cycle, where 2 mol of NADH are oxidised for the production of 1 mol succinate. NADH is the cofactor of other metabolic pathways associated to the production of lactic acid and ethanol as end-products. In this study, ethanol production was not observed and lactic acid production followed an increasing trend with increasing dilution rates when SSL was used in *B. succiniciproducens* cultures (Fig. 5a). The higher lactic acid concentrations may have led to insufficient intracellular pool of reductive energy (NADH) causing lower succinic acid concentrations with increasing dilution rates.

Fig. 5b presents the ratio of SA:AA, FA:AA and LA:AA for both strains cultivated on SSL. In the case of *B. succiniciproducens* cultures on SSL, the SA:AA ratio on SSL is approximately stable (3.6–3.9 g/g) at all dilution rates as it was observed in the case of xylose-based cultures (Fig. 3b). In the case of *A. succinogenes* cultures on SSL, the SA:AA ratio follows a declining trend (4.1–1.6 g/g) with a much faster reduction than the respective *A. succinogenes* cultures on xylose for the same dilution rates (0.02, 0.04 and 0.06 h^{-1}). The FA:AA ratio (Fig. 5b), did not vary at a wide value range for either *A. succinogenes* cultures (0.6–1.0 g/g) or *B. succinciproducens* cultures (0.54–0.70 g/g). The LA:AA ratio observed in *B. succiniciproducens* cultures on SSL followed a slightly increasing trend (0.21 – 0.85 g/g) with higher values than respective cultures on xylose (Fig. 3b) at each dilution rate used. The lowest succinic acid to by-product ratio (SA:FA:AA:LA) was observed at the lowest dilution rate (0.02 h^{-1}) used for both strains when cultivated on SSL with respective values of 1:0.16:0.24 (on g basis) for *A. succinogenes* and 1:0.15:0.27:0.06 for *B. succiniciproducens*. Pateraki et al. [9] reported SA:FA:AA ratio of 1:0.3:0.42 for *A. succinogenes* and SA:FA:AA:LA ratio of 1:0.12:0.26:0.35 for *B. succiniciproducens* when nanofiltrated SSL was used in fed-batch cultures.

3.4. Metabolic flux analysis

Fig. 6a and b present the biomass concentration achieved in continuous cultures carried out on xylose and nanofiltrated SSL using both bacterial strains. In the case of *A. succinogenes*, the biomass concentration remains almost constant (8.45 ± 0.25 g/L) at dilution rates up to 0.1 h^{-1} when it is cultivated on xylose, while it is gradually reduced from around 8.2 g/L at dilution rate of 0.02 h^{-1} to 5.2 g/L at dilution rate of 0.06 h^{-1} when it was cultivated on nanofiltrated SSL. This indicates that SSL influences biomass formation by *A. succinogenes*. In the case of *B. succiniciproducens*, biomass concentration is reduced with increasing dilution rates when either

xylose or SSL are used. The biomass concentration achieved by *B. succiniproducens* is lower than *A. succinogenes* at all dilution rates used contrary to succinic acid concentration where higher values are achieved by *B. succiniciproducens* at all dilution rates used (Figs. 2a and 4a).

Fig. 6c and d present the succinic acid yield as related to biomass yield in the continuous cultures carried out by *B. succiniciproducens* and *A. succinogenes* on either xylose or SSL at different dilution rates. The consumed xylose or sugars contained in SSL have been used in the estimation of the yields presented in Fig. 6c and d. Carbon balances were not complete when biomass formation was not taken into consideration. The continuous and dashed lines in Fig. 6c and d designate the maximum formation of succinate for a given production of biomass when 1 mol of xylose or sugars in SSL is consumed by *B. succiniciproducens* and *A. succinogenes*, respectively. In fermentations carried out with *B. succiniciproducens* cultivated on xylose, increasing dilution rates lead to decreasing biomass yield (22.73–9.90 g/mol_{xylose}) (Fig. 6c) with a slightly increasing trend of succinic acid yield (0.85–0.90 mol_{SA}/mol_{xylose}). In the case of *B. succiniciproducens* continuous cultures on nanofiltrated SSL, both biomass yield (21.17–12.98 g/mol_{xylose}) and succinic acid yield, based on consumed sugars, (0.88–0.83 $mol_{SA}/mol_{SSLsugars}$) showed a decreasing trend with increasing dilution rate (Fig. 6d).

When *A. succinogenes* was cultivated on xylose, it was observed that at 0.03–0.1 h^{-1} the biomass yield remained almost constant at around 36.14 g/mol_{xylose} that coincided with an almost constant biomass concentration (Fig. 6a). However, at dilution rates higher than 0.1 h^{-1}, biomass yield followed a reducing trend to 12.18 g/mol_{xylose} (Fig. 6c) that coincided with reduction of biomass concentration to 1.82 g/L (Fig. 6a). When *A. succinogenes* was cultivated on SSL, the biomass yield (36.85–29.67 $g/mol_{SSLsugars}$) was reduced faster than xylose-based cultures at dilution rates of 0.02–0.06 h^{-1}. The succinic acid yield was also reduced faster at cultures carried out on SSL (0.69–0.51 $mol_{SA}/mol_{SSLsugars}$) for dilution rates of 0.02–0.06 h^{-1}, whereas at the same dilution rates in xylose-based cultures the succinic acid yield was higher than 0.68 mol_{SA}/mol_{xylose}.

The results presented in Figs. 4 and 6d show that *B. succiniciproducens* is a more robust strain than *A. succinogenes* when it is cultivated on SSL, as higher succinic acid concentration, yield based on total sugars used and productivity are achieved for a wider range of dilution rates. Furthermore, although biomass concentration (Fig. 6b) and yield based on consumed sugars (Fig. 6d) are gradually reduced with increasing dilution rate, the productivity of succinic acid (Fig. 4c) is almost doubled between the dilution rates of 0.04 h^{-1} and 0.1 h^{-1} when *B. succiniciproducens* is used. Regarding the optimum biomass concentration that should be maintained in continuous cultures, biofilm formation creates problems in controlling biomass concentration in continuous cultures. However, in the case of *B. succiniciproducens* the reduction of biomass concentration up to dilution rate of 0.1 h^{-1} did not affect significantly the succinic acid production efficiency.

The two bacteria mainly differ on the fact that *A. succinogenes* has an incomplete TCA cycle [19], while *B. succiniciproducens* has a complete TCA cycle [17]. The carbon flux through the oxidative branch of TCA cycle in *B. succiniciproducens* is low compared to the reductive branch and mainly serves anabolic purposes. It is hardly considered as a cycle as it operates in two separate branches. The carbon flux towards the reductive branch of TCA cycle that leads to the production of succinate is 75%. The oxidative part of the TCA cycle was also found active *in vivo*, although at much lower flux [17]. *A. succinogenes* does not produce lactic acid extracellularly, while *B. succiniciproducens* does, in lower concentrations compared to acetic and formic acids. Both microorganisms get the NADPH from the pentose phosphate pathway. Both microorganisms use malic enzyme and oxaloacetate decarboxylase for the carbon flux between the C4 and C3 pathways. Taking into consideration the minor differences between the two bacteria, it is hard to explain why *B. succiniciproducens* achieves higher yields compared to *A. succinogenes*. The regulation of the oxidative branch of TCA cycle in *B. succiniciproducens* is not sufficient to explain the higher carbon flux to succinate than *A. succinogenes*.

4. Conclusion

This study demonstrated that SSL could be used for the production of succinic acid via continuous cultures of either *A. succinogenes* or *B. succiniciproducens*. Continuous cultures were maintained at three different dilution rates for both strains demonstrating stability when cultivated on nanofiltrated SSL. *B. succiniciproducens* was generally a more efficient succinic acid producer than *A. succinogenes* when cultivated in continuous cultures using SSL.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.bej.2018.05.015.

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