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ORIGINAL ARTICLE

Continuous succinic acid production from corn fiber hydrolysate by immobilized *Actinobacillus succinogenes* in a hollow fiber membrane packed-bed biofilm reactor

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Abstract

Succinic acid is one of the most useful intermediate chemicals that can be produced in a biorefinery approach. In this study, *Actinobacillus succinogenes* was immobilized to produce succinic acid using non-detoxified corn fiber hydrolysate (CFH) and a control mimicking the sugars in CFH. Tests were carried out in a hollow fiber membrane packed-bed biofilm reactor (HFM–PBR) operated in a continuous mode. Under steady-state conditions, the bioconversion process was characterized in terms of sugar consumption, succinic acid and other organic acid production. Steady states were obtained at dilution rates of 0.025, 0.05, 0.075, 0.1, 0.2, and 0.3 h^{-1}. The optimal results were achieved at the dilution rate of 0.05 h^{-1} and recirculation rate of 50 ml/min with a maximum succinic acid concentration, yield and productivity of 31.1 g/L, 0.61 g/g and 1.56 g/L h, respectively, when control was used. Succinic acid concentration, yield and productivity of 23.4 g/L, 0.51 g/g and 1.17 g/L h, respectively, were obtained when CFH was used. Productivity in the HFM–PBR was between 1.3 and 1.9 times higher than productivities for succinic acid production from CFH stated in the literature. The results demonstrated that immobilized *A. succinogenes* has the potential for effective conversion of an inexpensive biomass feedstock to succinic acid. Furthermore, the process has the potential to serve as a means for value-added chemical biomanufacturing in an integrated corn biorefinery.

Keywords Corn fiber · Succinic acid · Continuous fermentation · Hollow fiber membrane · Biofilm · Packed-bed reactor

Introduction

Growing concerns over the contribution of fossil fuels-based processes to global warming and the strong demand for environmentally friendly energy sources have inspired a deep interest in developing more sustainable processes with lower cost and energy consumption that affords the same product using renewable biomass [1, 2]. Succinic acid, an important building-block chemical, was featured as one of the top value-added chemicals from biomass by the US Department of Energy [3]. The development of bio-based succinic acid processes has gained traction due to the environmental impact of fossil-fuel dependent processes, which has been the traditional method to produce succinic acid [4]. Another major driver is succinic acid's key role in the synthesis of many large-volume chemicals and many consumer products such as 1,4 butanediol [5, 6].

Succinic acid can be obtained by microbial fermentation of sugars by *Actinobacillus succinogenes*, a native high-succinic acid producer that possesses the ability to use a wide variety of sugars and biomass hydrolysates for succinic acid production [7–10]. Moreover, the process captures the greenhouse gas CO_2, which is a substrate in succinic acid production [11–13]. Significant steps for succinic acid production by *A. succinogenes*, such as the metabolic flux of carbon and the activity of phosphoenolpyruvate (PEP) carboxykinase, are regulated by pH and dissolved CO_2 concentration in fermentation media. When gaseous CO_2 is supplied during fermentation, it possesses poor solubility in media at 1 atm, negatively affecting succinic acid production. In addition, due to the low affinity of the enzymes responsible for CO_2 fixation, high CO_2 partial pressures may be required

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succinic acid fermentations [14]. To further enhance CO_2 concentration in media, different strategies such as the addition of many kinds of carbonate salts as indirect supplementation of CO_2, high-pressure fermenters, and conventional bioreactors with sparging and intense agitation have been utilized [15–17]. Nevertheless, supplying high concentrations of carbonate salts is not economically feasible from an industrial point of view, and safety precautions during full scale operation of high-pressure fermenters requires further attention [18]. Also, due to both the damage to cells and high power consumption caused by excessive agitation speed or gas lift mechanisms, conventional stirred tank bioreactors need reconsideration. Another strategy could be the use of hollow fiber membranes (HFMs) to enhance the dissolved CO_2 concentration in succinic acid fermentations. HFMs diffuse gases through their micropores without forming bubbles yielding a large surface area for both gas–liquid transfer and, therefore, enhancing the amount of dissolved gas in fermentation broth [19]. HFMs have been employed successfully to enhance mass transfer of gases in wastewater and water treatments and ethanol and syngas fermentations [19–21].

Most *A. succinogenes* fermentations have been performed in batch and fed-batch mode. However, *A. succinogenes*' growth is inhibited by acids produced during fermentation [22, 23], which reduces volumetric productivity of batch processes [24]. Continuous fermentations offer an improvement in product production rates and productivity. However, biofilm formation is prevalent *A. succinogenes* fermentations [25, 26], which makes establishing steady-state conditions in a continuous stirred tank reactor very difficult due to cells attaching to internal surfaces within the reactor [25].

Actinobacillus succinogenes' proclivity for biofilm formation can be taken advantage of by allowing the biofilm to attach to support in a continuous packed bed reactor (PBR) [24–27]. Ferrone et al. [24] used a mixture of glucose, xylose, and arabinose that mimicked lignocellulosic biomass hydrolysate to produce succinic acid in a 166 mL PBR operated in continuous mode for five months. They achieved a succinic acid concentration of 43.0 g/L, a glucose conversion of 88%, and a volumetric productivity of 22 g/L h at the dilution rate 0.5 h^{-1}. Maharaj et al. [27] reported a continuous fermentation by *A. succinogenes* in a PBR with Poraver® beads using pure glucose. The authors reported a succinic acid volumetric productivity of 10.8 g/L h at a dilution rate = 0.7 h^{-1}.

In view of the above context, it is valid to explore processes that use renewable materials that have lower costs than pure sugar feedstocks, which is one of the major bottlenecks to establishing a successful industrial succinic acid bioconversion [28]. To the best of the authors' knowledge, there are no published studies investigating continuous succinic acid production from renewable feedstocks using a PBR and a HFM for supplying CO_2 to the fermentation media. Corn fiber, an inexpensive source of sugars, has been used previously in batch fermentations to achieve succinic acid yields similar to yields obtained from a pure sugar mixture [29]. The bioconversion process could be integrated into a corn bioethanol facility as corn fiber and gaseous CO_2 are low-value coproducts generated in these facilities. Using such coproducts at the point of production eliminates logistical costs of raw material supply. In this study, continuous succinic acid production in a PBR coupled with a HFM was used to produce succinic acid yield from corn fiber hydrolysate (CFH). This work presents important insights into the operation of a hollow fiber membrane packed bed reactor (HFM–PBR) and the effect of dilution rate (D) and recirculation flow rate on CFH sugars consumption and succinic yield and productivity.

Materials and methods

Microorganism, inoculum, and medium

Actinobacillus succinogenes 130Z was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and was used to produce succinic acid from CFH. The culture in the form of freeze-dried pellets was reactivated according to the procedure suggested by ATCC and stored following the recommendations of Long et al. [30]. Stock cell tubes were preserved at − 80 °C in 5% DMSO in 1.5 mL culture tubes and used for the inoculation. *A. succinogenes* cells were inoculated in anaerobic culture tubes containing seed medium (30 g tryptic soy broth/L and 15 g glucose/L) and incubated in a shaker at 37 °C, 250 rpm for 14–16 h. The culture was washed with sterile 0.89% sodium chloride solution and resuspended with fermentation medium and inoculated to the reactor. The growth medium based on Maharaj et al. [27], with some modifications, and had the following composition (per L): 16.0 g yeast extract, 1.0 g NaCl, 1.36 g $NaC_2H_3O_2$, 0.20 g $MgCl_2{\cdot}6H_2O$, and 0.20 g $CaCl_2{\cdot}2H_2O$. The carbon sources were a control that was a sugar solution mimicking the sugars contained in CFH, and CFH, a sugar solution obtained from corn fiber as explained in the next section. All media were sterilized at 121 °C for 20 min before use.

Preparation of CFH

The preparation of CFH was according to Vallecilla-Yepez et al. [29] and proceeded as follows: ground corn fiber provided by E-Energy Adams, LLC, (Adams, NE, USA) was subjected to liquid hot water pretreatment in a 7.5 L Parr reactor as described below (Parr Reactor Model 4552, Parr Instrument Co., Moline, IL, USA). Samples of 0.9 kg ground corn fiber (dry basis) were mixed with 5.1 kg water

to achieve 15% solids loading. The mixture was agitated at 300 rpm, heated to 180 °C, and held at 180 °C for 10 min. After the pretreatment, pretreated corn fiber was separated from the liquid by filtration and washed with 9 kg of water. Enzymatic hydrolysis of the pretreated corn fiber was done by with Ctec2 enzyme (Novozymes, Inc., Franklinton, NC, USA) in the ratio of 20 FPU/g glucan (Filter Paper Units enzyme/g glucan) and water to achieve 10% solids loading. The hydrolysis was carried out in a 42 L Techfors-S bioreactor (INFORS HT, Basel, Switzerland) at a mixing rate of 1000 rpm and at a temperature of 50 °C for 72 h. The pH was controlled at 5.0 with 5 M NaOH and 5 M H_2SO_4 solutions using the Techfors-S and Eve control software (INFORS HT). After hydrolysis, solid residues were separated by filtration and the filtrate was sterilized by passing the resultant solution through a 0.22 µm bottle top filter (Nalgene™ Rapid-Flow™ Sterile Single Use Bottle Top Filters, Thermo Fisher Scientific, USA). The filtered solution containing the sugars was designated as CFH, and it did not undergo detoxification procedures. CFH was kept at 4 °C until further use for fermentation.

pH control and buffer selection

pH is a key parameter in the succinic acid bioconversion process because both intracellular enzymatic activities and cellular maintenance in *A. succinogenes* are strictly pH dependent. The optimal pH range for succinic acid productivity was found to be between 6.0 and 7.2 with a maximum production of succinic acid achieved at 6.7 [15]. In addition, for succinic acid producing bacteria, the optimal pH for the PEP–carboxykinase activity and a higher effect of CO_2 on succinic acid yield have been observed at pH 6.5 [31]. Tests have been reported elsewhere in the literature to assess the best pH regulation strategy in succinic acid fermentation in attempts to improve its feasibility at industrial scale [16, 32]. In this study, a mixture of $Mg(OH)_2$ (5 M) and NaOH (5 M) in a mass ratio of 1:1 was used to control pH at 6.6, which is between the enzymes' optimal pH for succinate formation and for maximum CO_2 fixation.

Biofilm carriers

Two biofilm carriers were used during this study. Preliminary tests were performed with carrier 1, which was 238 g of packing material comprised of 568 HDPE plastic rings (model BCN 030, GEA 2H Water Technologies GmbH, Germany, Europe) used in a previous study that used a PBR to produce aryl alcohol oxidase with filamentous fungi [33]. The length and diameter of the rings were equal to 30 mm and 30–36 mm, respectively, with a surface of 320 m^2/m^3 and a protected surface of 259 m^2/m^3. The rings occupied a volume of ± 3.3 L (approx. 45% of the column volume). The second carrier, carrier 2, consisted of a constant mass of 5.8 kg of solid soda lime spheres (Avogadro's Lab Supply, Inc., Shamong, NJ, USA) to ensure an approximately constant volume and surface area available for attachment. The diameter and density of the spheres were equal to 0.01 m and 2.49 kg/m^3, respectively. The support beads occupied a volume of ± 6.7 L (approx. 91% of the column volume).

HFM–PBR design

An HFM–PBR photographed in Fig. 1 and shown schematically in Fig. 2 was designed for succinic acid fermentation. The novel reactor consisted of a 0.71 m tall glass column (Ace Glass Incorporated, Vineland, NJ, USA) with an internal diameter of 0.08 m that was jacketed for heat exchange. The glass column was incompletely filled with the packing material to provide a head space that allowed a thermowell to be in contact with the fermentation medium. A perforated borosilicate glass support plate (3 mm rectangular slits) (Ace Glass Incorporated, Vineland, NJ, USA) was inserted below the packing to support it while allowing flow of liquid and gas. The system contained a polydimethylsiloxane hollow fiber membrane module (PermSelect®-Silicone Gas Exchange Membranes, Ann Arbor, MI, USA). The module was a polycarbonate shell which envelops a polyurethane potting with 2500 cm^2 surface area, based on the outside area of the hollow fibers. Figure 3 shows the module connection to the HFM–PBR, which was configured to add gas to the liquid. In this type of configuration, the liquid (fermentation medium) flows in the shell side contacting with CO_2 that flows on the lumen side (inside the hollow fibers).

The inlets for fresh and recycled CFH were placed on the top of the reactor, as well as a thermocouple to measure the column temperature, and a 169-kPa (5 psig) safety pressure release valve to protect the glass column. A bioreactor (BioFlo 115, New Brunswick Scientific Co., NJ, USA) equipped with a 1.3 L vessel containing a 6-Blade

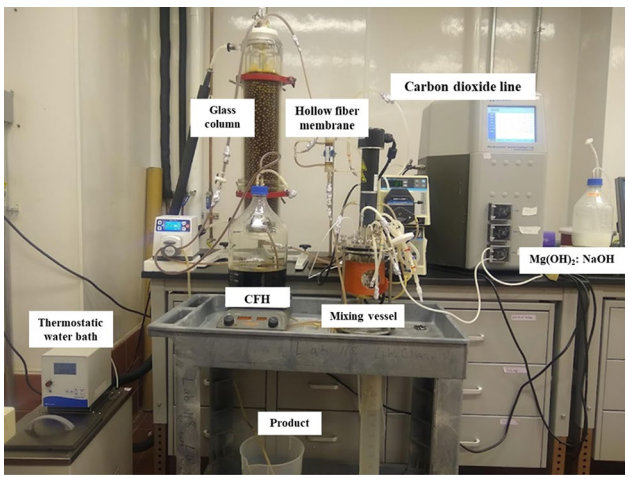

Fig. 1 The HFM–PBR during fermentation of CFH

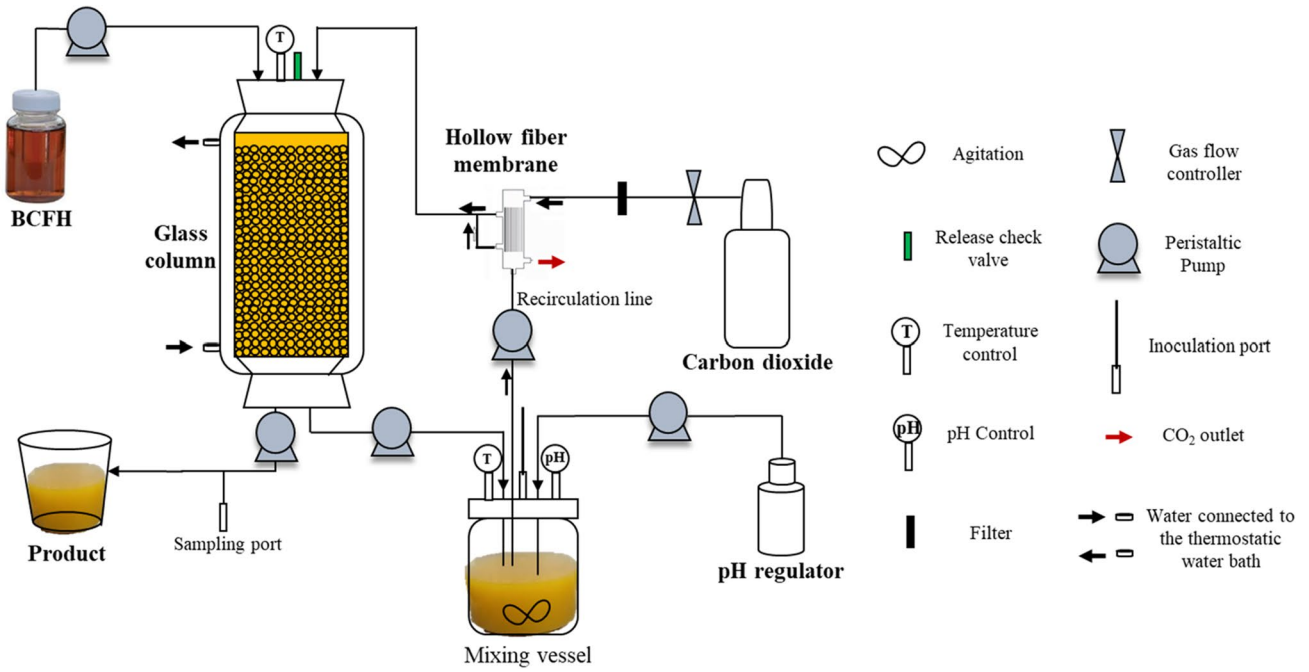

Fig. 2 Schematic representation of the HFM–PBR for continuous production of succinic acid

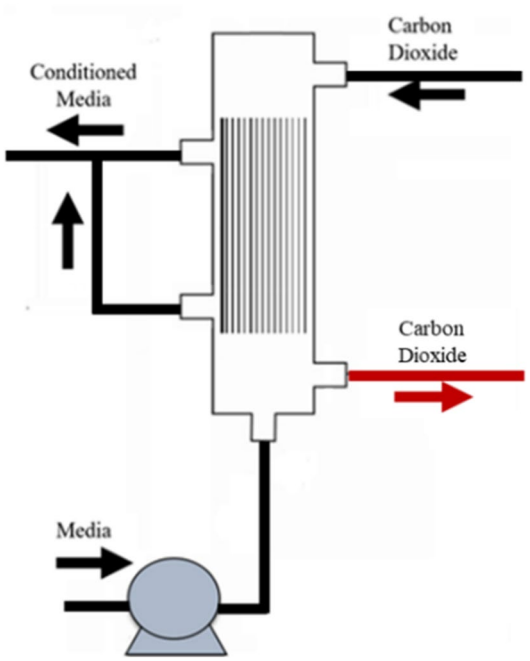

Fig. 3 HFM configuration for succinic acid fermentation

Rushton impeller was used as a mixing vessel and connected to the reactor. A sampling port at the bottom of the column allowed periodic sampling of the liquid broth. The inoculation port, pH probe, and a second thermocouple were located in the mixing vessel. The product line was connected at the bottom of the reactor and a sampling port in the product line allowed periodic sampling. Two peristaltic pumps (MasterFlex 7523-20, Barnant Co., Barrington, IL, USA) were used for media recirculation to provide mixing in the reactor. One pump took media out from the mixing vessel and into the HFM–PBR. The second pump took medium from the HFM–PBR to the mixing vessel at the same rate as the first pump. When the HFM–PBR operated in a continuous mode, a dual channel peristaltic pump (Masterflex® L/S® Digital Miniflex® Pump Systems, Barnant Co., Barrington, IL, USA) was used to add fresh medium into the system at the same rate that the product was collected. Filter-sterilized CO_2 (0.2 mm PTFE filter, Pall Corporation, NY, USA) was sparged into the CFH through the HFM membrane. Temperature was controlled using column's water jacket connected to a thermostatic water bath.

HFM–PBR fermentation

The HFM–PBR was sterilized by filling it with deionized water and autoclaving it at 121 °C for 45 min. The 1 L mixing vessel, tubing and fittings for the system were autoclaved separately and assembled later. After sterilization, the reactor was drained and refilled with sterile fermentation medium that was autoclaved separately. The working volume of the reactor (including the recirculation line) was 2.6 L. CO_2 was fed into the reactor at 169 kPa (5 psig) through the HFM to maintain anaerobic conditions. Medium pH was monitored by a pH-mV controller (Metter

Toledo 405-DPAS-SC-K8S/225) in the mixing vessel and maintained at 6.6 by automatic addition of the mixture of $Mg(OH)_2$:NaOH described earlier. The stirring speed in the mixing vessel was set as 70 rpm, and mixing vessel temperature was controlled at 37 °C with a heating blanket. Once temperature and pH were stabilized in the system, inoculum (10% v/v based on the total HFM–PBR volume) was added into the mixing vessel. Unless otherwise indicated, the recirculation flow rate was kept constant at 50 mL min^{-1} in all fermentations to maintain similar shear conditions.

Design of experiments and data analysis

Preliminary experiments were carried out in the HFM–PBR using a sugar control solution to select a biofilm carrier to establish the best operation conditions and dilution rates for the process of succinic acid production from CFH. The HFM–PBR was operated in batch mode until all glucose and xylose were consumed and biofilm was observed on the carrier. When this occurred, continuous operation of the HFM–PBR started with varying dilution rate. Profiles of sugar consumption, succinic acid and other organic acids production and succinic acid yield were determined for each dilution rate. In this paper, only steady-state results are reported. Steady state was assumed when the absolute deviation of the succinic acid concentration, captured over a period of at least 12 h, did not exceed 10% of the mean value. All dilution rates except 0.2 and 0.3 h^{-1} were evaluated in duplicate.

Analytical methods

Concentrations of glucose, xylose, succinic acid, lactic acid, formic acid, acetic acid, and ethanol in the samples were monitored by high-performance liquid chromatography (HPLC). A 10 µL cell-free sample was injected into a Bio-Rad chromatographic column (Aminex HPX-87H, 7.8 mm 300 mm, Biorad, Hercules, CA, USA), using 0.01 N H_2SO_4 as the mobile phase at a pump rate of 0.6 mL/min. The temperature of the refractive index detector, RI101 (Shodex, New York, NY, USA) and column were maintained at 50 and 65 °C, respectively.

Results and discussion

Biofilm start-up and selection of carrier for *A. succinogenes* immobilization

Nine separate fermentations were independently performed with the sugar control solution in the HFM–PBR. Three independent fermentations were performed with the biofilm carrier 1. Initially, the bioreactor was operated in batch mode to allow cells to grow and attach to the carrier; however, after 2 days, cell attachment to carrier 1 was not developed and continuous fermentation could not be started. Next, a series of repeated-batch succinic acid fermentations were performed over 4 days using carrier 1 to develop an *A. succinogenes* biofilm on the supports. Although succinic acid production was achieved (see Table 1), biofilm was not established on carrier 1 (see Fig. 4a).

A second support was then employed, and seven independent fermentations were performed with carrier 2. It took approximately 24 h in batch fermentation for carrier 2 to be covered with biofilm (see Fig. 4b). Once the biofilm was attached to carrier 2 and the sugars in the media were depleted, the operation of the reactor was switched from

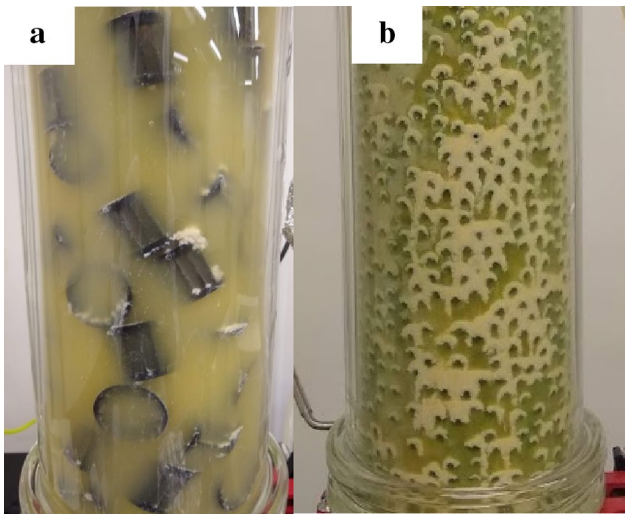

Fig. 4 **a** Biofilm attachment in the carrier 1 after 4 days of succinic acid fermentation, **b** biofilm attachment in the carrier 2 after 24 h

Table 1 Metabolite production and sugars consumption in batch succinic acid fermentation in the HFM–PBR with two different carriers (initial glucose and xylose concentrations of 47 and 4.7 g/L, respectively)

Packing material	Time of biofilm attachment (h)	Batch fermentation time (h)	Glucose (g/L)	Xylose (g/L)	Succinic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)
Carrier 1	–	96	23.0	1.2	15.0	4.1	6.6
Carrier 2	24	24	21.5	0.87	15.3	4.7	6.7

batch to continuous mode. It is worth noting that for carrier 2, succinic acid and other organic acids production and sugars consumption after 24 h were in the same range of the values obtained for carrier 1 after 96 h (Table 1). Although similar operation conditions were used with both packing materials, biofilm attachment only developed on carrier 2. Several factors can affect the performance of packing materials in cell attachment. Microorganisms naturally inhabit the outer and inner surfaces of gravel, sand, or stone [34] and this could be a plausible explanation of why *A. succinogenes* biofilm was established on glass beads (carrier 2) rather than on plastic rings. Moreover, some factors are also inherent to the biofilm carrier, such as surface area (a very large surface area for microbial growth is needed), pore size, porosity, surface roughness, orientation of the packing material, and appropriate contact between liquid and gas phases [35, 36]. In fixed-bed reactors, organic matter removal efficiency is directly related to the support material used for immobilization of anaerobic microorganisms [37]. Furthermore, common bioreactor operation factors, such as media recirculation rate, dilution rate, and pressure drop are important in cell attachment [38, 39]. Packing structure is also a critical characteristic in supporting microbes' growth. For example, carrier 1 are packing rings that are less structured materials that result in higher pressure drops and therefore lower mass transfer efficiencies than structured packings, such as carrier 2, which makes carrier 2 more desirable for biofilm formers [35].

Continuous fermentations in the HFM–PBR using sugar control

The recirculation rate for the initial continuous operation of the HFM–PBR was set at 50 mL/min and was kept at that value during the experiment evaluating different dilution rates. For all steady states, multiple HPLC samples were taken and accordingly averaged. Steady-state data on succinic acid and other organic acids production and glucose and xylose consumption at dilution rates from 0.025 to 0.3 h^{-1} are shown in Fig. 5a, b. The reproducibility of the steady states was tested at all dilution rates in duplicate with four samples taken at each steady-state condition.

Succinic acid was the major product produced in fermentations using the control and lactic, formic and acetic acid were also produced. Metabolite concentrations increased with corresponding decreases in dilution rate. The initial operation of the continuous fermentations was performed at a low dilution rate (0.025 h^{-1}) to allow the culture to adapt to the medium. After steady state at that D was achieved, D was subsequently increased to $D = 0.05$, 0.075, 0.1, 0.2, and 0.3 h^{-1}. At dilution rate of 0.025 h^{-1}, nearly all glucose and xylose were consumed, succinic acid concentration was 32.3 g/L (Fig. 5a) and lactic, formic and acetic

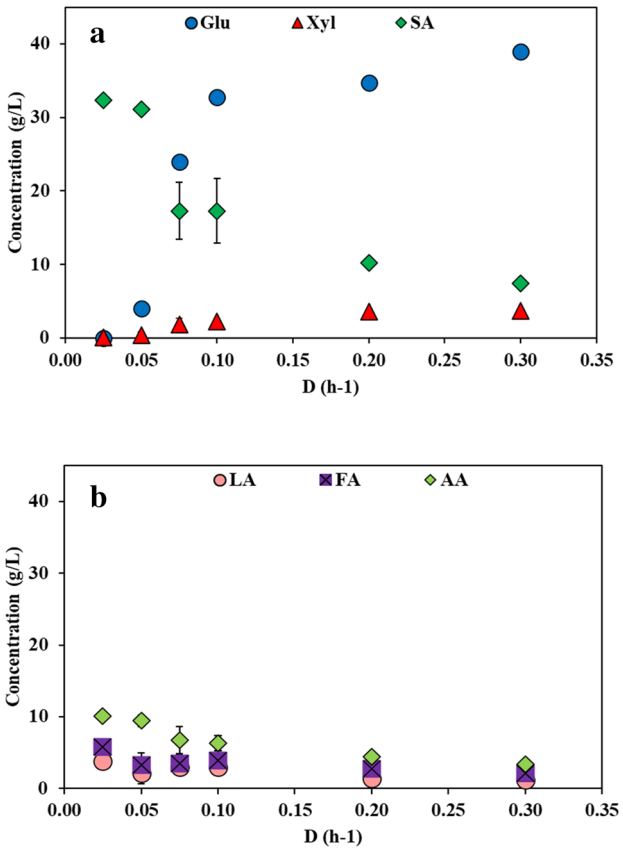

Fig. 5 **a** Steady-state succinic acid (SA), glucose (Glu) and xylose (Xyl) concentration and **b** formic acid (FA), lactic acid (LA) and acetic acid (AA) concentrations at various dilution rates. Data are average values of duplicate experiments, and error bars represent compound standard deviation. *Glu* glucose, *Xyl* xylose, *Ara* arabinose, *succinic acid* succinic acid, *LA* lactic acid, *FA* formic acid, and *AA* acetic acid. Error bars are not shown for dilution rates of 0.2 and 0.3 h^{-1} where repeat runs were not performed

acids concentrations were 3.9, 5.9, and 10.1 g/L, respectively (Fig. 5b). The % consumption of sugars at $D = 0.025$ h^{-1} was 100% of glucose and 98.6% of xylose.

The second highest succinic acid and organic acid concentrations were found at $D = 0.05$ h^{-1}, where succinic acid concentration was 31.1 g/L (Fig. 5a) and lactic, formic and acetic acids concentrations were 2.2, 3.3, and 9.4 g/L, respectively (Fig. 5b). At this operation condition, 91.3% of glucose and 90% of xylose were consumed. The consumption of glucose and xylose decreased gradually with increasing dilution rate from 100% of glucose and 98.6% of xylose at $D = 0.025$ h^{-1} to 17.0% of glucose and 45.7% of xylose at $D = 0.3$ h^{-1} (Fig. 5a). An order of preference in sugar utilization by *A. succinogenes* was not observed as all sugars were consumed simultaneously suggesting the absence of carbon catabolite repression of fermentation of sugar control in the HFM–PBR, which agrees with previous research [40]. The achieved succinic acid yield for both dilution rates, 0.025

and 0.05 h^{-1}, were 0.61 g/g, however, a higher productivity of 1.56 g/L h was observed at $D = 0.05$ h^{-1} compared to productivity of 0.81 g/L h at $D = 0.025$ h^{-1}. Therefore, 0.05 h^{-1} was selected as the dilution rate to carry out succinic acid production from CFH. Moreover, the maximum succinic acid concentration in this study (32.3 g/L) was higher than that of our previous batch study (28.7 g/L) [29].

Effect of the mixing recirculation rate in succinic acid production

The effect of the mixing recirculation rate was studied in order to evaluate if changing the shear conditions affected biofilm attachment, succinic acid production, and substrate utilization by *A. succinogenes* in the HFM–PBR. The mixing recirculation rate varied from 25 to 75 mL/min at constant dilution rate of 0.05 h^{-1}. The maximum rate allowed by the peristaltic pump for the tubing used in the recirculation line was 80 mL/min.

As can be seen in Fig. 6, production of succinic acid and other organic acids and consumption of sugars with variations in the mixing recirculation rate. The highest succinic acid concentration of 23.4 g/L and sugar utilization of 75.4% for glucose and 81.3% for xylose were achieved at the recirculation flow rate of 50 mL/min. At the highest recirculation rate (75 mL/min), it was observed that biofilm attachment in the packing media decreased and the succinic acid concentration was 21.1 g/L and sugar utilization was 62.0 and 67.7% for glucose and xylose, respectively. Previous investigations in biofilm reactors have increased the recirculation rates to scrub loose biofilm and remove cell segments from the packing material [28]. Also, gas–liquid mass transfer in reactors for syngas fermentation decreased with higher recirculation rates due to an increase in liquid hold-ups in a PBR [20]. It was expected that increasing the recirculation rate would decrease substrate utilization and succinic acid production due to decreased gas–liquid mass transfer and increased shear force preventing biofilm formation, and that is what was observed. At the lowest recirculation rate (25 mL/min), succinic acid concentration was 19.5 g/L and sugar utilization was 58.3% for glucose and 67.7% for xylose. Moreover, it was noticed that biofilm formation increased in the mixing vessel and the HFM rather than in the packing material. In general, media recirculation rate in processes that involve conversion of gas substrate by microorganisms can be crucial since mass transfer is limiting. Sugar utilization and succinic acid production decreased when recirculation rate was decreased from 50 to 25 mL/min while maintaining a fixed $D = 0.05$ h^{-1}. It was expected that decreasing the recirculation rate would enhance substrate utilization and succinic acid production since the media broth spends a longer time in contact with the biofilm, thus enhancing mass transfer time, which improves diffusion of substrate into the *A. succinogenes* cells on the carrier. However, biofilm attached to the glass walls and internals of the mixing vessel and attachment to the column packing was reduced compared to what was observed in the dilution rate experiment (Fig. 7). Another observation was the lower succinic acid concentration and sugar utilization achieved during the recirculation rate experiment compared to the values obtained during the dilution rate experiment. At 25 mL/min, biofilm attachment occurred not only in the mixing vessel, but also in the HFM, affecting both gas–liquid transfer and the amount of dissolved gas in fermentation broth and therefore this could result in lower succinic acid yields. As recirculation rates were increased, some biofilm was removed from the HFM, but much still remained. After the recirculation rate experiment was completed, a new HFM was installed in the system. An experiment with the new HFM was conducted at $D = 0.05$ h^{-1} and 50 ml/min and succinic acid concentration and sugar utilization was similar to the dilution effect experiment were achieved at 50 ml/min (data not shown here).

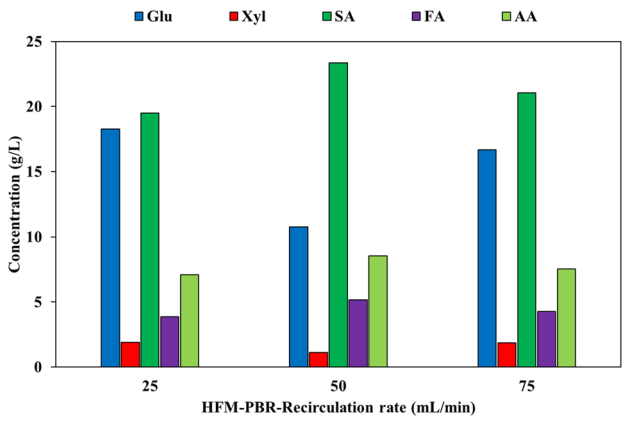

Fig. 6 Succinic acid (SA), glucose (Glu) and xylose (Xyl), formic acid (FA), and acetic acid (AA) concentrations at $D = 0.05$ h^{-1} and various recirculation rates

During all the experiments, biofilm attachment in the HFM internals was observed (Fig. 8); however, the system tubing was not clogged and continuous fermentations were performed without any impediments. The highest succinic acid concentration and sugar utilization was found at 50 mL/min, suggesting that at this recirculation rate there are more suitable conditions such as residence time, homogeneous media distribution through the packing bed, and better gas–liquid transfer that allow a higher succinic acid production and biofilm attachment to the packing material. Once the recirculation rate for the highest succinic acid production was established, continuous succinic acid production in the HFM–PBR from CFH was carried out at $D = 0.05$ h^{-1} and 50 mL/min.

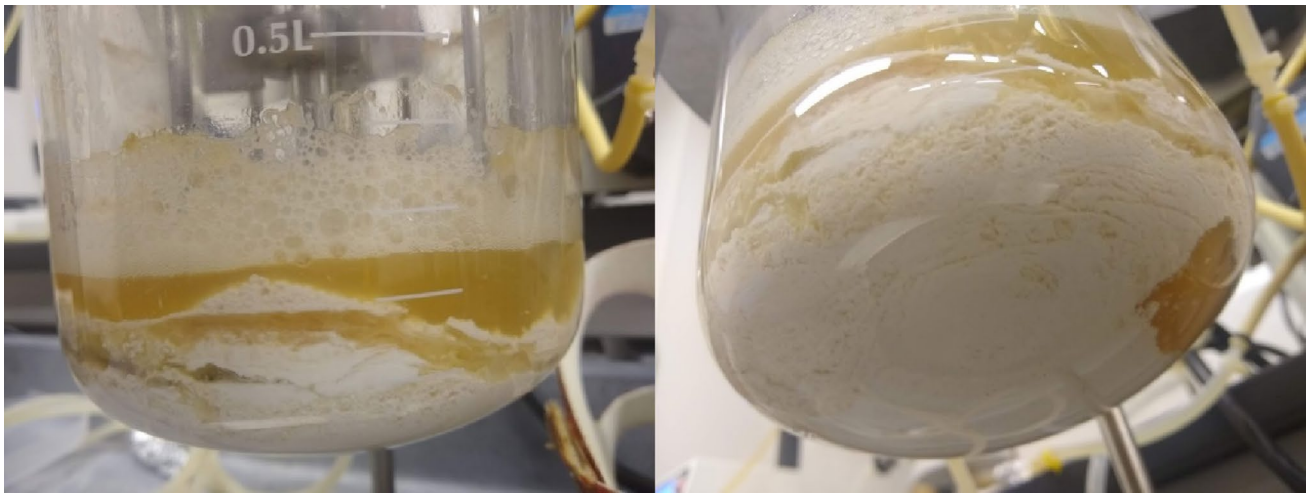

Fig. 7 *A. succinogenes* biofilm formation in the mixing vessel wall that occurred at recirculation rate of 25 mL/min

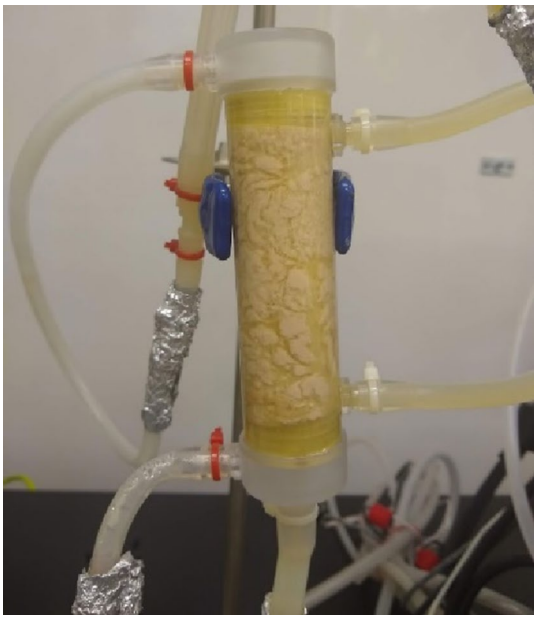

Fig. 8 *A. succinogenes* biofilm attached to the HFM internals in the fermentation of the sugar control

Continuous succinic acid production in the HFM–PBR using corn fiber hydrolysate

A fermentation was performed in the HFM–PBR using CFH. The HFM–PBR was able to operate continuously for 6 days without encountering any clogging or contamination problems. At 24 h of batch operation, a stable biofilm was developed in the packed column; however, HPLC analysis showed high formic acid and low succinic acid concentrations (2.8 and 11.0 g/L, respectively). Analysis of the CFH showed that while furfural and hydroxymethylfurfural compounds were not present in the hydrolysate, formic acid and acetic acid were detected at initial concentrations of 12.8, and 1.8 g/L, respectively. These compounds are known inhibitors of *A. succinogenes* [25]. Batch fermentation was continued until 36 h, after which biofilm formation was observed, the concentrations of succinic, lactic, formic, and acetic acids were 18.3, 2.7, 11.8, and 9.7 g/L, respectively, while sugars in the medium were depleted. *A. succinogenes* adapted to the CFH from 24 to 36 h. Once sugars were consumed at 36 h, the system was switched to continuous mode at a dilution rate of 0.05 h^{-1}; however, after 12 h of continuous operation biofilm attachment on the packing material was not developed. The dilution rate was reduced to 0.025 h^{-1}, which was a strategy used in a previous study of continuous succinic acid production from dilute acid pretreated corn stover hydrolysate [40]. After 12 h operation at $D = 0.025$ h^{-1}, biofilm attachment was observed. Then, the operation was held at $D = 0.025$ h^{-1} for 12 more h to allow the culture to adapt to the CFH and develop further biofilm formation. After 24 h, dilution rate was increased to 0.05 h^{-1} and the performance of the system was assessed under steady-state conditions at $D = 0.05$ h^{-1}. Figure 9a shows the biofilm development on the HFM–PBR around carrier 2 at $D = 0.05$ h^{-1} and 50 mL/min recirculation rate using the CFH. In Fig. 9b, biofilm formation can be observed in the HFM internals using the CFH, but it did not impede the recirculation of media through the system or normal operation of the HFM–PBR.

The maximum amount of succinic acid produced from CFH was 23.4 g/L, with a yield of 0.51 g/g and productivity of 1.17 g/L h. The productivity achieved in this study represents the highest succinic acid productivity achieved from CFH in literature (between 1.3 and 1.9 times) [8, 29, 41, 42]. Lactic, formic, and acetic acid concentrations were 4.1, 11.7, and 11.5 g/L, respectively, and this includes the

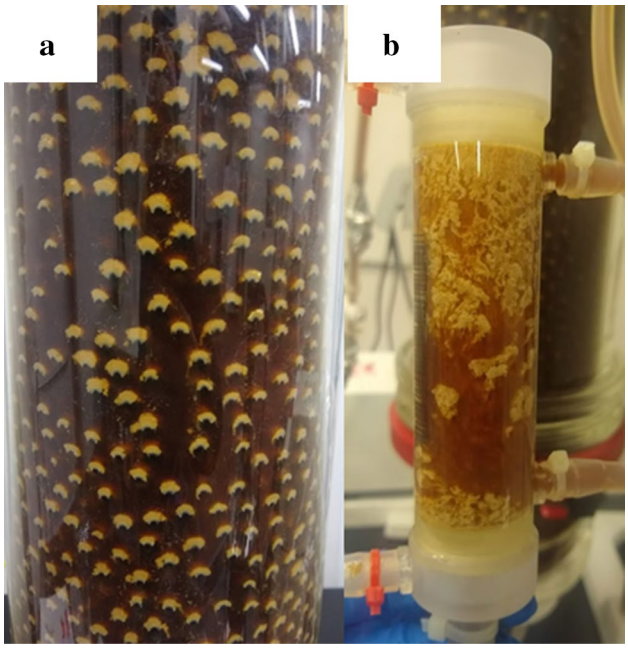

Fig. 9 **a** Biofilm formation in HFM–PBR by fermenting CFH during continuous operation at $D = 0.05\ h^{-1}$. **b** Biofilm attachment in the HFM internals from CFH fermentation

acids that were initially in the CFH. In agreement with the results of continuous succinic acid fermentation using the control, the sugars were consumed simultaneously but at different rates with no utilization preferences as seen in the present study. Succinic acid production and the conversion of glucose (86.3%) and xylose (79.4%) at $0.05\ h^{-1}$ in the CFH fermentation were lower than that of the sugar control fermentation at the same dilution rate. The concentration of produced succinic acid decreased by 24.7% with respect to the control, which could be attributed to the presence of inhibitors. As it was stated previously, formic acid was detected in the CFH at initial concentration of 12.8 g/L. Weak organic acids, such as formic acid, have inhibited the conversion efficiency of anaerobic bacteria used in succinic acid, acetone–butanol–ethanol, and H_2 fermentation [25, 43, 44]. When microbial cells are exposed to fermentation media with formic acid produced during a physicochemical pretreatment of biomass, there is a higher maintenance cell requirement compared to media without formic acid. This occurs because the cells spend more energy (ATP) in translocating outside anions and protons across the plasma membrane, which results in a lower molar growth yield with respect to the carbon source [45]. However, succinic acid was the major compound produced. Moreover, it was found that *A. succinogenes* adapted to the hydrolysate, suggesting a strong evolutionary response to the toxic compounds in the CFH.

Other analysis

The total accumulative time span of HFM–PBR operation was 1200 h. This time includes the establishment of biofilm during start-up, steady state conditions and recovery from undesirable events such as reactor drainage, setting of the recirculation rate and constant media level in the mixing vessel, and adjusting flow lines in the system. Furthermore, high mixing and submerged spargers were not required to feed CO_2 to the bacteria as was required in the two previous immobilized reactors for succinic production [28, 40]. Since HFMs yield a large surface area for both liquid and mass transfer without forming bubbles, no foaming was observed during the fermentations and therefore antifoaming agents were not necessary during the HFM–PBR operation. Moreover, strategies such as foam traps or CO_2 feeding into recycle lines were not used in the configuration of the HFM–PBR.

Conclusions

In this study succinic acid was produced continuously by immobilized *A. succinogenes* in a novel HFM–PBR converting almost 100% of sugars present in a sugar control with a succinic acid concentration of 31.1 g/L, yield of 0.61 g/g, and productivity 1.56 g/L h. Succinic acid productivity of 1.17 g/L h, yield of 0.51 g/g, and concentration of 23.6 g/L were achieved when a non-detoxified CFH was the carbon substrate, which was a 24% reduction in succinic acid concentration compared to the control. Formic acid, a known inhibitor of *A. succinogenes*, in the CFH likely inhibited succinic acid production.

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Author contributions Both authors contributed to the study conception and design. LVY was responsible for experiment execution, data collection and analysis, and writing the original draft of the article. MRW was responsible for securing funding, project administration, and review and editing of the article.

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Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest Lisbeth Vallecilla-Yepez is now employed by Novozymes A/S, whose enzymes were used in this study.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Authors' information Mark R. Wilkins is now employed by Kansas State University and Lisbeth Vallecilla-Yepez is now employed by Novozymes A/S. The study described was performed at the University of Nebraska-Lincoln, thus the authors' affiliations on the title page are with the University of Nebraska-Lincoln.

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