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Succinic acid production from cheese whey by biofilms of *Actinobacillus succinogenes*: packed bed bioreactor tests

Short title: Succinic acid production from cheese whey by Actinobacillus succinogenes biofilms

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Abstract

BACKGROUND. Succinic acid (SA) biotechnological production represents a promising alternative to the fossil-fuel based chemical production route. The goal of this study was to develop a SA production process conducted with biofilms of *Actinobacillus succinogenes* and fed with cheese whey, a lactose-rich by-product of the cheese-making processes.

RESULTS. The screening between five commercial biofilm carriers, based on a statistical analysis of the process rates and yields, led to the selection of Glaxstone®, a sintered glass porous material. The attached-cell performances obtained when SA production was fed with cheese whey or with pure lactose were equivalent. The feasibility of a repeated batch process of SA production by biofilms of *A. succinogenes* was demonstrated in a Glaxstone®-filled 1-L packed bed bioreactor, and an effective sequence of biofilm growth and SA production phases was identified. A SA productivity of 0.72 g_{SA} $L^{-1}_{packed\ bed}$ h^{-1}, a SA specific production rate of 0.18 g_{SA} $g_{protein}^{-1}$ h^{-1} and a biofilm concentration of about 4 g $L^{-1}_{packed\ bed}$ were obtained.

CONCLUSIONS. SA bioproduction under biofilm conditions from organic by-products such as cheese whey is a feasible and promising process. This work represents the first attempt to develop a biofilm-based process of SA bioproduction from cheese whey.

Keywords: succinic acid; biofilm process; cheese whey; packed bed bioreactor; *Actinobacillus succinogenes*; carrier selection.

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INTRODUCTION

Succinic acid (SA), an intermediate of the tricarboxylic acid cycle, was recently recognized as one of the top 10 building blocks according to the U.S. Department of Energy [1]. SA has a large number of industrial – related applications in the cosmetic, chemical, agriculture and food sectors. In particular, it is used as reagent for the production of lacquers and perfumes [2], as well as in the manufacture of coatings, plastic bumpers, surfactants and dyes [3]. It is also used as acidity regulator, flavor enhancer (E363) and stimulating agent in plant and animal growth [4]. Moreover, succinic acid can be easily converted into many industrially important chemicals as adipic acid, γ-butyrolactone, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, 1,4-butanediol and other succinate salts [5]. 180 kt_{SA}/y are utilized in the synthesis of new generation renewable polymers such as poly-1,3-propylene succinate and polybutyrate succinate (PBS) [4]. The global revenues associated to SA production increased from $183 million in 2010 to an expected $469 million in 2016 [6].

SA is traditionally manufactured through multiple high temperature and high pressure fossil fuel-depending synthetic procedures. The liquid-phase Ni – Pt – Ru – mediated catalytic hydrogenation of maleic anhydride to succinic anhydride, followed by the hydration to succinic acid, is the most direct and used reaction [7].

Due to the depletion of fossil fuels and to the environmental impact of the chemical route, there is a deep interest on the biotechnological alternatives for SA production. Indeed, SA is the end product of different anaerobic metabolism pathways. Therefore, SA can be easily produced by anaerobic fermentations, that fix 1 mole of CO_2 for each mole of produced SA (corresponding to 0.37 $kg_{CO2\ fixed}/kg_{SA\ produced}$). This provides a further advantage in terms of environmental interest and carbon footprint of the process.

Different microorganisms were recognized as natural high-yield succinic acid producers: *Actinobacillus succinogenes*, *Anaerospirillum succiniproducens* and *Mannheimia succiniproducens*. Moreover, *E. coli* strains were engineered to produce succinate. Table 1 reports the SA yields, productivities and maximum concentrations obtained in the main literature studies conducted with *Actinobacillus succinogenes*, the strain tested in the present work [8-24].

In the metabolic pathway to SA, phosphoenolpyruvate (PEP) is the key intermediate. It is produced by sugar glycolysis and converted into either pyruvate, with the formation of acetate, formate, ethanol and lactate as end products (*C_3 pathway*), or oxaloacetate, with the formation of SA (*C_4 pathway*) [25]. Many articles report that the addition of carbon dioxide to the culture medium leads to increases in terms of final SA concentration, yield and productivity [25, 26]. Van der Werf et al. [25] observed a direct relationship between the amounts of CO_2 added to the fermentation medium and SA productivity. More recently, McKinlay et al. [26] gave more insights into *Actinobacillus succinogenes* fermentative metabolism and showed that, when CO_2 is used to shift the key intermediate PEP from the C_3 pathway (acetate and formate) to the C_4 pathway (succinate), the acetic acid (AA):formic acid (FA):SA molar ratios are equal to about 1. The authors gave a plausible explanation for these molar ratios based on a redox balance, and suggested that the reducing power (NADH and NADPH) is generated in excess by the *C_3 pathway* and consumed by the *C_4 pathway*. This provides an upper limit to the SA/substrate yield, equal to 0.66 g g^{-1} if glucose is used as substrate [19]. On the other hand, if an electron donor such as H_2 is added as a source of reducing power, higher SA/substrate yields can be obtained thanks a decrease of the C_3 pathway, and therefore of the AA:SA and FA:SA ratios [25]. Bradfield and Nicol [19] suggested that pyruvate can be metabolized by *A. succinogenes* according to two pathways: the pyruvate formate-lyase pathway (PFL) with formation of both AA and FA, and the pyruvate dehydrogenase pathway (PDH) with the formation of AA and CO_2 (no production of FA). If the PDH pathway is present, 2 moles of SA are produced per mole of AA formed, with a potential increase from 0.66 to 0.87 g g^{-1} in the SA/glucose yield, neglecting biomass production. In conclusion, according to Bradfield and Nicol [19], the AA:SA and FA:SA molar ratios are expected to be 0.5:1 and 0:1 respectively (corresponding to 0.25:1 and 0:1 g g^{-1}, on a mass basis).

When the operational conditions are optimized for SA production, the substrate flux directed towards biomass formation is almost negligible [19 20]. Maharaj et al. [20] performed a glucose mass balance which closed to 99.3 ± 3.2% without considering biomass formation, and concluded that the steady-state carbon flux to biomass is negligible compared to the flux to metabolites. Under optimized conditions the maximum SA/glucose mass yield – occurring when no glucose is utilized for biomass formation - is 0.66÷0.87 g g^{-1}, depending on how much glucose is transformed into acetate and formate [19].

Three elements can significantly contribute to increase the productivity and profitability of the SA biotechnological production process: i) the use of high concentrations of substrate, ii) the use of low-cost renewable feedstocks, such as cheese whey, and iii) the use of attached-cell biomass systems.

With regard to the first point, high substrate concentrations generally lead to higher reaction rates, and therefore to higher productivities. On the other hand, high substrate concentrations can lead to more intense phenomena of substrate and product (succinic, acetic and formic acid) inhibition, as documented by several studies. Corona-Gonzalez et al. [10] observed no significant inhibition effects by either substrate or products up to 30 $g_{glucose}$/L. At higher glucose concentrations, substrate inhibition led to a decrease of SA/glucose yield, glucose uptake rate, SA production rate, biomass growth rate, AA:SA ratio and FA:SA ratio. With regard to product inhibition, when the SA and acid mixture concentrations reached 13 and 20 g L^{-1} respectively, biomass production stopped, whereas products formation slowed down. Above 30 g L^{-1} of SA and 45 g L^{-1} of total acids biological activity stopped completely. Corona-González et al. [10] suggested a possible strategy to avoid growth inhibition and improve the process productivity: the development of a two-stage process with a first biomass growth phase followed by a SA production phase. Yan et al. [27] studied product inhibition phenomena in a continuous biofilm reactor. They processed their data with the Jerusalimsky equation, previously successfully used by Corona-Gonzalez et al. [10] to fit the specific SA production rate with product and substrate inhibition. They showed a strong deviation from the model at 60 g_{SA} L^{-1}, which may be a critical concentration in their continuous biofilm reactor. Finally, other studies pushed the maximum SA concentration even further, showing that *A. succinogenes* can tolerate up to 140 g L^{-1} of pure glucose and 100 g L^{-1} of succinate [28, 29]. The use of continuous flow perfectly mixed systems, where the entire bioreactor works at the exit substrate and products concentrations, can be useful to reduce substrate inhibition.

As for renewable feedstocks, in addition to allowing for a relevant decrease of the process operational cost, they can significantly reduce inhibition phenomena, thus allowing the use of higher substrate concentrations. Indeed, sugar-rich feedstocks generally contain complex sugars which are transformed into simple sugars. For example, in the case of cheese whey, lactose, the main constituent, is turned into glucose and galactose, which are the actual inhibitors. As the latter are intermediates of the reaction, there concentrations tend to be very low, thus minimizing inhibition phenomena. Biological SA production from renewable feedstocks and agro–industrial byproducts achieved the threshold of profitable process, leading different companies (e.g. Myriant, BioAmber, BASF&Purac) to build the first production plants [30, 31]. Among the renewable feedstocks, cheese whey, a waste stream from the dairy industry, is particularly interesting and can be used in the cost-effective fermentative production of succinic acid [15].

Lastly, biofilm reactors can further improve SA productivity, considering that the optimal conditions for SA production may lead to a very low biomass formation rate. Indeed, in an attached-cell system, once the biofilm is established the required growth rate needed to replace the dead and detached cells is lower than in a suspended-cell system, if the latter is not associated to a biomass concentration and recycle section. Nevertheless, most of the studies on biological SA production were conducted with suspended biomass (Table 1, upper section), whereas only few works focused on biofilm–based process (Table 1, lower section). As shown in Table 1, the SA productivities vary in the 0.8-1.4 g L^{-1} h^{-1} range in the suspended cell tests, and in the 1-10 g L^{-1} h^{-1} range in the biofilm studies. In particular, Urbance et al. [24, 32], tested a tailored-made Plastic-Composite-Support (PCS) in repeated-batch biofilm fermentations achieving a SA productivity of 2.1 g L^{-1} h^{-1} and a yield of 0.72 g_{SA} $g_{glucose}^{-}$

[1]. Other continuous experiments involved the utilization of commercial supports as Poraver® beads [20], Genulite® Groperl particles [23], polypropylene protruding arms [21], and stainless-steel wool backbones [19]. All the attached-cell studies used glucose as substrate, with the exception of Bradfield et al. [21], who used pure xylose and the xylose-enriched fraction of a non-detoxified, xylose-rich corn stover hydrolysate stream produced from dilute acid pretreatment. In that work the authors used a standard fermenter outfitted with a novel porous polypropylene agitator to support attached-cell biofilm. Yan et al. [18] performed batch and fed-batch fermentations with *Actinobacillus succinogenes* and glucose and showed that high SA concentrations (98 g L^{-1}), yields (0.89 g_{SA} $g_{glucose}^{-1}$) and productivities (2.77 g L^{-1} h^{-1}) can be simultaneously achieved in an internal fibrous bed batch bioreactor. In a later study, the same authors reported the results of a continuous process of SA production in a fibrous bed bioreactor [27]. At the optimum operating conditions (glucose 80 g L^{-1} in the feed, dilution rate equal to 0.05 h^{-1}, pH=6.0-6.5), they obtained 55 g L^{-1} of SA, a 0.80 g_{SA} $g_{glucose}^{-1}$ yield, a productivity equal to that obtained in batch conditions (2.77 g L^{-1} h^{-1}) and a AA:SA molar ratio of 0.29. The final SA yields were estimated between 0.7 and 0.9 g g^{-1} and the final SA concentrations between 10 and 100 g L^{-1}.

The general goal of this study was to develop a SA production process conducted with biofilms of *A. succinogenes* ATCC 55618 and fed with cheese whey, a lactose-rich by-product of the cheese-making processes. The specific goals were: i) to carry out a screening between 5 different types of commercial carriers for SA production by biofilms of *A. succinogenes*, and to set up an ANOVA-based carrier screening procedure; ii) to evaluate the performances of SA production from cheese whey by *A. succinogenes* biofilms immobilized on the selected carrier; iii) to evaluate the feasibility of a repeated-batch process in a 1 L packed-bed column bioreactor. The first part of the tests were fed with lactose, the main constituent of de-proteinated cheese whey, whereas the final assays were conducted with commercial de-proteinated cheese whey. Preliminary tests were conducted to evaluate the SA production performances of suspended cells of *A. succinogenes*, to be used as an internal benchmark to rate the performances of the subsequent attached-cell tests.

The novelties of this work are i) the development of a biofilm process of SA production from cheese whey, and ii) the screening of commercial carriers for SA production. Indeed, to the best of our knowledge the present work is the first to deal with the development of a process for SA production from cheese whey with attached-cell biomass, whereas only 1 paper focused on suspended cell SA production from cheese whey using *A. succinogenes* [15].

MATERIALS AND METHODS

Chemicals and strain cultivation

All the chemicals used in this study were purchased from Sigma – Aldrich (Milan, Italy), except the Molkolac Instant® de-proteinated cheese whey that was provided by Milei GmbH (Hamburg – Germany). Molkolac Instant is a lyophilized powder with the following composition: lactose 85%, proteins 4%, fats 1%, ashes 7%, water 3%. Before every use it was dissolved in water and sterile-filtered with 0,22 μm VacuCap® filters (Pall Corporation, USA).

Actinobacillus succinogenes (ATCC 55618) was obtained from the DSMZ collection (Braunschweig, Germany) and initially grown on 2.5 g/L of trypticase soy broth (TSB) for 24 h on a rotary shaker (37°C, 100 rpm). The strain was stored at – 80 °C in 2 mL cryovials containing 0.5 mL of culture suspension and 0.5 mL of fresh sterile 545 DSMZ medium, supplemented with 20% v/v of sterile glycerol.

Carrier main features

The main features of the five commercial biomass carriers investigated are reported in Table 2, whereas their pictures are shown in Fig. S1 in the Supplementary Material. Kaldnes K1® and BioFlo 9 Sinker® are new generation high-density polyethylene carriers with high surface area, mainly used for denitrification processes in moving bed biofilm reactors [33, 34]. Biomax® and Glaxstone® are respectively inert ceramic rings and sintered glass fragments, commonly used for aquarium water treatment. In recent studies, they were tested as biofilm carriers for innovative processes of on-site groundwater treatment and biohydrogen production [35-39]. Poraver®, commercially used as aggregate in dry mortar, tile adhesive, lightweight concrete and thermal insulator, was previously used as biofilm carrier in a limited number of studies [20].

0.12-L batch tests

In the first part of this work, small-scale experiments were carried out in batch mode with suspended and attached biomass in 0.12 L glass anaerobic bioreactors (microcosms) in order to assess the performances of the process of SA production from cheese whey and to select the best-performing biofilm carrier. Three initial lactose concentrations were tested with suspended biomass (7, 11 and 15 g L^{-1}). In the case of attached-cells, the 5 candidate carriers were tested at a single condition lactose initial concentration (7 g L^{-1}). Additional tests at lactose concentration ranging from 2 to 14 g L^{-1} were performed only with the two best performing carriers (Glaxstone® and Poraver®). Lastly, the same concentration range was used with the best performing carrier (Glaxstone®), using cheese whey instead of lactose.

In the suspended biomass tests, 60 mL of fermentation medium (Table 3) were added, leaving about 60 mL of headspace (containing either N_2 or CO_2). The attached-cell tests contained 60 mL of carriers and enough fermentation medium (Table 3) to cover the carriers (70 mL for Kaldnes K1® and Bio-Flo 9 sinker®, 50 mL for the other carriers), leaving 40-60 mL of headspace (Table 3).

In both types of tests, three subsequent steps were designed and optimized to establish a reliable procedure for the biomass growth, biomass immobilization in the attached-cell tests and SA production: 1) an inoculum activation phase, 2) a growth phase, and 3) an SA bioproduction phase.

Each of these steps was characterized by specific fermentation media and headspace compositions, as specified in Table 3. The media were sterilized by filtration, using 0.22 VacuCap® filters (Pall Corporation, USA).

In the inoculum activation step (phase 1), 1 mL of frozen inoculum was suspended in sterile tubes supplemented with 9 mL of the activation medium (Table 3). The inoculated tubes were closed in an anaerobic jar equipped with an AN0035 - AnaeroGen bag (ThermoFisher Scientific, USA) to create anaerobic conditions and a BR0055 – AnaeroIndicator (ThermoFisher Scientific, USA) to check the oxygen absence. The tubes were then incubated for 24 hours (37°C, 150 rpm). 1 mL of liquid culture was then sampled to assess the final biomass concentration. For the cell growth step (phase 2), sterile 116 mL bottles filled with fermentation medium (Table 3) and - in the case of attached-cell tests - autoclaved carriers were inoculated with 10% (v/v) of the inoculum tubes. Then the vials were sealed with teflon-coated butyl stoppers and aluminum crimp sealers and anaerobic conditions were established by flushing sterile–filtered nitrogen for 30 min. Finally, vials were incubated for 24h (37°C, 100 rpm) to grow the suspended or attached biomass. In the case of suspended biomass, a single growth microcosm was used to inoculate the triplicate SA production vials. In the attached-cell tests, at the end of the growth phase 5 carriers for each bottle were sampled and analyzed to quantify the attached biomass. Then, the exhausted growth medium was discharged from the microcosm and replaced with the same volume of production medium (Table 3). It should be noted that during the growth phase some formation of succinic, acetic and formic acids occurs, and that in the attached-cell tests the medium replacement from the growth to the production step is not complete.

For these reasons, low concentrations of SA, AA and FA are already present at the beginning of the production step.

At the end of the growth procedure, microcosms were flushed for 30 min with 100% sterile – filtered CO_2 to ensure anaerobic conditions (GVS 0,22 μm cellulose acetate filters, GVS Filter Technology, Stanford, USA) and to obtain a CO_2-saturated solution suitable to shift the metabolism towards SA production. Microcosms were then connected to a 100% CO_2 bag to provide the CO_2 required for SA production while maintaining atmospheric pressure. The SA production phase was monitored for 8 h (37°C, 100 rpm), and the pH was measured every 2 h and corrected to 6.6 by adding a 2M sterile NaOH solution. Microcosms were sampled hourly to analyze substrate, products and suspended biomass. At the end of the attached-cell experiments, 5 carriers were sampled and analyzed to quantify the attached biomass.

pH control and buffer selection

The optimal pH for the PEP – carboxykinase activity is 6.6 [4] and the operating pH range is narrow (6.2-7.2) [10, 15, 25]. A strict pH control is therefore necessary to neutralize the produced acids, maintain a high CO_2/HCO_3^- concentration in the liquid, thus ensuring an optimal incorporation rate of the CO_2-derived carbon in the SA backbone. Thus, several tests were performed in the literature to assess the best pH control method in small scale batch reactors where an automatic pH control by NaOH addition is not easily applicable [17]. The best results were obtained with the bis(2-Hydroxyethyl) iminotris(hydroxymethyl) methane (BIS–TRIS) buffer under continuous CO_2 supply, and with $MgCO_3$ with no CO_2 supply. As the BIS-TRIS pKa (6.5) is very close to the optimal pH for CO_2 fixation into SA (6.6), this buffer system was used in the 0.12 L batch tests.

1-L packed bed bioreactor tests

Bioreactor configuration. In the last part of this study, the feasibility of a repeated-batch biofilm process of SA production from lactose was evaluated in a 1 L packed-bed column bioreactor. The plant flow-sheet is shown in Fig. 1. The core of the plant was formed by a 1.04 L glass column (0.049 m internal diameter, 0.585 m length) packed with 840 grams of Glaxstone®, the selected best performing carrier, with sampling ports at the column inlet and outlet. Three additional sampling ports were evenly spaced along the column. The packed column had a bulk density of 808 $g_{carrier}$ $L_{reactor}^{-1}$. A Masterflex L/S 0.1 HP 1-100 rpm peristaltic pump and an autoclaved Norprene® tube (Cole-Parmer, Vernon Hill, USA) were used to connect the bottom of the column to a 2 L bottle filled with 50-100 mL of growth or SA production 3 medium (Table 3). This bottle, closed with a tailor-made screw cap equipped with a gas sampling port and a valve for manual liquid addition, acted as medium reservoir. The liquid was fed to the bottom of the column, through the carrier bed and, then, recirculated into the bottle. The plant was kept at 37°C in a temperature controlled hood. In order to mimic the operational conditions of an industrial SA production process, the BIS-TRIS buffer was not included in the growth and production media used in the PBBR tests. pH was monitored hourly and a 2M NaOH solution was used to correct the pH to 6.6 whenever necessary.

In order to sterilize the packed bed bioreactor (PBBR), all the components (column, reservoir bottle, tubes and connections) were autoclaved (121°C, 20 min). After sterilization, the column was immediately connected to the tubes and connections in a sterile environment in order to avoid contaminations. The reservoir bottle, instead, was placed under a laminar flow hood and the growth or production medium (Table 3) was sterile-filtered into the bottle, flushed with sterile N_2 for 30' and immediately inoculated (10% v/v) with the pre-culture. The connection of the bottle to the plant was performed in a sterile environment as previously reported.

At the beginning of the test, before filling the column with the growth medium, the entire plant was flushed with sterile 100% N_2 to strip away all the oxygen. Both the reservoir headspace and the top of the column were then connected to an anaerobic bag (88% N_2 - 12% CO_2 during growth phases, 100% CO_2 during production phases), to maintain atmospheric pressure and to ensure the required CO_2 for the SA bioproduction phase. Sterility was provided by a GVS 0.22 μm cellulose acetate filter (GVS Filter Technology. Stanford, USA) placed in the bag connection. A gas sampling port was added at the top of the column to monitor the gas composition. The medium in the bottle was agitated with a magnetic stirrer at 500 rpm, to maintain a suitable gas–liquid mass transfer.

Packed bed fluid-dynamic test. A conventional perturbation/response tracer test (50 mM NaCl) was initially used to study the fluid dynamic behavior of the packed bed. The whole plant was filled with deionized H_2O and recirculated for 30 min (2.4 L/h, 37°C) while maintaining the stirring of the reservoir bottle at 500 rpm. Then, the bottle was changed with the NaCl solution, and the electrical conductivity (μS/cm) was measured at the column exit every 30 s. If the normalized conductivity values are plotted versus time, the resulting profile is a sigmoid function, whose inflection point corresponds to the average hydraulic residence time of the column (HRT). The HRT was used to calculate the PBBR effective porosity. More details on the tracer tests and HRT calculation are provided by Frascari et al. [40] and Pinelli et al. [41].*PBBR inoculation, biofilm growth and production tests.* In the 1-L bioreactor tests the inoculum activation, biofilm growth and bioproduction phases were modified as follows. The inoculum activation phase was incubated under anaerobic conditions for 24 h (37°C, 50 rpm) in a sterile 250 mL bottle with 100 mL of total liquid volume, inoculated with 10% v/v of inoculum (90 mL of medium and 10 mL collected from different frozen criovial stocks). After 24 h, the sterile 2 L reservoir bottle was filled with the amount of growth medium (Table 3) required to fill the porosity of the packed bed and to guarantee a residual 50 mL volume in the reservoir bottle (about 570 mL), and inoculated (10% v/v) with the inoculum activation phase suspension. The bottle was then connected under sterile condition to the plant. The plant was kept in continuous recirculation at 37°C for 24 h, in order to ensure enough time for biofilm formation. No carriers were sampled to maintain the sterility. pH was monitored and manually adjusted at 6.6 every hour. To switch from biofilm growth to production phase, the column liquid medium was discharged in the reservoir, and the bottle containing the exhausted culture medium was replaced with an identical 2 L sterilized bottle filled with production medium and a 12-15 g L^{-1} lactose concentration. The growth phase gas bag was replaced with a new one containing 100% CO_2. The plant was then monitored every hour for medium composition. Whenever required, a further biofilm growth phase was performed according to the above-described procedure, to restore biofilm activity before the following repeated batch bioproduction phase. At the end of the last experiment, 5 carriers were sampled from the bottom, the middle and the top part of the column to quantify biofilm concentration.

Analysis

The headspace composition was monitored using an Agilent Technologies 5890 SERIES II Plus gas-chromatograph with a TCD detector (injector temperature 230°C, column temperature 150°C, carrier gas N_2) and a 30 m capillary 1010 plot Carboxen column with a 0.53 mm diameter. Organic acids and sugars were monitored by HPLC, using H_2SO_4 0.01 N as the mobile phase (0.8 mL/min, 40°C), a Transgenomic Coregel-87H3 HPLC column (a negatively charged mixture of totally sulfonated polystyrene/divinylbenzene, stable up to 90°C and in the 0-14 pH range) and a Shimadzu 10A RID detector. Suspended cell concentration was quantified using the simplified version [42] of the Lowry method [43] after two centrifugation steps (14000 rpm, 4°C, 10 min) and two subsequent washings in saline solution. The results were then expressed as $g_{protein}$ L^{-1} through a linear interpolation of absorbance (540 nm) with a Bovine Serum Albumin analysis with deionized H_2O as blank. For each attached biomass determination, five carriers were sampled and treated with 7 mL of NaOH 1 M (1.5 h, 30°C, 90 rpm) to detach and hydrolyse the biofilm. 500 μL of the obtained supernatant was

analysed with the Lowry method. The 5 carriers were then weighted after drying at 105°C for one night and heating at 600°C for 1 h to determine the actual carrier dry weight. The attached-cell biomass concentrations were thus expressed as $g_{protein}\ g_{dry\ carrier}^{-1}$. The total mass of the attached cells in the vial or PBBR was then calculated by multiplying this concentration by the total mass of carriers. Finally, the volumetric attached-cell concentrations ($g_{protein}\ L_{reactor}^{-1}$) were obtained dividing the total biomass mass by the total volume of the bioreactor (liquid + solid). In the PPBR, large flocks of suspended biomass remained entrapped in the void spaces between carriers and were not detected either as suspended biomass or as attached cells. This biomass fraction, referred to as "entrapped suspended biomass", was estimated after the last bioproduction test as follows: the column was emptied by gently and carefully removing the carriers with a pair of sterile tweezers. The empty column was then washed with 580 mL of demi water, strongly agitated in order to remove all the entrapped biomass, and this was analyzed as suspended biomass.

Rate evaluation and statistical analysis (ANOVA)

The rates of lactose consumption ($-r_{LAC}$) and SA production (r_{SA}) were obtained by dividing the slope of the linear part of the plots of total mass versus time by the liquid volume (in the suspended-cell tests) or by the packed bed volume (in the biofilm tests). For each experimental condition, the average rates and yields were associated to the corresponding standard deviation, obtained from the triplicate tests. In order to statistically evaluate the differences among the average rates obtained with different carriers, an analysis of variance (ANOVA) was applied. An F test with 0.05 significance level was applied to the $s^2_{between}/s^2_{within}$ ratio, where $s^2_{between}$ expresses the sample variance between the different carrier tests, whereas s^2_{within} indicates the sample variance within each triplicate test [44]. The F test was applied with k–1 degrees of freedom for $s^2_{between}$, and 2k degrees of freedom for s^2_{within}, where k is the number of tested carriers. The result of the F test indicates the probability that the null hypothesis is true, i.e. that there are no statistically significant differences between the tested experimental conditions as far as the selected parameter is concerned [44]. When such probability was < 0.05, the null hypothesis was rejected. In addition, multiple comparisons between all the possible pairs of experimental conditions included in the assay were performed (t–test), to identify possible statistical clusters of experimental conditions not significantly different among each other. A 0.05/*n* significance level was adopted on the basis of a Bonferroni correction (*n* = number of comparisons made) [45]. More details on the statistical analysis are described by Cappelletti et al. [35].

RESULTS AND DISCUSSION

Preliminary suspended-cell tests of SA production

Preliminary tests were performed in 0.12 L batch bioreactors in order to assess the performances of suspended cells of *A. succinogenes*, to be used as a reference condition for the comparison with the attached-cell tests. Three SA productions were performed in triplicate using relatively low initial lactose concentrations (7, 11 and 15 g L^{-1}) to avoid inhibition problems and to better control pH. As expected, no significant differences were observed between the three tested lactose concentration in terms of SA/lactose yield ($Y_{SA/LAC}$), that varied in the 0.60-0.62 $g_{SA}\ g_{LAC}^{-1}$ range (Table 4). Conversely, a slightly higher SA productivity was obtained in the test at 11 $g_{LAC}\ L^{-1}$ (1.06 g $L^{-1}\ h^{-1}$) in comparison with those at 15 $g_{LAC}\ L^{-1}$ (1.00 g $L^{-1}\ h^{-1}$) and 7 $g_{LAC}\ L^{-1}$ (0.90 g $L^{-1}\ h^{-1}$). As an example, Figs. 2a and 2b show the time profiles of lactose, succinic acid and other products relative to the test conducted at the best-performing lactose concentration (11 $g_{LAC}\ L^{-1}$). In all the three tests lactose was hydrolyzed into glucose and galactose with no lag phase. Glucose and galactose partly accumulated during the initial 4 h, then they were completely consumed. As expected, *A. succinogenes* preferentially consumes glucose, but it is capable to metabolize also galactose. Succinic, acetic and formic acids

were formed. No other organic acid, in particular lactic acid, was produced. The average values of $Y_{SA/LAC}$ and SA productivity obtained in these tests (0.61 g_{SA} g_{LAC}^{-1} and 0.99 g L^{-1} h^{-1}, respectively: Table 4) are in very good agreement with the corresponding average values reported in the literature for SA production from different substrates using *A. succinogenes* (0.70 g_{SA} g_{LAC}^{-1} and 1.0 g L^{-1} h^{-1}, respectively: average of the values reported in the upper part of Table 1). The biomass/lactose yield ($Y_{X/LAC}$) was equal to 0.15 ± 0.04 $g_{protein}$ g_{LAC}^{-1}, the AA:SA ratio was 0.50 ± 0.04 g g^{-1} (0.99 ± 0.08 mol mol^{-1}), and the FA:SA ratio was 0.50 ± 0.06 g g^{-1} (1.29 ± 0.16 mol mol^{-1}). The SA/LAC yield, AA:SA ratio and FA:SA ratio are in good agreement with the observations and hypothesis of McKinlay et al. [26], who predicted a maximum yield equal to 0.66 g_{SA} $g_{glucose}^{-1}$, an AA:SA molar ratio of 1 and an FA:SA molar ratio of 1 in case of negligible biomass formation and balanced C_3 and C_4 pathways. Conversely, other studies of SA production with *A. succinogenes* obtained lower AA:SA and FA:SA ratios: Liu *et al.* [46] report an AA:SA equal to 0.08-0.13 g g^{-1} and FA:SA of 0.04-0.07 g g^{-1}, in batch and fed-batch tests fed with glucose; Wan *et al.* [15] found an AA:SA of 0.38 g g^{-1} and FA:SA of 0.24 g g^{-1}, in batch tests fed with lactose-rich cheese whey; Bradfield and Nicol [19] attained an AA:SA of 0.20 g g^{-1} and a negligible FA production, in continuous tests fed with glucose. Interestingly, while in two of these studies the lower AA and FA productions led to a higher SA/substrate yield in comparison with our data [19,46], in the study of Wan *et al.* [15] the final SA/substrate yield resulted lower than ours, probably due to a larger biomass/substrate yield. Even though the examination of the literature studies on SA bioproduction with *A. succinogenes* does not indicate a final explanation on the experimental approaches that lead to the metabolic pathways characterized by minimum AA and FA production, low AA:SA and FA:SA yields are generally associated with the following operational conditions: non-growth cell state; high substrate and/or SA concentrations; continuous or fed-batch process; presence in the medium of other reducing power sources in addition to the main growth substrate [15,19,46]. This observation can provide a tentative explanation for the relatively high AA:SA and FA:SA yields obtained in this study, which was not conducted under the above-listed operational conditions.

Selection of a suitable carrier for attached-cell SA production

One of the specific goals of this work was to select a commercially available, cost-effective, suitable carrier to form a biofilm of *A. succinogenes* effective in the SA production process. Five different carriers, listed in Table 2, were tested in triplicate. Biofilm growth was carried out using a 20 g L^{-1} lactose solution (Table 3). Conversely, the SA production tests were performed at an initial lactose concentration of 7 g L^{-1}. Indeed, even if the SA productivity obtained in the preliminary suspended-cell tests at 11 g_{LAC} L^{-1} was slightly higher than that of the tests at 7 g_{LAC} L^{-1} (1.06 versus 0.90 g L^{-1} h^{-1}, respectively), the latter lactose concentration was selected for the carrier selection tests in order to reduce the entity of pH decrease during these tests, and therefore to minimize the possible influence of pH variations on the carrier selection process. This choice is based on the observation that the rate of SA production by *A. succinogenes* decreases rapidly if pH drops below 5.5 [46].

Three performance indexes were chosen to compare the tested carriers: i) SA production rate, ii) LAC uptake rate, and iii) SA/LAC yield ($Y_{SA/LAC}$). The average values obtained for each carrier are shown in Fig. 3. Glaxstone® resulted in the highest value for all the 3 performance indexes taken into consideration, whereas Poraver® was the second best performing carrier. The statistical significance of the observed performance differences between the 5 tested carriers, and in particular between Glaxstone® and Poraver®, was evaluated by means of an analysis of variance (ANOVA) and of multiple t-test comparisons between each couple of carriers. As shown in Table S1 in the Supplementary Material, the ANOVA indicated that the LAC/SA yields obtained with the 5 carriers were not statistically different ($p < 0.05$), whereas for the LAC and SA rates the carrier performances were characterized by statistically significant differences. As illustrated in Table S2 in the Supplementary Material, a further analysis consisting in multiple t-test comparisons indicated that

the performances of Glaxstone® and Poraver® were statistically different only in the case of the LAC uptake rate, whereas no statistical differences were found in terms of SA rate and LAC/SA yield.

On the basis of this analysis, further SA production tests were conducted, in order to compare in more detail the SA production performances of Glaxstone® and Poraver®. The tests were performed at 5 different initial LAC concentrations in the 2-12 g L^{-1} range. As shown in Fig. 4, where the initial rates relative to these tests are shown versus initial LAC concentration, the LAC and SA rates obtained with Glaxstone® were always slightly higher than those obtained with Poraver® (average increase in performance of Glaxstone® over Poraver® = 27%). Moreover, as shown in Table 4, the fraction of attached biomass at the end of these tests was similar for the two carriers (64-68%), whereas Poraver® was characterized by a slightly higher attached biomass final concentration (4 versus 3.1 g $L^{-1}_{packed\ bed}$). Poraver® was also characterized by a higher tendency to produce biomass during the SA production phase. Lastly, the two carriers performed similarly in terms of AA/SA and FA/SA ratios: indeed the first ratio resulted equal in average terms to 0.50±0.02 g g^{-1}, or 0.99±0.04 mol mol^{-1}, whereas the average FA/AA ratio was equal to 0.43±0.05 g g^{-1} (1.09±0.13 mol mol^{-1}). Both values are statistically compatible with the observations and hypothesis of McKinlay et al. [26].

Overall, even if the choice of the best carrier for scaling up the process should be made on the basis of a comprehensive economic analysis that goes beyond the scope of this work, Glaxstone® was selected for testing the use of actual cheese whey instead of lactose, and for evaluating the feasibility of a repeated batch process of SA production in a 1-L packed bed reactor.

As shown in Table 4, even if the SA/LAC yields obtained with Glaxstone® and Poraver® are higher than those measured with suspended cells, the attached-cell SA productivity is about half of that obtained in the suspended cell tests, and about 10% of the average value reported in the literature studies of SA production with biofilms of *A. succinogenes* (equal to about 5 g L^{-1} h^{-1}; Table 1). This result suggests that higher LAC concentrations should be applied, and that the growth procedure used in this work should be optimized in order to obtain a thicker biofilm.

Cheese whey fed attached-cell tests

After the identification of Glaxstone® as best carrier for SA production by biofilms of *A. succinogenes*, another series of tests at different substrate concentrations (2-15 g_{LAC} L^{-1}) was carried out, using actual waste (de-proteinated cheese whey, CW), instead of lactose, that represents its main constituent. For comparison sake, the cheese whey concentrations were expressed in terms of actual lactose concentration. No lag phase was observed at any concentration. The initial LAC uptake rates and SA production rates are plotted in Fig. 4 versus initial LAC concentration, whereas yields and productivities are shown in Table 4. No significant differences in performance were detected between CW and LAC. No substrate or product inhibition occurred using LAC concentrations up to 15 g L^{-1}. This finding is in agreement with Corona-Gonzalez et al. [10], who report inhibition on biomass formation for glucose concentrations > 30 g L^{-1} for a SA production process conducted with suspended cells of *A. succinogenes*. The performances obtained in this study with CW (maximum SA productivity 0.48 g_{SA} L^{-1} h^{-1}, SA/LAC yield 0.68 g g^{-1}) slightly exceed those obtained in the only published work of SA production from CW, relative to a suspended-cell process at much higher cheese whey concentrations [15] : SA productivity 0.44 g L^{-1} h^{-1} and $Y_{SA/LAC}$ 0.57 g g^{-1}, in the best fermentation conditions (pH 6.8, inoculum 5% v/v and initial cheese whey concentration 50 g L^{-1}, corresponding to about 40 g L^{-1} of LAC).

Batch feasibility tests in a 1-L packed bed bioreactor

In the last part of this work, the process of SA production from lactose by attached cells of *A. succinogenes* was scaled up to 1-L PFR – type reactor, packed with Glaxstone® as biofilm carrier

(Fig. 1). The goal was to perform a preliminary evaluation of the feasibility of an industrial process based on repeated-batch tests conducted in a biofilm reactor under fluid-dynamic conditions close to those of the industrial process (packed column instead of small vial with orbital shaking; flowing liquid medium instead of still medium). In order to perform repeated-batch tests, the packed bed bioreactor (PBBR) was operated under perfectly mixed phase conditions, by imposing a packed bed hydraulic retention time (HRT) < 1/10 of the bioreaction characteristic time [47]. The latter was cautiously assumed as the time corresponding to a 75% lactose conversion in the small-scale 15 g L^{-1} test (equal to about 3 h). Therefore, the maximum HRT (referred to the sum of the PBBR liquid volume and reservoir liquid volume) was assumed equal to 0.3 h. A perturbation/response tracer test was used to assess the effective porosity (56%) and liquid volume (580 mL) of the packed bed, whereas the liquid volume in the reservoir varied between 50 and 100 mL, depending on the tests. The flow rate was therefore set to 2.4 L h^{-1} (corresponding to a 1.3 m h^{-1} surface velocity), so as to yield a 0.26-0.28 h total HRT. In order to stimulate biofilm formation, two consecutive pulses of growth medium (Table 3) with 20 g L^{-1} of lactose were provided, following the procedure illustrated in the Materials and methods.

A crucial issue to be solved in order to develop an industrial-scale repeated-batch process is how to maintain biofilm activity and avoid significant lag phases after a period of bioreactor inactivity, which could be due to plant closure during the week-end or to the maintenance of a plant component. Indeed, preliminary evaluations performed in 0.12 L batch biofilm tests showed that if a biofilm of *A. succinogenes* is left for 2 days under anaerobic conditions without any glucose or lactose supply, it is then characterized by a SA production rate significantly smaller than the value observed before the inactivity period (data not shown). A tentative explanation for this behavior is that the cells run out of reducing power (NADH) during the resting phase. Therefore, the first experiment conducted in the 1 L PBBR had the following structure: 2 subsequent SA production phases (phase 3 medium, Table 3, 12 g_{LAC} L^{-1}) → 2-day resting phase at 37°C with a 100% CO_2 headspace → 2 further SA production phases (phase 3 medium, Table 3, 12 g_{LAC} L^{-1}). During the first 2 production phases, the SA production rate resulted equal to about 50% of the value obtained in the Glaxstone-filled 0.12 L tests fed with the same lactose concentration, possibly due to the lower biofilm concentration attained in the 1 L PBBR. During the 3rd production phase, operated after a 2-d resting phase, the LAC uptake rate and SA production rate dropped by 50-60% in comparison with the initial values. The first experiment was therefore stopped before the operation of the 4th production phase. In order to recover microbial activity and to increase biofilm thickness, two further pulses of growth medium (Table 3) with 20 g $_{LAC}$ L^{-1} were provided.

In the second experiment conducted in the 1-L PBBR, the LAC concentration was slightly increased, and an additional phase of biofilm growth with phase 2 medium was added after the 2-d resting phase, in order to avoid the drop in biofilm performance. Indeed, in the phase 2 medium the absence of biotin – a crucial vitamin for SA production – and of scarcity of CO_2 drives a larger fraction of the consumed lactose towards biofilm growth and reducing power (NADH) regeneration. The experiment structure was thus: 2 subsequent SA production phases (phase 3 medium, Table 3, 14-15 g_{LAC} L^{-1}) → 2-d resting phase at 37°C with a 100% CO_2 headspace → 1 growth phase (phase 2 medium, Table 3, 20 g $_{LAC}$ L^{-1}) → 2 further SA production phases (phase 3 medium, Table 3, 14-15 g_{LAC} L^{-1}). The profiles of LAC and SA concentration versus time relative to the SA production phases of the 2nd test are shown in Fig. 5, whereas the average performances of this test are reported in the last line of Table 4 and the average LAC and SA rates are graphically represented in Fig. 4. It can be observed that the additional growth phase after the 2-d rest allowed the operation of 2 further SA production phases without any decrease in SA rate. The design and experimental approach applied to the 1-L PBBR allowed the attainment of a very low fraction of suspended biomass (6% of the total biomass, versus 32% in the Glaxstone-filled 0.12 L reactors; Table 4). The average LAC and SA rates relative to this experiment were significantly higher than the corresponding values obtained at similar lactose concentrations in the Glaxstone-filled 0.12-L batch tests (150% increase for LAC, 75% increase for

SA), whereas a 24% decrease in SA/LAC yield was observed. Considering that the attached-cell concentration attained in the PBBR was 25% higher than that of the 0.12 L bioreactors, the marked increase in LAC and SA rate indicates that an actual increase in *specific* rates, and therefore in biofilm efficiency, occurred. Notwithstanding this, the average SA productivity obtained in these tests (0.72 $g_{SA}\ L^{-1}_{packed\ bed}\ h^{-1}$) is significantly lower than the average value reported in the literature studies on SA production by biofilms of *A. succinogenes* (5.3 $g_{SA}\ L^{-1}_{packed\ bed}\ h^{-1}$; Table 1), as a result of the lower biofilm concentration: for example, Maharaj and Bradfield [20] obtained biofilm concentrations in the 21-28 $g_{dw}\ L^{-1}$ range, in a glucose-fed process of SA production from glucose by Poraver®-attached cells of *A. succinogenes*. Interestingly, the specific SA rate obtained in our PBBR (about 0.1 $g_{SA}\ g_{dw}^{-1}\ h^{-1}$, under the assumption 1 $g_{protein} = 2\ g_{dw}$; [48]) falls within the range obtained by Maharaj et al. [20] in a study of SA production from glucose conducted in a Poraver®-filled PBBR (0.1-0.4 $g_{SA}\ g_{dw}^{-1}\ h^{-1}$, for hydraulic residence times ranging from 1.4 to 10 h). These results indicate that the Glaxstone-attached biofilm is working in an efficient way, and that further work is needed to increase the initial substrate concentration and biofilm thickness.

CONCLUSION

- The screening between 5 commercial biofilm carriers, based on a statistical analysis of the SA production rates, SA/LAC yields and LAC uptake rates, led to the selection of two highly-effective supports for SA production by biofilms of *A. succinogenes*. Among these, Glaxstone® was selected for the testing of an innovative substrate (cheese whey) and of the feasibility of a repeated-batch process in a packed bed bioreactor.
- De-proteinated cheese whey resulted an effective substrate for SA production by biofilms of *A. succinogenes*. This represents the first study of SA production from cheese whey by *A. succinogenes* attached cells.
- The feasibility of a repeated-batch process of SA production by biofilms of *A. succinogenes* was demonstrated in a 1-L packed bed bioreactor, and an effective sequence of growth and production phases was identified. The efficiency of the Glaxstone®-attached biofilm was satisfactory, but higher substrate and biofilm concentrations must be reached in order to attain SA productivities similar to those reported in the literature for glucose-fed processes.
- Overall, this work indicates that SA bioproduction under biofilm conditions from organic by-products such as cheese whey is a feasible and promising process. Further research is needed to perform a model-based scale-up of the process and to attain higher SA productivities.

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Supporting Information

Supporting information may be found in the online version of this article.

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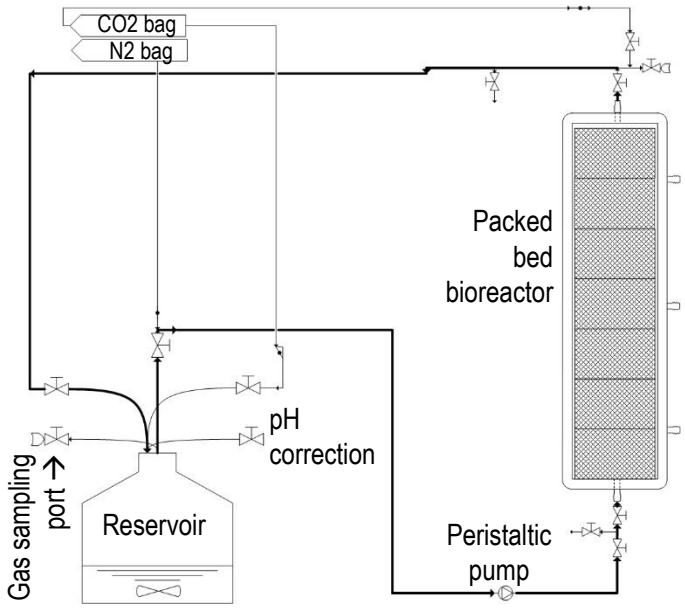

Figure 1. Schematic flow-sheet of the packed bed bioreactor plant (PBBR) used for succinic acid production.

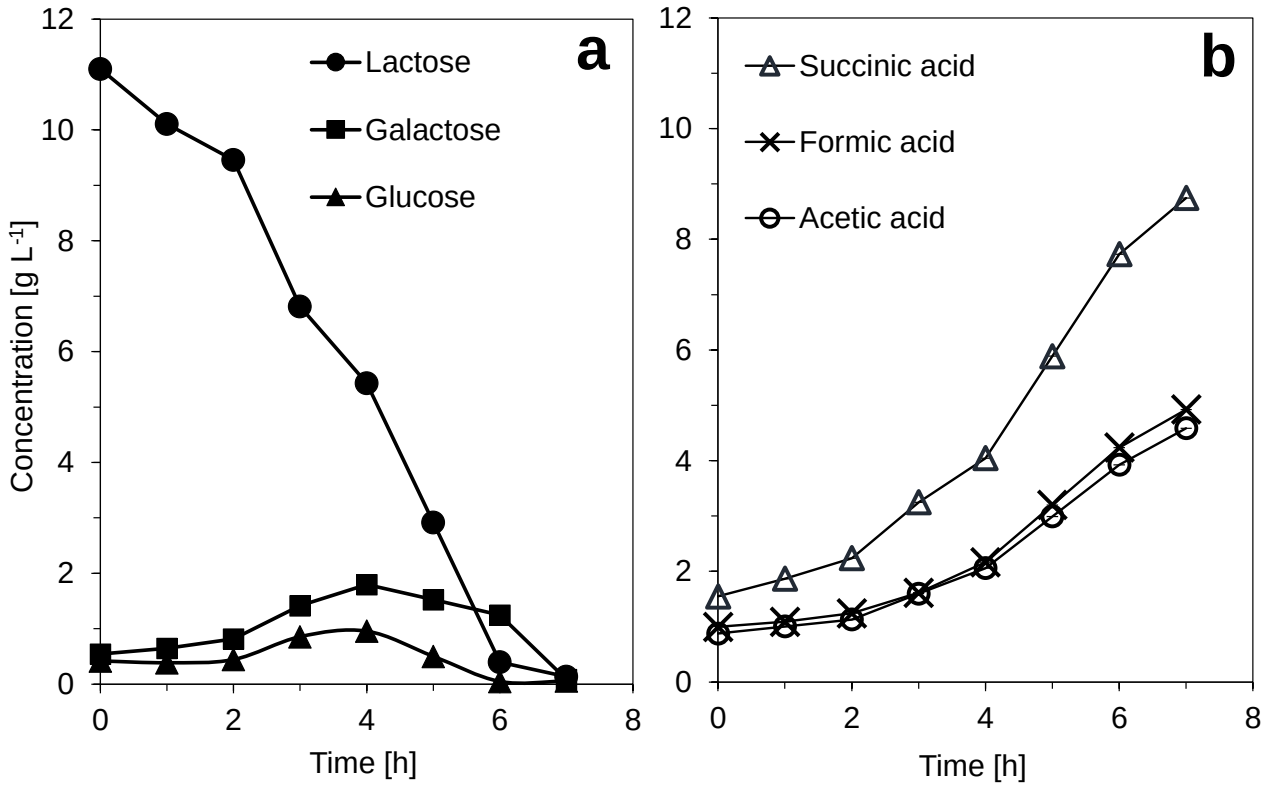

Figure 2. Concentration profiles in the suspended-cell tests with initial LAC concentration of 11 g L^{-1}: (a) lactose and intermediate sugars, (b) SA and other acids. Ethanol and lactic acid, not shown in the graph, were always below the detection limit.

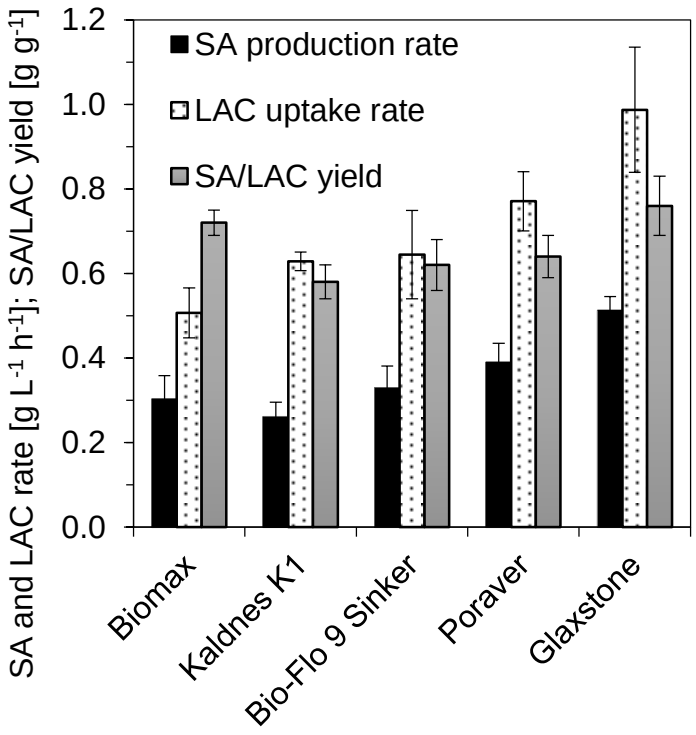

Figure 3. SA production rate, LAC uptake rate and SA/LAC yield obtained in the carrier selection tests. Average values with standard deviations.

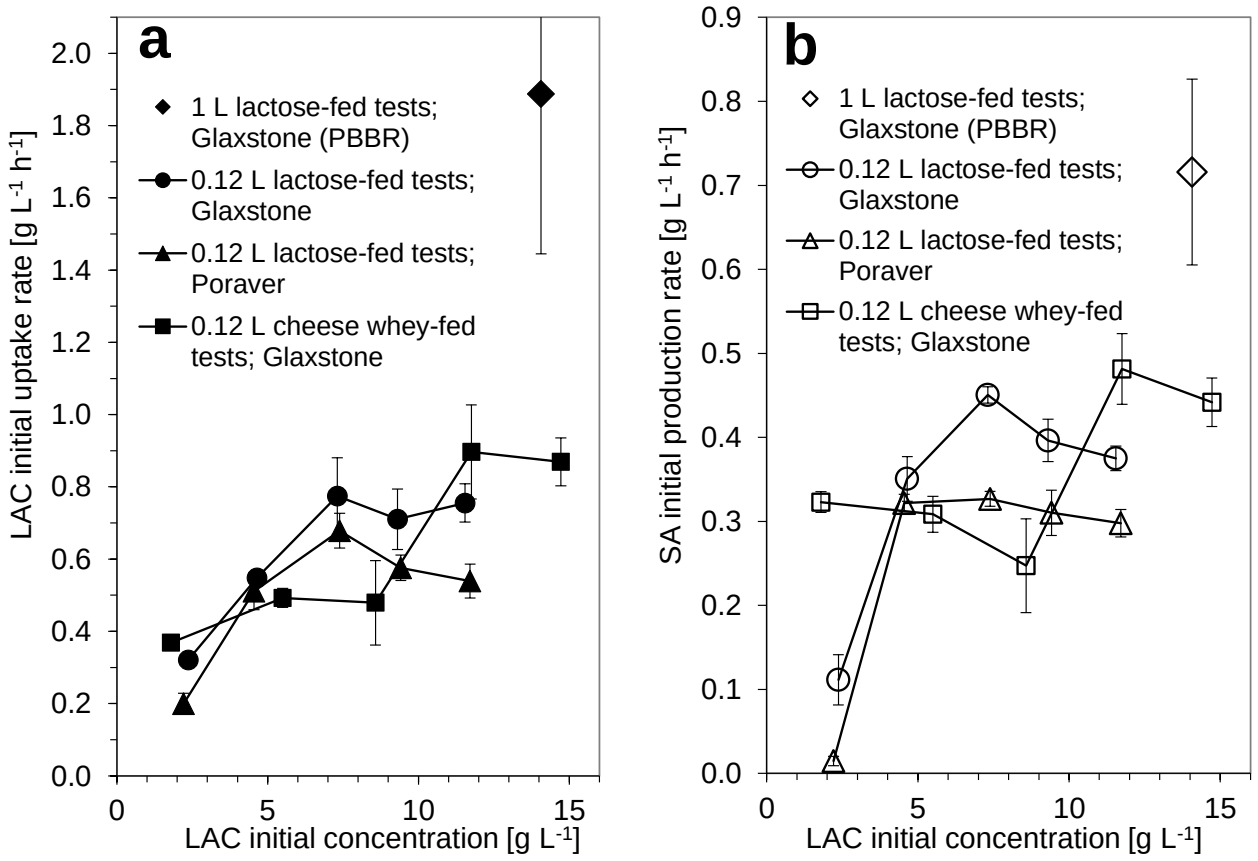

Figure 4. LAC initial uptake rates (a) and SA initial production rates (b) obtained at different LAC initial concentrations in the 0.12 L and 1 L biofilm tests filled with Glaxstone or Poraver, and fed with lactose or cheese whey.

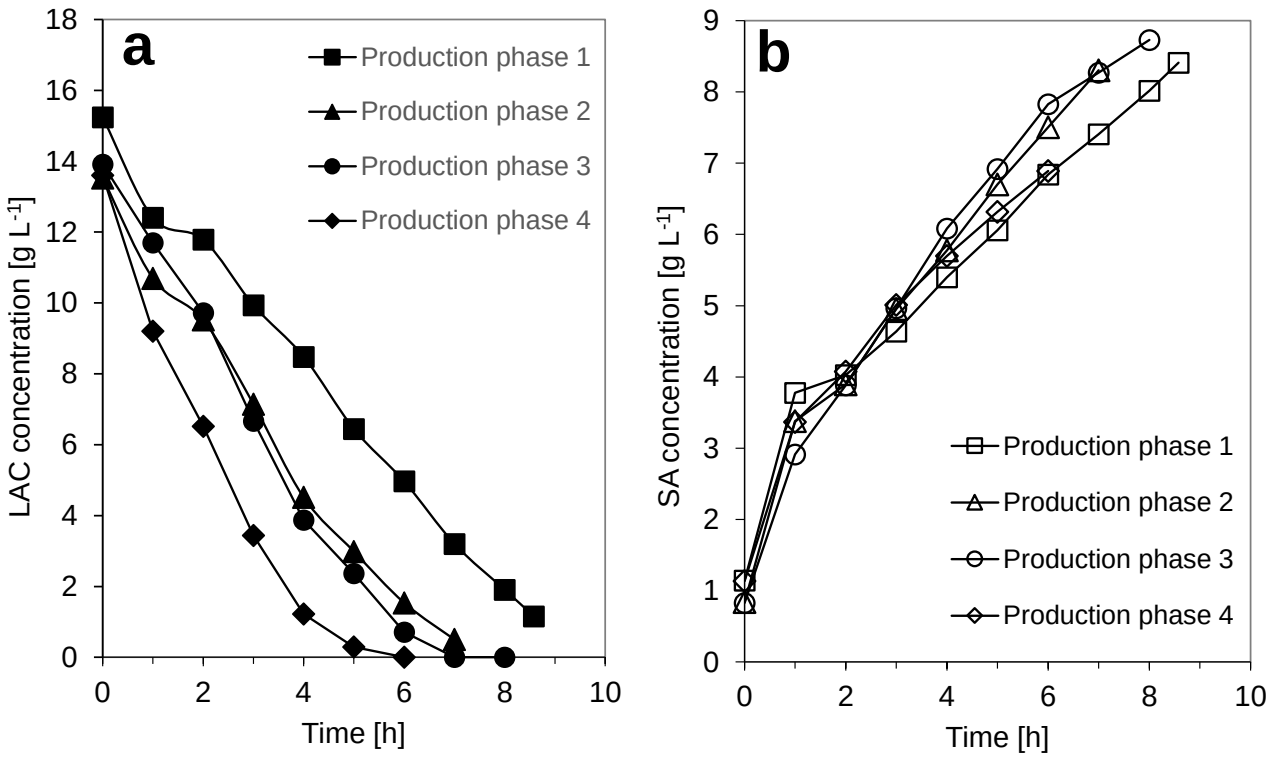

Figure 5. LAC (a) and SA (b) concentrations obtained in the second experiment conducted in the 1 L PBBR.

Table 1. Operational conditions and performances relative to the main literature works on SA bioproduction with different types of *A. succinogenes*

Cell condition[a]	Process type[b]	Substrate	*A. succinogenes* strain	SA max conc. [g L^{-1}]	P_{SA}[c] [g $L^{-1}h^{-1}$]	$Y_{SA/S}$[d] [g g^{-1}]	Reference
Suspended	B	Glucose	FZ53	105.8	1.4	0.83	[8]
Suspended	B	Glucose	130Z	67.2	0.8	0.70	[9]
Suspended	B	Glucose	ZT-130	33.8	1.0	0.62	[10]
Suspended	FB	Glucose	CGMCC 1593	53.2	1.2	0.83	[11]
Suspended	B	Xylose	CIP 106512	22.5	1.0	0.43	[12]
Suspended	B	Glucose	ATCC 55618	27.9	1.0	0.65	[13]
Suspended	B	Glucose	ATCC 55618	64.2	1.2	0.81	[14]
Suspended	B	Cheese whey	130Z	21.5	0.4	0.57	[15]
Suspended	B	Bakery waste	130Z	24.8	0.8	0.80	[16]
Suspended	B	Cane molasses	CGMCC 1593	50.6	0.8	0.80	[17]
Biofilm (fibrous-bed)	B, FB	Glucose	CCTCC M2012036	98.0	2.8	0.89	[18]
Biofilm (stainless-steel wool)	C	Glucose	130Z	48.5	[e]	0.91	[19]
Biofilm (Poraver ® beads)	C	Glucose	130Z	32.5	10.8	0.90	[20]
Biofilm (polypropylene protruding arms)	C	Xylose	130Z	39.6	1.8	0.78	[21]
Biofilm (Silicone tube)	C	Glucose	130Z	9.7	9.0	0.74	[22]
Biofilm (Genulite TM Groperl)	C	Glucose	130Z	12.0	6.4	0.69	[23]
Biofilm (PCS disks)	RB	Glucose	130Z	35.1	0.9	0.86	[24]

[a] In case of biofilm processes, the carrier type is specified in parenthesis.
[b] B, batch. FB, fed batch. C, continuous. RB, repeated batch.
[c] SA productivity.
[d] SA/substrate yield.
[e] Parameter not available.

Table 2. Main features of the carriers tested for A. succinogenes immobilization

Carrier	Supplier	Material	Surface area [$m^2\ m^{-3}$]	Bulk density [$g\ L^{-1}$]	Inner porosity [%]	Average diameter[a] [cm]	Average height[a] [cm]
Kaldness K1®	EvolutionAcqua	HDPE	950	165.5	[b]	1.06 ± 0.05	0.75 ± 0.01
Bio-Flo 9 Sinker®	SmokyMountain	HDPE	800	190.9	[b]	1.05 ± 0.10	0.80 ± 0.01
Glaxstone®	WaveAcquaristic	Sintered glass	270	717.9	76.4	1.99 ± 0.28	1.00 ± 0.01
Biomax®	Askoll	Ceramic	250	753.2	65.9	0.91 ± 0.04	1.10 ± 0.04
Poraver®	Poraver	Expanded glass	[c]	205.5	38.1	0.29 ± 0.05	0.29 ± 0.05

[a] Diameter and height, measured for 20 carriers of each type, are expressed here as average values ± standard deviation.
[b] Kaldness K1 and Bio-Flo 9 Sinker do not have an inner porosity (see Fig. S1 in the Supplementary Material).
[c] Information not available.

Table 3. Fermentation medium and headspace composition for the pre-culture, growth and bioproduction phases

	Phase 1: inoculum activation	Phase 2: strain growth	Phase 3: SA bioproduction
Bacteriological peptone [$g\ L^{-1}$]	10	10	10
Yeast extract [$g\ L^{-1}$]	10	10	10
K_2HPO_4 [$g\ L^{-1}$]	3	3	3
NaCl [$g\ L^{-1}$]	1	1	1
$(NH_4)_2SO_4$ [$g\ L^{-1}$]	3	3	3
$MgCl_2 \cdot 6H_2O$ [$g\ L^{-1}$]	0.3	0.3	0.3
$CaCl_2 \cdot 2H_2O$ [$g\ L^{-1}$]	0.3	0.3	0.3
$FeSO_4 \cdot 7H_2O$ [$g\ L^{-1}$]	0.005	0.005	0.005
Na_2CO_3 [$g\ L^{-1}$]	5	5	0
Biotin [$g\ L^{-1}$]	0	0	0.0002
BIS-TRIS (buffer) [$g\ L^{-1}$]	20	20	20
Glucose [$g\ L^{-1}$]	20	0	0
Lactose [$g\ L^{-1}$]	0	20	2-15
Cheese whey [$g\ L^{-1}$]	0	0	2-15[a]
pH	6.6	6.6	6.6
Headspace composition [%]	100% N_2	88% N_2 12% CO_2	100% CO_2

[a] Referred to the actual lactose concentration. Lactose constitutes 85% of the tested de-proteinated cheese whey.

Table 4. Overview of the main performances obtained in the batch tests conducted under different cell conditions (suspended / attached), substrates (lactose / cheese whey) and bioreactor volume (0.12 / 1 L)

Cell condition[a]	Substrate	$V_{reactor}$ [L]	Initial LAC [$g\ L^{-1}$]	$Y_{SA/LAC}$[b] [$g\ g^{-1}$]	$Y_{X/LAC}$[c] [$g_{protein}\ g^{-1}$]	Attached biomass concentration		Attached biomass fraction [%]	P_{SA}[d] [$g\ L^{-1}\ h^{-1}$]
						[$g\ L^{-1}_{packed\ bed}$]	[$g\ kg^{-1}_{carrier}$]		
Suspended cells	Lactose	0.12	7-15	0.61±0.01	0.15±0.04	[e]	[e]	[e]	0.99±0.08
Biofilm (Glaxstone®)	Lactose	0.12	7-12[f]	0.76±0.07	0.01±0.01	3.1±0.2	3.1±0.7	68%	0.41±0.04
Biofilm (Poraver®)	Lactose	0.12	7-12[f]	0.64±0.05	0.04±0.02	4.0±0.5	13.1±2.2	64%	0.31±0.01
Biofilm (Glaxstone®)	Cheese whey	0.12	9-15[g]	0.68±0.06	0.02±0.01	3.9±0.3	3.9±0.4	67%	0.46±0.03
Biofilm (Glaxstone®)	Lactose	1.04	14	0.58±0.02	[h]	3.9±0.9	4.8±1.1	94%	0.72±0.11

[a] In case of biofilm tests, the carrier type is specified in parenthesis.
[b] SA/ LAC yield.
[c] Biomass / LAC yield, referred to the SA production phase.
[d] SA productivity.
[e] Not applicable to the suspended-cell tests.
[f] The LAC-fed small scale tests with Glaxstone® and Poraver® as carriers were conducted over the 2-12 $g_{LAC}\ L^{-1}$ concentration range. However, the average values reported in this table are referred to the 3 tests conducted at the highest concentrations (7-12 $g_{LAC}\ L^{-1}$), more meaningful from an application point of view and characterized by smaller uncertainties.
[g] The cheese whey-fed small scale tests with Glaxstone® as carrier were conducted over the 2-15 $g_{LAC}\ L^{-1}$ concentration range. However, the average values reported in this table are referred to the 3 tests conducted at the highest concentrations (9-15 $g_{LAC}\ L^{-1}$), more meaningful from an application point of view and characterized by smaller uncertainties.
[h] Information not available.

Succinic acid production from cheese whey by biofilms of *Actinobacillus succinogenes*: packed bed bioreactor tests

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SUPPLEMENTARY MATERIAL

Table S1

ANOVA relative to the 3 performance indexes utilized in the batch tests conducted with the 5 tested carriers[a].

Performance index	Degrees of freedom relative to $s^2_{between}$	Degrees of freedom relative to s^2_{within}	$s^2_{between}$	s^2_{within}	$\frac{s^2_{between}}{s^2_{within}}$	P[b]	Significance level	Result
SA production rate	4	10	$2.86 \cdot 10^{-2}$	$6.06 \cdot 10^{-4}$	47.1	$1.9 \cdot 10^{-6}$	$5 \cdot 10^{-2}$	Statistically different
LAC uptake rate	4	10	$9.96 \cdot 10^{-2}$	$2.48 \cdot 10^{-3}$	40.2	$3.9 \cdot 10^{-6}$	$5 \cdot 10^{-2}$	Statistically different
SA/LAC yield	4	10	$1.57 \cdot 10^{-2}$	$8.97 \cdot 10^{-3}$	1.8	$2.1 \cdot 10^{-1}$	$5 \cdot 10^{-2}$	Not statistically different

[a] N. of replicates for each performance index: 3. N. of compared experimental conditions: 5. Degrees of freedom: 4 for $s^2_{between}$, 10 for s^2_{within}.

[b] Probability that the variance of the entire test is *not* statistically different from the variance of each test, i.e. that there are *no* significant differences among the means of the tested parameter in the different experimental conditions compared.

Table S2

Multiple t-test comparisons relative to the 3 performance indexes utilized in the batch tests conducted with the 5 tested carriers. Significance level: 0.05/10 (Bonferroni correction). Each tabulated value expresses the probability that there are no differences among the means of the tested performance index in each couple of carriers compared. The probabilities lower than the significance level, corresponding to statistically different means, are highlighted in pink.

Parameter	Carrier	Kaldnes K1®	Bio-Flo 9 Sinker®	Poraver®	Glaxstone®
SA production rate	Biomax®	0.111	0.343	0.018	0.001
	Kaldnes K1®		0.027	0.002	0.000
	Bio-Flo 9 Sinker®			0.049	0.001
	Poraver®				0.002
LAC uptake rate	Biomax®	0.004	0.022	0.001	0.001
	Kaldnes K1®		0.667	0.004	0.002
	Bio-Flo 9 Sinker®			0.033	0.004
	Poraver®				0.014
SA/LAC yield	Biomax®	0.000	0.001	0.005	0.112
	Kaldnes K1®		0.095	0.021	0.000
	Bio-Flo 9 Sinker®			0.518	0.000
	Poraver®				0.001

Figure. S1. Pictures of the five tested carriers. From left to right: Poraver®, Glaxstone®, Bio-Flo 9 Sinker®, Kaldnes K1®, Biomax®.