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Well Knowledge of the Physiology of *Actinobacillus succinogenes* to Improve Succinic Acid Production

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Abstract: The anaerobic fermentation of glucose and fructose was performed by *Actinobacillus succinogenes* 130Z in batch mode using three different volume of bioreactors (0.25, 1 and 3 L). The strategy used was the addition of $MgCO_3$ and fumaric acid (FA) as mineral carbon and the precursor of succinic acid, respectively, in the culture media. Kinetics and yields of succinic acid (SA) production in the presence of sugars in a relevant synthetic medium were investigated. Work on the bench scale (3 L) showed the best results when compared to the small anaerobic reactor's succinic acid yield and productivity after 96 h of fermentation. For an equal mixture of glucose and fructose used as substrate at 0.4 mol L^{-1} with the addition of FA as enhancer and under proven optimal conditions (pH 6.8, T = 37 °C, anaerobic condition and 1% *v*/*v* of biomass), about 0.5 mol L^{-1} of SA was obtained, while the theoretical production of succinic acid was 0.74 mol L^{-1}. This concentration corresponded to an experimental yield of 0.88 (mol-C SA/mol-C sugars consumed anaerobically) and a volumetric productivity of 0.48 g-SA L^{-1} h^{-1}. The succinic acid yield and concentration obtained were significant and in the order of those reported in the literature.

Keywords: platform molecules; succinic acid; fermentation; metabolic pathway; mass balance; scale-up

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1. Introduction

Succinic acid (SA), which is a biomolecule, is well-known as a platform chemical and as a highly versatile building block used in a variety of industrial applications namely, surfactants, green solvents and pharmaceutical compounds, because of its linear and saturated structure [1,2]. SA is at the top of the list of 12 building blocks and holds the title of being the only special product with an annual market demand of about 710 kilotons, with a net value of USD 115.2 million, expected to exceed USD 1.8 billion (768 million MT at USD 2.3/kg) by 2025 [3,4]. Previously, SA was made primarily by catalytic hydrogenation of maleic anhydride, a finite fossil-derived chemical at a current price of USD 2.94/Kg [5]. In fact, society and industry are becoming more aware of petrochemical processes' effects on environmental footprint and increasing concern has resulted in a search for alternative routes for the durable production of chemicals [6]. Nowadays, the world is at a critical changeover point as we move ahead into a bio-economy, while simultaneously reducing dependence on finite fossil fuels and leaving behind the petroleum-based economy [7]. According to this scenario, petroleum refineries could be progressively replaced by bio-refineries as governments pursued the generation of renewable energy, bio-fuels

and bio-derived chemicals [8,9]. The preferable approach for bio-renewable chemicals is the fermentation process, which microbial-based generation and cheap renewable resources and which requires simple conditions of pressure and temperature to produce chemicals through several pathways [10]. There is great interest in producing biologically derived SA, potentially at commercial scale, with possible process implementation at different levels (such as feedstock selection, process and operation setup, microbial strain and a novel bio-way) and leading to high concentrations of the accumulated product [11,12]. Emphasis has been put on the use of pure or simple fermentable substrates in bio-derived chemical production; however, to reduce the processing fees, low-cost and/or waste organic materials can be promisingly considered [13]. A tremendous amount of research has been performed to develop a biological process for SA production as an end-product of energy metabolism under aerobic [2], micro-aerobic [14] and/or anaerobic conditions [15]. For this purpose, several natural or mutant microorganisms were considered, mainly including *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens* and *Escherichia coli*, especially *A. succinogenes* 130 Z, a facultative anaerobe isolated from the bovine rumen [16]. Conventional production of SA involves the anaerobic bacterial fermentation of pure or a mixture of sugars with the addition of carbon dioxide (CO_2) sources, such as *A. succinogenes* [17]. This latter produces SA as an end-product at higher concentrations than other strains without genetic tool modification [18]. An *A. succinogenes* native strain could be used but requires an anaerobic environment (nitrogen bubbling) and the presence of concentrated dissolved CO_2 in the fermentation broth as $MgCO_3$, which definitely controls the metabolic flux of carbon and the activity of enzymes including phosphoenolpyruvate carboxykinase (PEPCK), which are the crucial steps for SA bioproduction by succinate-producing bacteria [19].

SA is produced as an end-fermentation product via some intermediate compounds of the tricarboxylic acid (TCA) cycle reductive branch, including oxaloacetate (OAA), malate (MAL) and fumarate (FUM) under anaerobic fermentation [20]. The reduction of fumarate is the main source of succinate accumulation during fermentation and in *A. succinogenes*, the formation of succinate is strictly required, taking advantage of the unique incomplete TCA cycle, which natively terminates at SA [21,22].

The main features to exploit *A. succinogenes* to produce SA are the utilisation of a wide variety of feedstock containing carbon sources. Indeed, *A. succinogenes* can consume a broad range of C_5 and C_6 sugars and other carbon sources, including mono and disaccharides, such as glucose, xylose, arabinose, mannose, galactose, fructose, sucrose, lactose, cellobiose, mannitol, maltose and glycerol [23–25]; it shows an efficient fermentative pathway even with sustainable raw materials [26], as well as suitable tolerance to inhibitors.

The objective of the current study was to improve the high-value products from native strain of *A. succinogenes*, SA by the addition of one of C_4 TCA metabolites. The consumption of substrate (glucose and fructose) and conversion of carbon to optimize the output of the metabolic network were used as a means to improve SA production as follows. Based on the conversion rate of the substrate, the C_4 TCA metabolites and the central path anaerobic fermentations were carried out to upgrade the native strain of *A. succinogenes*, to verify its tolerance and to confirm the inhibitory action of *A. succinogenes* against SA. In the following section, FA was used as enhancer to improve the production of SA from *A. succinogenes and*, according to the consumption rate of carbohydrates, the production rate and the metabolic pathway for products with biomass formation, a mass balance of experimental data obtained from anaerobic batch fermentation is presented. Finally, a set-up of a small scale-up system in the laboratory using three different volumes (0.25 L, 1 L and 3 L with and without pH control) for SA production is also designed and the process is described in detail.

2. Material and Methods

2.1. Chemical and Gas

All chemicals used were purchased from various supplier as Sigma-Aldrich (Saint Louis, MO, USA) or Merck (Kenilworth, NJ, USA). Ultra-high purity Nitrogen (N_2) gas was supplied from Linde-GAS, Saint-Priest, France.

2.2. Microorganism and Inoculums Preparation

Native *Actinobacillus succinogenes* DSM 22257 in pellet form was purchased from DSMZ German Collection of Microorganisms and Cell Cultures and used in all experiments. Cells were incubated in 15 mL of Tryptic Soya Broth (TSB) in 25 mL sterilized glass vials at 37 °C for 24–48 h in an incubator (VWR® INCU-Line, Radnor, PA, USA), which was the time required for the microorganism to enter the exponential growth phase and then centrifuged aseptically (1800 g, 4 °C and 5 min) in a centrifuge (HERAEUS Megafuge 16R, Thermo-Fisher Scientific, Waltham, MA, USA) and then resuspended in 10 mL KCl (150 mM). For the inoculum preparation, 2 mL of suspended culture cells were inoculated into 250 mL anaerobic bottle containing 200 mL of broth and placed in an incubator shaker (INNOVA 40, New Brunswick, NJ, USA) at 37 °C and 150 rpm for 48 h. Cells from the preculture were centrifuged aseptically in the same conditions mentioned above and the suspension obtained after harvesting cells and re-suspending in 10 mL of KCl 150 mM was used for inoculation.

2.3. Fermentation Media and Experimental Design

Fermentation media consisted of major and minor components. The major components included the carbon sources (glucose, fructose) at various concentrations and the nitrogen source, 0.53 g L^{-1} NH_4Cl. The minor components included inorganic salts, buffers, cofactors, namely (per liter): 3 g K_2HPO_4, 3 g KH_2PO_4, 1.25 g NaCl, 0.3 g $MgCl_2{\cdot}6H_2O$, 0.3 g $CaCl_2{\cdot}2H_2O$, 1.5g NaH_2PO_4, 1.5g Na_2HPO_4. Initial fermentation pH media was adjusted to 6.8, then, the media prepared was sterilized by filtration on 0.22 μm sterile membrane filter (Sartorius, Göttingen, Germany), when setting up the fermenters. A series of experiments were conducted using a modified fermentation medium added with FA as co-factor in various concentrations (from 0.0008 to 0.2 mol L^{-1}) and the relative percentage change which refers to the difference of SA concentration between the experiments was calculated using the following formula:

Relative percentage: % change = (final SA−initial SA)/initial SA × 100

All equipment was sterilized before use. Experiments were carried out in glass bottles 250 mL volumes capacity. A working volume of 200 mL was used and operated at a temperature of 37 °C, pH between 6.8 and 7 and an agitation speed of 150 rpm. The inoculum size for the batch fermentation was 1% (*v*/*v*).

Experiments for determining the inhibitory effects of the products were carried out in Petri dishes (PD), each containing 15 mL of defined medium TSA with several concentrations of succinic acid.

2.4. Fermentation Conditions and Sampling

Carbon dioxide (CO_2) fixation is required to enhance SA production. Therefore, CO_2 was supplied to the fermentation broth by the addition of $MgCO_3$. Two sets of $MgCO_3$ concentrations (0.1 and 0.2 mol L^{-1}) were added as a buffer medium to maintain the pH between 6.8 and 7. $MgCO_3$ also acts as a carbon source and the concentration present in the media was determined by a simple titration of CO_3^{2-} ions by H_2SO_4 at a concentration of 0.2 mol L^{-1}at the first fermentation time (T_0) and at the end of fermentation. A fermentation temperature of 37 °C and an agitation speed of 150 rpm were maintained throughout the incubation period. During the fermentation, samples of 5–10 mL were withdrawn aseptically from the gloss bottles at regular time intervals in order to follow the optical cell densities, sugar consumption and the production or uptake of acids (e.g., succinic,

formic, acetic and lactic acid) and other compounds (e.g., ethanol). Batch fermentations consisted of microaerobic and anaerobic phases. Under microaerobic conditions, the dissolved oxygen level was 100% of air saturation at the time of inoculation and under anaerobic conditions, nitrogen gas was sparged into the bottles for 5 min to remove oxygen before inoculation; however, SA production phase occurs in anaerobic conditions.

2.5. Analytical Methods and Data Analysis

Cell growth was followed by measuring the optical cell density at 660 nm (OD_{660}) using a Spectronic 601 spectrophotometer (Milton Roy, Ivyland, PA, USA). From each flask, all the culture medium (50 mL) was loaded into a pre-weighed centrifuge tube (M1) and centrifuged (Thermo-Fisher Scientific, Waltham, MA, USA) at 1800× *g* for 5 min. The supernatant was decanted and the tube with cells pellet was then dried in an incubator at 105 °C overnight. The tube with the pellet was weighed in order to determine cell weight (M2). This drying/weighing was continued until a constant weight was achieved. It turned out that a sampling interval of 24 h was difficult to sustain operationally over a total culture period of around 4 days, it was decided that a 96 h sampling interval would be considered.

Cell concentration was monitored by spectrophotometry using OD_{660} correlated to dry cell weight (DCW); the relationship between optical density and Dry cell weight is described by:

$Y = 0.0003x + 0.0001$

$R^2 = 0.98$

where $Y = OD_{660}$; x = (dry weight of cells) mg L^{-1}

DCW assessments were used as a rough indication of the biomass concentration. An OD_{660} value of 1 (Absorbance) corresponded to 333 mg L^{-1} DCW *A. succinogenes,* deduced from a standard calibration curve. Samples were centrifuged and filtered through a 0.22 µm membrane filter and the concentrations of fermentation products and carbon substrate were determined according to the protocol described in [27] and verified by NMR. Feedstock consumption and platform molecules formation were compared in terms of the key performance factors. Some factors were assigned in terms of percentage; the dependent variables used were final SA concentration, productivity and yield expressed in mol-C SA/mol-C sugars consumed. Information about the key performance parameters were collected from various literature sources. Based on the data collection, each key parameter was quantified using the parameter equations. The key performance parameters are defined as follows:

% of glucose consumption = ((Ci glu−Cf glu)/Ci glu) × 100

% of fructose consumption = ((Ci fru−Cf fru)/Ci fru) × 100

% of difference = ((FA consumed−SA produced)/FA consumed) × 100

Yield SA= molC-SA produced/molC-Sugars consumed

Productivity SA = g of succinic acid L^{-1} h^{-1}

dS/dt = Substrate consumption rate during fermentation time (S initial−S final)/t final

dP/dt = production rate during fermentation time (P final−P initial)/t final

Yp/s (dP/dS) = yield coefficient of succinic acid on sugars (g SA/g sugars)

Yx/s (dX/dS) = yield coefficient of biomass on sugars (g biomass/g sugars)

Yp/x (dP/dX) = yield coefficient of succinic acid on biomass (g SA/g biomass)

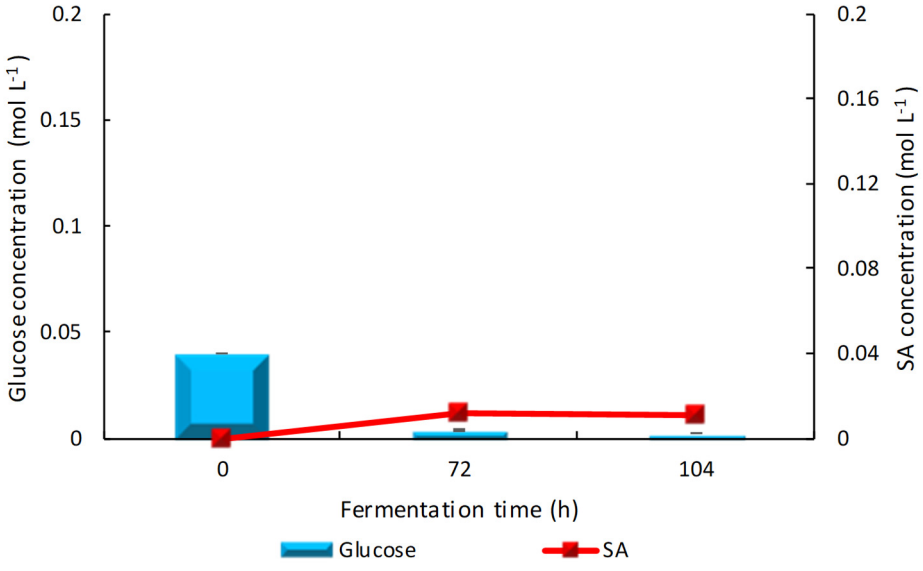
3. Results and Discussion

3.1. Results from 250 mL Shake Flask Reactors Using Glucose as Carbon Source in Micro-Aerobic and Anaerobic Conditions

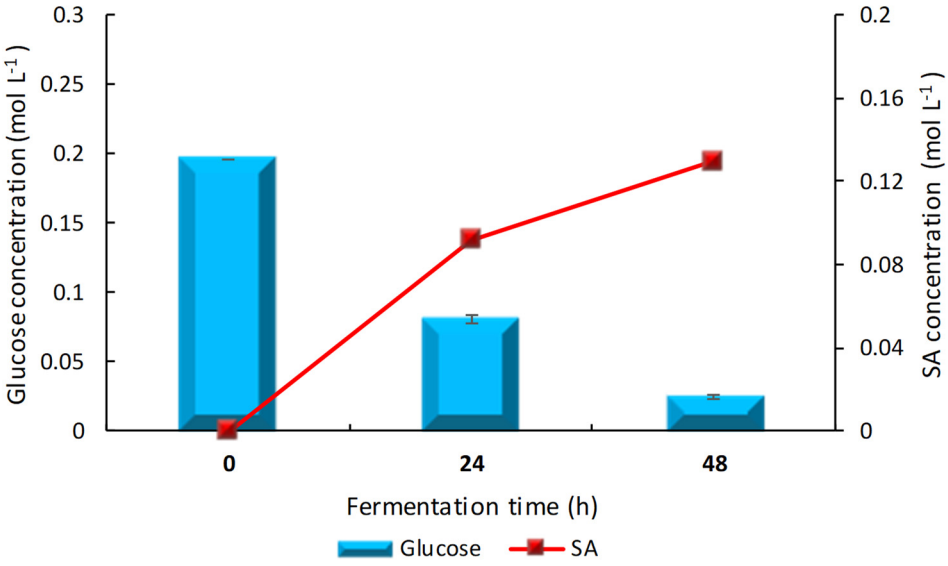
Fermentations of *A. succinogenes* in micro-aerobic conditions did not show any SA production, while it revealed low yields of (<0.25 mol-C SA/mol-CGLU) and productivities (<0.012 g-SA/L/h), when 0.04 mol L^{-1} of glucose was used as the only carbon source in anaerobic conditions, SA was produced at low levels, recording a value of 0.01 mol L^{-1}

(Figure 1a). Further evidence is provided in previous study [28,29] demonstrating that SA is formed even when this substrate was added as the sole carbon feedstock in anaerobic conditions. Furthermore, *A. succinogenes* cannot produce SA in a synthetic media that contains glucose as the sole carbon source in microaerobic and aerobic conditions [30]. Biomass yield was less than 0.03 g-DCW/g-GLU, while analogues run with different glucose concentrations displayed higher biomass yields (>0.06 g-DCW/g-GLU). A series of experiments were performed in synthetic medium to improve the process. $MgCO_3$ was screened to be a key factor in SA production [31] and, hence, it was added to the fermentation medium as a neutralizer and to redirect the metabolic flux at 0.1 mol L^{-1} concentration.

Figure 1b illustrates the results obtained from batch fermentation experiments in small anaerobic reactors (250 mL flask) during fermentation in the presence of $MgCO_3$. Fermentations were conducted with different amounts of initial glucose concentration (0.05, 0.19 and 0.26 mol L^{-1}). As shown in Figure 1b SA concentration reach it maximum value and record 0.14 mol L^{-1}, moreover, yield and productivity increased by 3.5, 0.8 times and exhibited maximum values of 0.93 molC-SA/molC-GLU, 0.17 g-SA/L/h, respectively, when compared to the results obtained in the absence of $MgCO_3$ (Figure 1a). In addition, biomass yield increased from 0.02 g-DCW/g-GLU to 0.05 g-DCW/g-GLU. The addition of $MgCO_3$ was beneficial to promote the biomass yield and succinic acid synthesis. This could be attributed to the necessity of the CO_2 and the cofactor Mg^{2+} provided by $MgCO_3$ for conversion pathway into biomass and products.



(**a**)



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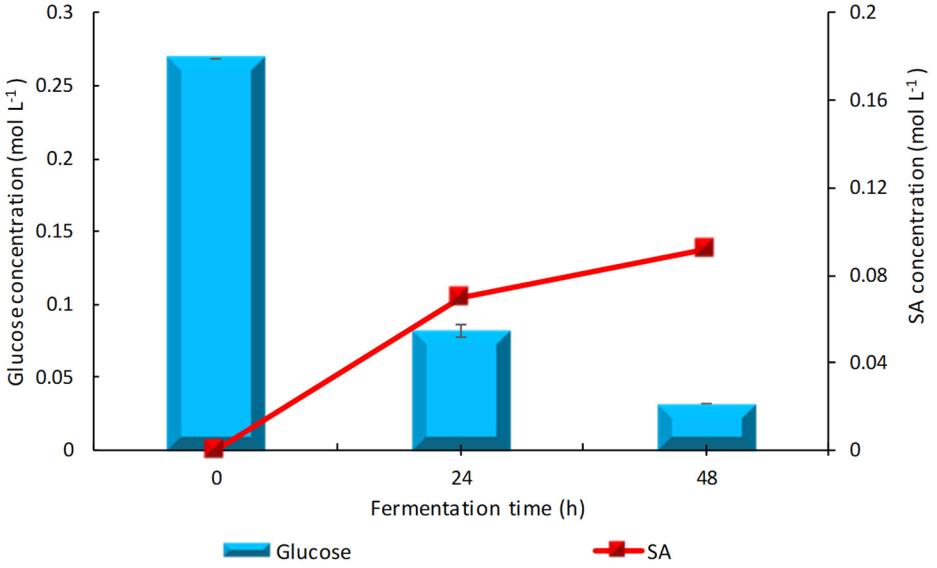


(**c**)

Figure 1. (**a**–**c**): succinate formation in 250 mL small shake flask for different glucose concentrations (0.04, 0.19 and 0.26 mol L^{-1} respectively) in anaerobic conditions at 37 °C.

As shown in Figure 1c, no further major progression was noticed in succinate concentration, yield and productivity at the end of the experiments when 0.26 mol L^{1} glucose was added. Zou et al., [32] reported for the first time the substitution of CO_2 gas by $MgCO_3$ in the fermentation of SA. Theoretically, 1.71 mol of SA is produced per mol glucose in the presence of CO_2 sources based on the available electron as per the following equation (Equation (1)) [33]:

$$Glucose + 0.86\ HCO_3^- \rightarrow 1.71\ succinate^{2-} + 1.74\ H_20 + 2.58\ H^+ \quad (1)$$

Experimental yields could be restricted by the pathways used and by the carbon converted to biomass and alternative products. The molar yield obtained from the experiments presented in Figure 1b was about 0.84 mol SA/mol glucose.

The percent yield was calculated and show a value of 49%, meaning that 49% of glucose was converted to SA after 48 h of fermentation.

When $MgCO_3$ was added, HCO_3^-, CO_3^{2-} and CO_2 would become in equilibrium in the fermentation broth according to the following equations:

$$MgCO_3 \rightarrow Mg^{2+} + CO_3^{2-} \quad (2)$$

$$H^+ + CO_3^{2-} \leftrightarrow HCO_3^- A = \pi \quad (3)$$

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3 \quad (4)$$

Consequently, the insoluble $MgCO_3$ supplement caused turbid broth, cells spreading which avoid cell flocculation and indeed it increases the dissolved concentrations of HCO_3^-, CO_3^{2-} and CO_2 in the broth which influence carbon flux and the activity of phosphoenolpyruvate carboxykinase enzyme, to redirect the flux toward SA biosynthesis [15] and because of these properties, $MgCO_3$ plays an essential role in improving SA production. The analysis of $MgCO_3$ concentration at the end of fermentation recorded a value of 0.016 mol L^{-1}, which means that the carbonate minerals were consumed when compared to the initial carbonate concentration. However, $MgCO_3$ could not be used as CO_3^{2-} donor because few reports indicate that CO_3^{2-} is directly used as substrate by SA producer's microorganisms [32].

3.2. Results from 250 mL Anaerobic Bottles Using Sole and Mixed Sugars as Carbon Source in Anaerobic Conditions

A fermentation profile of the fructose-SA system was shown in Figure 2. SA was the main product present in the media. Moreover, no ethanol nor lactic acid was detected in any sample. Yields, productivities, optical cell densities variation and succinate concentrations at the end of the fermentation time for several substrate concentrations are illustrated in Table 1.

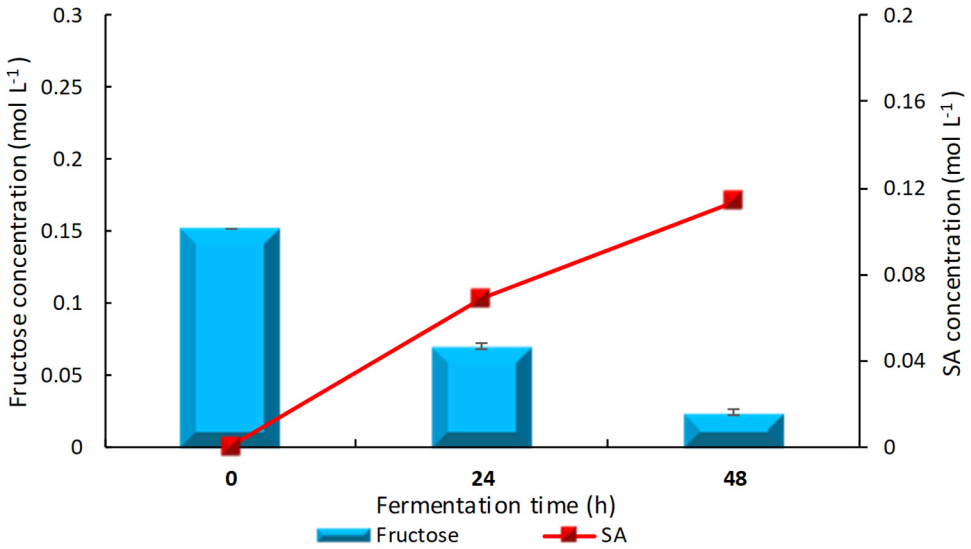
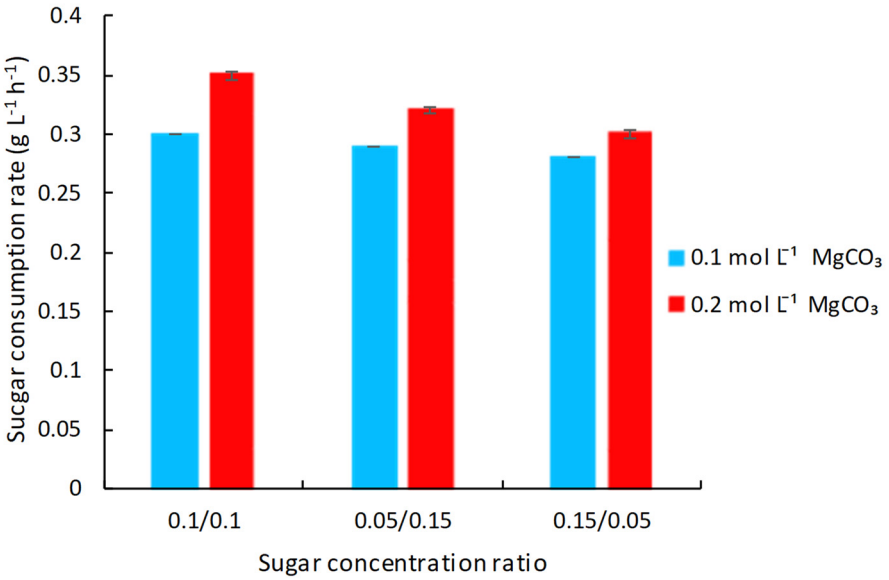


Figure 2. Fermentation profile of the fructose-succinic acid system in 250 mL anaerobic shake flask for a fructose concentration of 0.15 mol L^{-1} in anaerobic conditions at 37 °C.

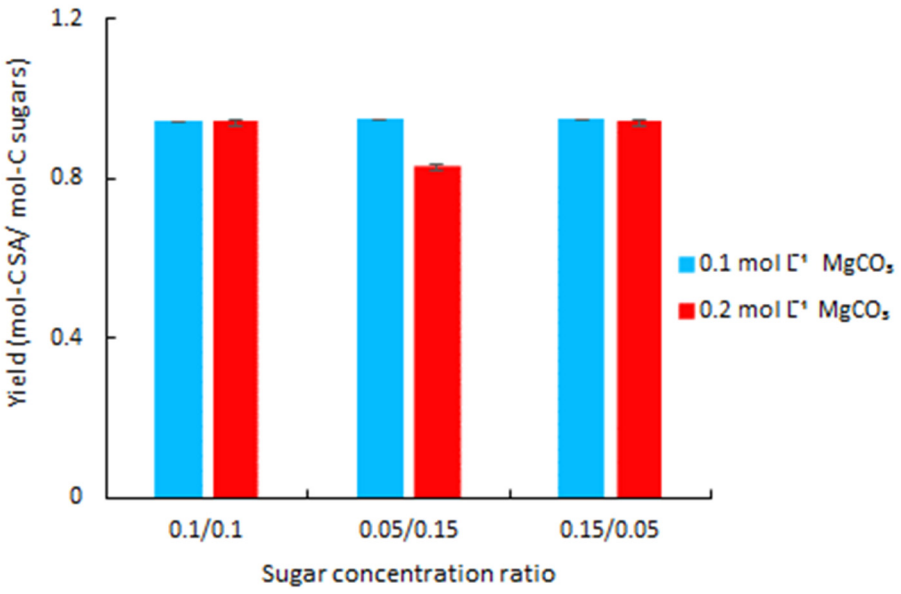
Table 1. Comparison of succinic acid produced from glucose and fructose after 48 h (A: medium containing glucose, B: medium containing fructose) in 250 mL anaerobic bottles with a volume of 200 mL.

Initial Sugar Concentration (mol L^{-1})		Consumed Sugar (%)		SA (mol L^{-1})		$A_{\lambda 660}$ nm		Yield (molC/molC)		Productivity (g L^{-1} h^{-1})	
A	B	A	B	A	B	A	B	A	B	A	B
0.05	0.04	91.90	99.70	0.04	0.025	0.13	0.17	0.90	0.67	0.33	0.06
0.19	0.13	87.24	92.44	0.14	0.07	0.41	0.18	0.94	0.59	0.39	0.17
0.26	0.15	88.05	83.44	0.09	0.11	0.49	0.38	0.40	0.89	0.22	0.27

Fructose was mostly consumed during the first 48 h. The highest carbon yield (C-mol/C-mol) was found at 0.15 mol L^{-1} initial fructose concentrations, with a maximum value of 0.92 molC-SA/molC-FRU. This should most likely be related to the highly reduced state of glucose and fructose in addition to glycerol, which unlike the other sugars, promotes the generation of SA [34]. Prior studies also pointed out a high yield in SA production when mutant bacterial strains were used [4,35,36]. The feasibility of SA production via *A. succinogenes* co-utilizing glucose and fructose simultaneously as carbon sources was evaluated. By combining these two sugars, they are consumed more quickly than when a single sugar was used (Figure 3a). Moreover, we demonstrate that the process can adapt to changing concentrations of these two sugars. As long as a substrate is present in the medium, biomass grew up exponentially till around 70 h; then, due to substrate limitation, biomass enters in the stationary phase. The highest SA production in co-fermentation was 0.22 mol L^{-1} (equivalent to a yield of 0.94 molC-SA/molC-sugars and a SA productivity of 0.27 g-SA/L/h) observed in a mixture with a ratio of 50% fructose and 50% glucose in the presence of 0.2 mol L^{-1} of $MgCO_3$ (Figure 3b). The presence of 0.2 mol L^{-1} of $MgCO_3$ was beneficial to promote SA production and giving the maximum of SA concentration (0.22 mol L^{-1}). For this reason, $MgCO_3$ at 0.2 mol L^{-1} was considered thereafter (Figure 3c).



(**a**)



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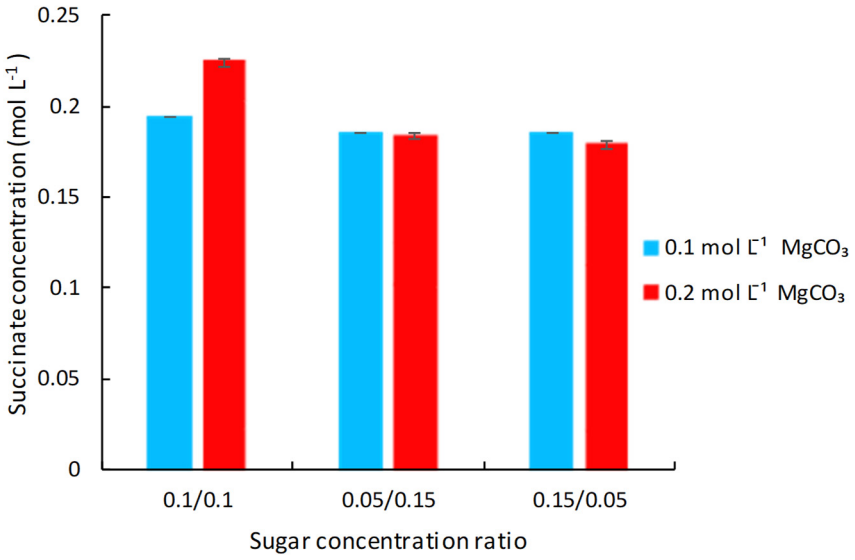


(**c**)

Figure 3. (**a**) sugar consumption rate; (**b**) Profile of conversion yield and (**c**) succinic acid concentration during *A. succinogenes* fermentations in 250 mL batch small anaerobic shake flask bottles at 96 h with different initial sugar concentrations in mol L^{-1} (with glucose to fructose ratios of 0.1/0.1, 0.05/0.15, 0.15/0.05) The apparent yield refers to the sugars consumed during fermentation.

3.3. Improvement of Succinic Acid Production by the Addition of Mediator in 250 mL Anaerobic Bottles

Most published studies on the improvement of SA production in *A. succinogenes* focused on manipulating enzyme activities through deletion of competing pathways and/or overexpression of the beneficial pathways using genetic tools [1,37,38]. Consequently, the purpose of this study was to generate succinate overage from *A. succinogenes* by increasing

fumarate reductase activity, through the addition of reduced mediators that transfer electrons into bacterial cells and act as an electron donor for the fumarate reductase. Reduction and oxidation between fumarate and succinate should create a loop into the TCA cycle. We worked on the availability of different fumarate concentrations and our concept was to prove its effect as a mediator. Table 2 present the effect of different initial fumarate concentrations on sugars consumption, pH, SA formation, yields, productivities and the relative percentage of succinic acid.

Table 2. Effect of FA addition on succinic acid formation at the end of fermentation in 250 mL anaerobic bottles with a volume of 200 mL.

Runs	0	1	2	3	4	5
FA (mol L^{-1})	0	0.0008	0.0017	0.0025	0.0034	0.0042
OD_{660} nm	0.28	0.25	0.24	0.23	0.18	0.13
Consumed glucose (%)	97.54	97.99	97.94	83.81	86.74	95.76
Consumed fructose (%)	97.04	97.60	97.60	93.83	92.94	94.41
SA (mol L^{-1})	0.18	0.22	0.22	0.23	0.23	0.23
pH	6.00	5.98	5.65	5.53	5.66	5.32
Yield (mol-C/mol-C)	0.47	0.57	0.57	0.66	0.56	0.62
Productivity (g L^{-1} h^{-1})	0.17	0.21	0.21	0.22	0.22	0.22
Relative (%)	0	18.9	22.1	25.9	27.56	28.0

The decrease of pH was attributed to the dissociated form of FA that was initially added into the medium. Results indicate that when FA concentration increased, the net production of SA was higher by 28% compared to experiment done without FA. In addition, SA production yield and productivities increased, achieving 0.62 mol-C SA/mol-C sugars and 0.23 g L^{-1} h^{-1}, respectively, with SA concentration up to 0.23 mol L^{-1} at the end of fermentation (120 h). The fumarate reductase has a key role because it allows electrons transfer from the reduced mediator into bacterial cells. One of the scenarios is that fumarate is adsorbed at the inner membrane and transfer electrons to the fumarate reductase producing succinate.

A. succinogenes is a wild type succinate producer, which produces SA anaerobically through the reductive branch of the TCA cycle [39]. Succinate production from anaerobic growth with C_4-dicarboxylates, such as fumarate requires transporters catalyzing uptake and efflux of C_4-dicarboxylates. As a result, a multiple sequence alignment (MSA) of protein sequence was done using UNIPROT databases with a protein sequence found in *E. coli*, which requires transporters for fumarate uptake, showed that *A. succinogenes* have a transport system of 3 transporters for fumarate uptake and expressed during anaerobic growth on fumarate; they are: Asuc_1063, Asuc_1999 and Asuc_0142.

A. succinogenes grows well on C_4-dicarboxylates such as fumarate under anaerobic conditions. Supplied fumarate is almost turned into succinate and more than 90% of the supplied sugars are also transformed to succinate. Under all tested conditions, microaerobic and anaerobic conditions with glucose and fructose or fumarate, SA level was markedly improved in the presence of fumarate (see Table 3), if compared to the fermentation using the C_6-sugars, leading to a SA concentration of about 0.5 mol L^{-1} with an experimental yield and productivity of 0.88 mol-C/mol-C, 0.48 g L^{-1} h^{-1}, respectively. Anaerobic growth on fumarate was stimulated by the transporter and the major product was succinate with a conversion yield of 95.4%, indicating the involvement of fumarate uptake in SA production, similar to succinate production from glucose and fructose. Fumarate uptake of 0.0008 mol. L^{-1} shows that the systems had substrate specificity for fumarate but not for succinate.

Table 3. Succinic acid formation from glucose and fructose and/or fumarate at the end of fermentation in 200 mL anaerobic bottles. Run 1: equal mixed sugars of glucose and fructose with 0.087 mol L^{-1} FA; run 2: 0.043 mol L^{-1} FA; run 3: 0.087 mol L^{-1} FA.

Runs	1	2	3
Glucose concentration (mol L^{-1})	0.2	-	-
Consumed glucose (%)	97.14	-	-
Fructose concentration (mol L^{-1})	0.2	-	-
Consumed fructose (%)	97.01	-	-
FA (mol L^{-1})	0.087	0.043	0.087
Consumed FA (%)	100	100	100
SA (mol L^{-1})	0.492	0.037	0.083
Yield *	0.99	0.88	0.91
Productivity (g L^{-1} h^{-1})	0.48	0.036	0.081
Conversion yield (%)	61.87	86.04	95.4

Yield *: Overall yield molC SA/molC substrate.

3.4. Conversion of FA into Succinic Acid in Actinobacillus succinogenes

In this study, different batch fermentation tests were carried out in 250 mL anaerobic bottles contained glucose and fructose and FA in different quantities, among which the concentrations of glucose and fructose were equal. On the other hand, a simulated medium, containing FA as described earlier except the sorts of mixed sugars were used for SA fermentation. In this case, batch fermentation tests were further chosen to compare with the fermentation ability of single carbon feedstock (FA). Each carbon source was added to keep a constant amount of carbon. Glucose, fructose and FA were almost consumed at the end of the fermentation (Table 4). The extra SA produced is normally corresponding to the consumed FA added in the media with an error percentage (Table 4). The first run corresponded to the control experiments done without the addition of FA. In this experiment the SA concentration obtained was about 0.053 mol L^{-1}. However, the maximum SA concentration (run 3) produced was approximately 0.24 mol L^{-1}, with almost a similar productivity (0.25 g L^{-1} h^{-1}), corresponding to sugars and FA consumption. According to this, the FA consumed was 0.192 mol L^{-1} and then the extra SA produced from FA when compared to control run was 0.187 mol L^{-1} with an error percentage of 2.6%, indicating that FA can be used to directly produce SA in the presence of mixed sugars. SA concentrations were about 0.24 mol L^{-1}, 0.16 mol L^{-1} and 0.05 mol L^{-1}, obtained from an initial equal concentration of a mixture of glucose and fructose (0.05 mol L^{-1}), with 0.2 mol L^{-1}, 0.1 mol L^{-1} or without FA, respectively. For the fermentation using FA as sole carbon source, SA concentration and productivity were relatively low when compared to conversion yield.

Table 4. Efficient succinic acid formation from glucose and fructose and/or FA at the end of fermentation in 200 mL culture medium, in anaerobic condition. Run 1: equal mixed sugars of glucose and fructose; Run 2: equal mixed sugars of glucose and fructose with 0.1 mol L^{-1} FA; Run 3: equal mixed sugars of glucose and fructose with 0.2 mol L^{-1} FA; run 4: 0.1 mol L^{-1} of FA; run 5: 0.2 mol L^{-1} of FA.

Runs	1	2	3	4	5
Glucose concentration (mol L^{-1})	0.05	0.05	0.05	-	-
Consumed glucose (%)	99.05	98.87	96.67	-	-
Fructose concentration (mol L^{-1})	0.05	0.05	0.05	-	-
Consumed fructose (%)	97.62	98.09	96.72	-	-
FA (mol L^{-1})	-	0.1	0.2	0.1	0.2
Consumed FA (%)	-	92.55	96.16	99.33	98.92
SA (mol L^{-1})	0.053	0.17	0.24	0.09	0.15
Extra SA (mol L^{-1})	0	0.11	0.187	-	-
Yield *	0.58	0.92	0.85	0.94	0.74
Productivity (g L^{-1} h^{-1})	0.05	0.18	0.25	0.09	0.16
Conversion yield (%)	54.63	89.47	85.71	100	76.14
% of difference (absolute value)	-	27	2.6	0	24

Yield *: Overall yield molC SA/molC substrate.

However, compared to the fermentation using glucose and fructose, the fermentation using 0.1 mol L^{-1} and 0.2 mol L^{-1} of FA (run 4 and 5, respectively) as the carbon source resulted in a final SA concentration of about 0.08 mol L^{-1} and 0.14 mol L^{-1}, respectively. These batch tests led to a yield of 0.94 and 0.74 molC-SA/molC-FA with 0.09 g L^{-1} h^{-1} and 0.16 g L^{-1} h^{-1} SA productivity, respectively, confirming that the carbon obtained from FA can be used for the biomass formation (growth) and is crucial and efficiently used to produce SA during fermentation culture. Fumarate reductase (FRD) is a key enzyme activated and synthesized under low oxygen conditions. In *Actinobacillus succinogenes*, fumarate reductase induced by anaerobic growth is expressed in the last step of anaerobic fermentation [28], allowing the released electrons to an awaiting fumarate to be reduced into succinate in the anaerobic pathway. The reduction of fumarate is the main source of succinate during fermentation and under anaerobic conditions, it is strictly required for the formation of succinate [40]. According to the metabolic flux of *Actinobacillus succinogenes*, OAA converts into succinate via MAL and FUM as intermediates [41]. The succinate yield is strongly related to nicotinamide adenine dinucleotide (NADH) availability [42] and, hence, to fumarate. Subsequently, a flux distribution that supports high fumarate and NADH presence is in favor of succinate production [43–45].

3.5. Mass Balance Analysis (MBA)

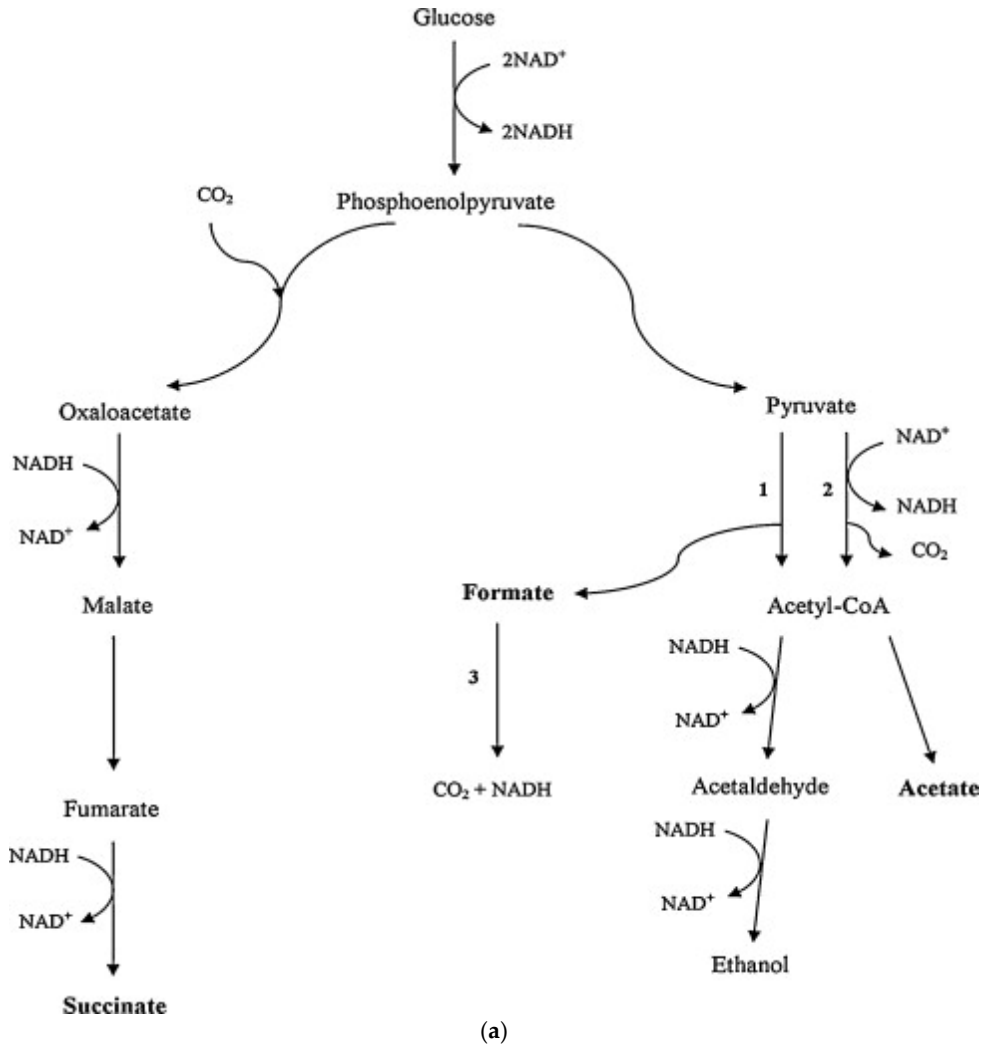
MBA is a way to determine what the maximum capabilities of an organism are in a steady state condition for a given carbon substrate. In addition, MBA are profitable for flux distribution, biomass formation and metabolite synthesis and it is influenced by environmental conditions. The accuracy of data was evaluated by performing general mass balances which consist of the calculation of stoichiometric amount of substrates required (glucose and fructose in our case) to produce the metabolite relying on elemental balances (Cin = Cout + Caccumulated) and compare this amount (theoretic) to the obtained (measured) amount of metabolites produced. Table 5 presents kinetics of multiple fermentation runs with the mass balance closure. Since glucose and fructose have the same C, H and O ratio, they were combined into a single amount in the calculation and the mineral carbon obtained from $MgCO_3$ was excluded. The percentage closure of the mass balance is calculated as the ratio of the experimental C_{SA} and DCW produced to the obtained amount of sugars consumed. A value under 100 indicates that more carbon was consumed than accounted for metabolites and biomass production.

The maximum production rate was seen when an equal mixture of sugars with FA addition was tested, recording a value of 0.48 g L^{-1} h^{-1}; while the maximum yield was obtained from the co-fermentation when the mass balance closed on average of 82%.

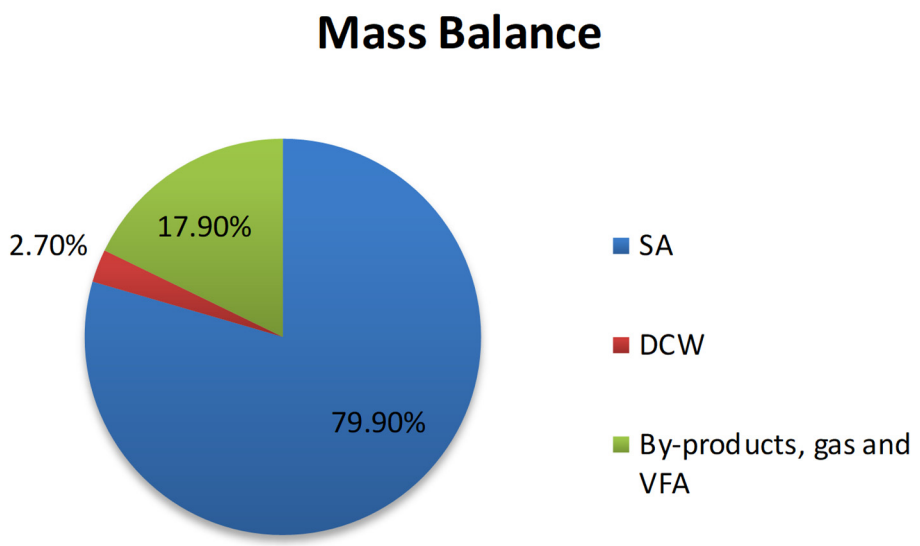
Table 5. Kinetics and mass balance analysis for different runs. Run 1: glucose in different concentrations: Run 2: Fructose: Run 3: equal mixed sugars of glucose and fructose; Run 4: equal mixed sugars of glucose fructose with FA; Run 5: with FA.

Runs		**1**		**2**	**3**	**4**	**5**
Fermentation time (h)	104	48	48	48	96	120	96
Glucose concentration (mol L^{-1})	0.05	0.2	0.26	-	0.1	0.2	-
Fructose concentration (mol L^{-1})	-	-	-	0.15	0.1	0.2	-
Fumarate concentration (mol L^{-1})	-	-	-	-	-	0.087	0.2
dS/dt (g L^{-1} h^{-1})	0.06	0.64	0.86	0.48	0.34	0.66	0.23
dP/dt (g L^{-1} h^{-1})	0.01	0.34	0.24	0.29	0.27	0.48	0.18
Yp/s	16.4	54.1	28.5	60.7	79.4	72.8	77.8
Yx/s	≈0	1.69	1.3	2.2	2.7	1.47	1.00
Yp/x	-	31.8	21.5	26.8	130.2	49.10	89.8
% closure of mass balance	16.4	55.7	29.8	62.9	82.1	74.2	78.8
Yield (mol-SA/g-DCW)	-	0.26	0.18	0.22	0.25	0.41	0.65
(SA) ratio (o/t)	14.7	48.2	25.6	54.5	71.0	65.3	76.1

Figure 4 present the mass balances in co-fermentation which closed to 79.9% on average suggesting that more sugars were consumed than metabolite produced. The sample taken at the end of fermentation (96 h) present 2.7% of DCW closed the MBA to 82.1% and a rest of 17.9% distributed between by-products, gas and volatile fatty acid (VFA). The incomplete mass balances closure attributed to undetected metabolites does not detract from the process relevance of the obtained results. Writing a mass balance for each intracellular metabolite leads to collect the metabolic reactions and pathways occurs in *Actinobacillus succinogenes* (see Appendix A) to understand the mechanism of production of the end products and the by-products. Natural *A. succinogenes* strain produces SA by Phosphoenolpyruvate (PEP) pathway and TCA cycle reductive branch (C_4 pathway, Figure 4). Through these pathways, NADH was completely consumed; thereby, flux toward SA is restricted due to satisfy the demand of redox balance leading to by-products formation such as formic and acetic acid through the pyruvate pathway to balance the carbon metabolism. *A. succinogenes* pyruvate metabolism can take place in two different pathways, particularly the pathways of pyruvate formate-lyase (PFL) or pyruvate dehydrogenase (PDH). When PFL flux is taking place, the Y_{AAForA} is 1.0 mol/mol because the Acetyl-CoA generated from pyruvate is transformed into acetic acid (Figure 4). However, when PDH route takes place, CO_2 and NADH were formed instead of formic acid; thus, Y_{AAForA} turned to zero and an extra reducing power (NADH) output is increased. Likewise, the Y_{AAForA} become zero when the PFL pathway occurs in concurrence with formate dehydrogenase (FDH), leading to CO_2 and NADH production resulted from formic acid breakdown by FDH.



(**a**)



(**b**)

Figure 4. Carbon flux (**a**) and mass balance (**b**) at the end of co-fermentations (96 h) carried out in 200 mL anaerobic bottle flasks using an equal mixture of 0.1 mol L^{-1} of glucose and fructose. The metabolic network of *A. succinogenes* representing the pathways of metabolites [46]. (1) pyruvate formate lyase (PFL), (2) pyruvate dehydrogenase (PDH) and (3) formate dehydrogenase (FDH).

The following reactions performed when pyruvate was transformed via the PFL pathway:

$$\mathrm{Glu} + 2\mathrm{CO2} + 2\mathrm{NADH} \rightarrow 2\mathrm{SA} \quad (5)$$

$$\mathrm{Glu} \rightarrow 2\mathrm{AA} + 2\mathrm{FA} + 2\mathrm{NADH} \quad (6)$$

Equation (5) presents SA formation and Equation (6) indicates acetic and formic acids formation. The integration of the two Equations (5) and (6) yields the net redox balance, Equation (7):

$$Glu + 2\mathrm{CO2} \rightarrow 2\mathrm{SA} + \mathrm{AA} + \mathrm{FA} \quad (7)$$

Theoretically, biomass formation is considered as zero and all the carbon is converted to products. Equation (7) presents the theoretic value for $Y_{SA,AA}$ which is 1.0 mol/mol = 1.97 g/g and 0.66 g/g for $Y_{SA,Glu}$. When pyruvate is converted through the PDH pathway, the reactions that take place are the following:

$$2Glu + 4CO2 + 4NADH \rightarrow 4\mathrm{SA} \quad (8)$$

$$Glu \rightarrow 2\mathrm{AA} + 2\mathrm{CO2} + 4\mathrm{NADH} \quad (9)$$

Basically, Equation (8) represent the Equation (5) times two, to fit the redox requirements and Equation (9) presents the acetic acid production. As above, integration of Equations (8) and (9) yields the net redox balance in Equation (10):

$$3\ Glu + 4CO2 \rightarrow 4\mathrm{SA} + 2\mathrm{AA} \quad (10)$$

Theoretically, no biomass was formed and from Equation (10), the $Y_{SA,AA}$ maximum value is 2.0 mol/mol equal to 3.93 g $_{SA}$/g $_{AA}$ and 0.87 g/g for $Y_{SA,Glu}$. Similarly, when PFL is active with concurrence of FDH, formic acid can be transformed into CO_2 and NADH giving 3.93 g/g as maximum value for $Y_{SA,AA}$ when biomass growth and maintenance state are negligible. When biomass is formed, a part of substrate (glucose and/or fructose) will

be addressed to the biosynthesis metabolism flux, affecting that way redox balance with a lower yield than mentioned before. The maximum yield observed in this study, ($Y_{SA,S}$) achieved 0.79 g/g, exceeding the theoretical yields for glucose, fructose (0.66 g/g) but not for the co-fermentation with (1.02 g/g) or without (1.12g/g) FA addition. The highest $Y_{SA,S}$ values published in *A. succinogenes* fermentation was 0.76 g/g [47], 0.69 g/g [48], 0.59 g/g [49] and 0.94 g/g [50]. It should be pointed out that in most cases, the literature related to the fermentation of *A. succinogenes*, sugar consumption, metabolite concentrations or yields, are not provided and thus metabolic network analysis cannot be assessed. The transhydrogenation also called the net formation of NADPH/NADH is related to biomass generation and may account for the redox imbalance. To settle the redox balance, it is helpful to determine the surplus "extra" NADH needed at high $Y_{SA,AA}$ as a function of substrate demand. The yield of "extra" NADH is expressed by moles of NADH produced per mole of substrate consumed mol/mol (pathway presented in Figure 4). In our study, at the end of fermentation, the carbohydrates consumption was 69.84 g L^{-1} h^{-1} and led to 1.18 g L^{-1} of DCW. Villadsen et al., [51] assumed the biomass molecular composition ($CH_{1.8}O_{0.5}N_{0.2}$) and from the metabolic pathway given in Figure 4, the NADH generated via biomass formation (0.0027 mol/mol), via glycolysis (0.77 mol/mol) and via PDH/FDH pathway (unknown in our case) but theoretically (1 mol/mol) is only 77.27% must require to produce 58 g/L of SA. The "extra" NADH implies the surplus reducing power formed by biomass and by PDH/FDH pathway, as a whole may considered the extra NADH needed and delivered to please the redox balance. Many papers reported the highest yield ($Y_{SA,Glu}$ to 1.12 g/g) and discuss the ability of generating NADH from TCA cycle oxidative branch by the glyoxylate shunt, increasing thereby the C_4 pathway flux without by-product formation [52].Yet, TCA cycle functional enzymes leading to the glyoxylate shunt (citrate synthase and isocitrate dehydrogenase) are lacked in *A. succinogenes* [53]; hence, these pathways are ruled out as NADH additional origins. A study done by Rühl et al., [54] discussed the ability of *Bacillus subtilis* to sustain the metabolic activity with non-significant biomass formation, leading that way to an overflow in NADPH through the pathway of pentose phosphate (PPP) which further transformation into NADH via transhydrogenase. Similarly, when (PPP) takes place, the carbon produced from glucose-6-phosphate and/or fructose-6-phosphate is completely recycled and channeled into this pathway in *A. succinogenes*, which possess the transhydrogenase as mentioned above, suggesting the conversion of excess NADPH to NADH. The present net reactions could occur:

$$3\ Glu \rightarrow 2CO2 + 8NADH + 4SA \quad (11)$$

$$\frac{1}{2}Glu + CO2 + NADH \rightarrow SA \quad (12)$$

Equation (11) presents the SA formed via (PPP) oxidative pathway assuming that the glyceraldehyde-3-phosphate (GA3P) formed from (PPP) is transformed into PEP with one NADH release and the PEP is thereafter being channelized to SA by C_4 pathway. Equation (12) presents the SA formed from TCA cycle reductive branch. By combining these two equations, the redox is balanced in the following reaction:

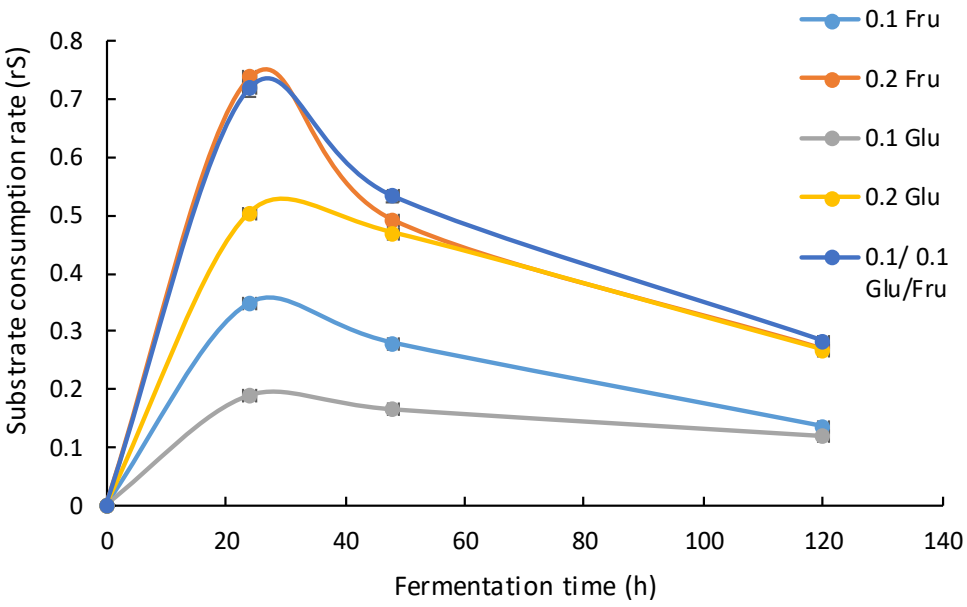
$$7\ Glu + 6CO2 \rightarrow 12SA \quad (13)$$

From Equation (13) SA on glucose maximum yield ($Y_{SA,Glu}$) is 1.12 g/g obtained with no biomass or by-products formation. Consequently, SA yield maximization can be achieved with an active oxidative PPP as it provides enough reducing power and eliminated the need of C_3 pathway. This mechanism could elucidate the non-detectable by-products observed in this study. Another piece of data published, dealing with *A. succinogenes* batch fermentations [55,56], suggest the shift of metabolic flux distribution in favor of SA production reflected by DCW decrease. In the present study, the yield $Y_{SA,S}$ obtained was between 0.16 g/g and 0.79 g/g and substrate consumption between 7.2 g L^{-1} and 79.93 g L^{-1}. DCWs in different situations were between (≈0 and 1.18 g L^{-1}) and $Y_{SA,X}$

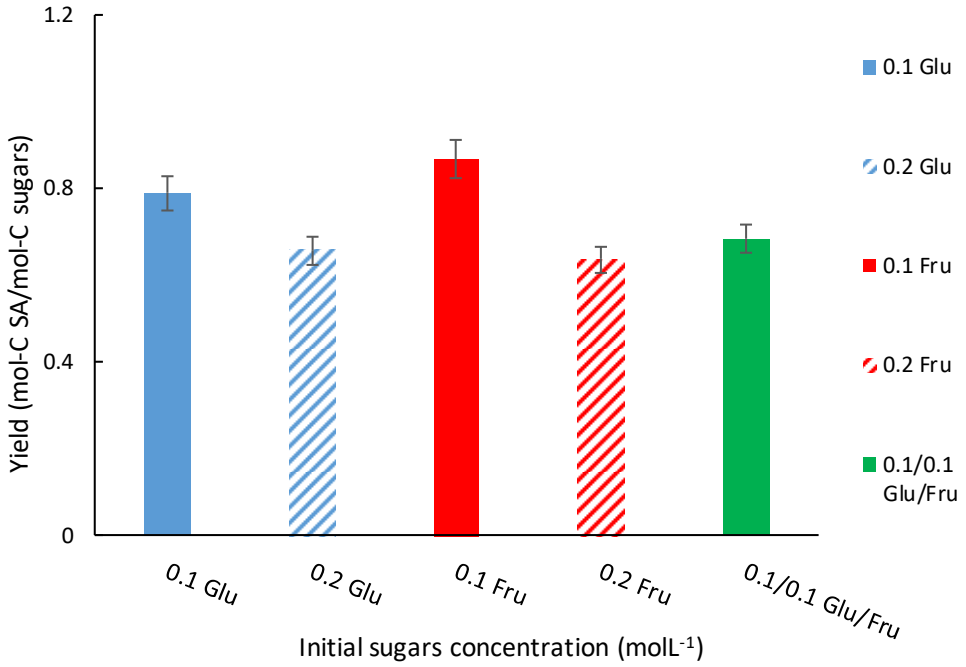
was also between 0.18 g/g and 0.65 g/g. The differences in degree imply a difference in the behavior of *A. succinogenes*. At a high concentration of SA, the $Y_{X,S}$ decrease could be explained by maintenance or non-growth state applied by the cells as mentioned above. The notion of *A. succinogenes* maintenance state entering with multiple flux distribution ties is discussed by [54]. However, an inquiry of PPP when it is active whether or not should be required.

3.6. Batch Fermentation Realized in 1 L Reactors

Previous experiments in 250 mL anaerobic bottles demonstrated that SA is produced regardless sugar species (Figure 5a). To more deeply investigate SA production, batch cultures were carried out under anaerobic conditions in 1 L reactor employing SM containing glucose and fructose and or mixtures of both sugars to have different initial concentration varied in total between 0.1 and 0.4 mol L^{-1}. Sugar consumption (r_S) for glucose, fructose and their mixtures at different initial sugar concentrations are illustrated in Figure 5b; sugar consumption rates (r_S) were for fructose (0.73 g L^{-1} h^{-1}), glucose (0.50 g L^{-1} h^{-1}) and sugars mixture (0.74 g L^{-1} h^{-1}) at 24 h. No sign of diauxic growth was observed, since glucose and fructose were simultaneously consumed. The amount of remaining sugar increased with the initial concentration of initial sugars concentration increased (Figure 5b). Regarding SA production (Figure 5b), maximum yield values were 0.79, 0.83 and 0.67 C-mol SA/C-mol substrate for glucose and fructose, respectively, at 0.1 mol L^{-1} for pure and/or mixed sugars.



(**a**)



(**b**)

Figure 5. Experimental substrate consumption rate (**a**) and succinic acid production yield (**b**) of *A. succinogenes* fermentation at different initial concentrations of glucose and fructose.

3.7. Fermentation in the Bench-Top Fermenters (B-TFs)

Fermentation runs were also performed in a 3 L capacity fermentor with 1800 mL working volume using the same medium as in 250 mL anaerobic bottles. Figure 6a displays the results from similar initial sugars concentrations (glucose and fructose equal mixture) with FA as a co-factor at the ending of experiments. Maintenance production towards the end of the fermentation is slow (Figure 6a), glucose and fructose were simultaneously consumed, however, they present a significant amounts of residual sugars present (0.01 mol L^{-1}). The SA concentration obtained at the end of fermentation was about 0.4 mol L^{-1}. The specific productivity was 14.6 g of succinic acid/g of dry cell weight at the end of fermentation (after 96 h). Fermentations in B-TFs demonstrated similar behavior as the 250 mL anaerobic bottles. At t = 0 h; 100% saturation level of DO indicated highly aerobic conditions; however, at t = 48 h, a situation between aerobic and anaerobic conditions was obtained and a slight decrease in the pH was observed (Figure 6b).

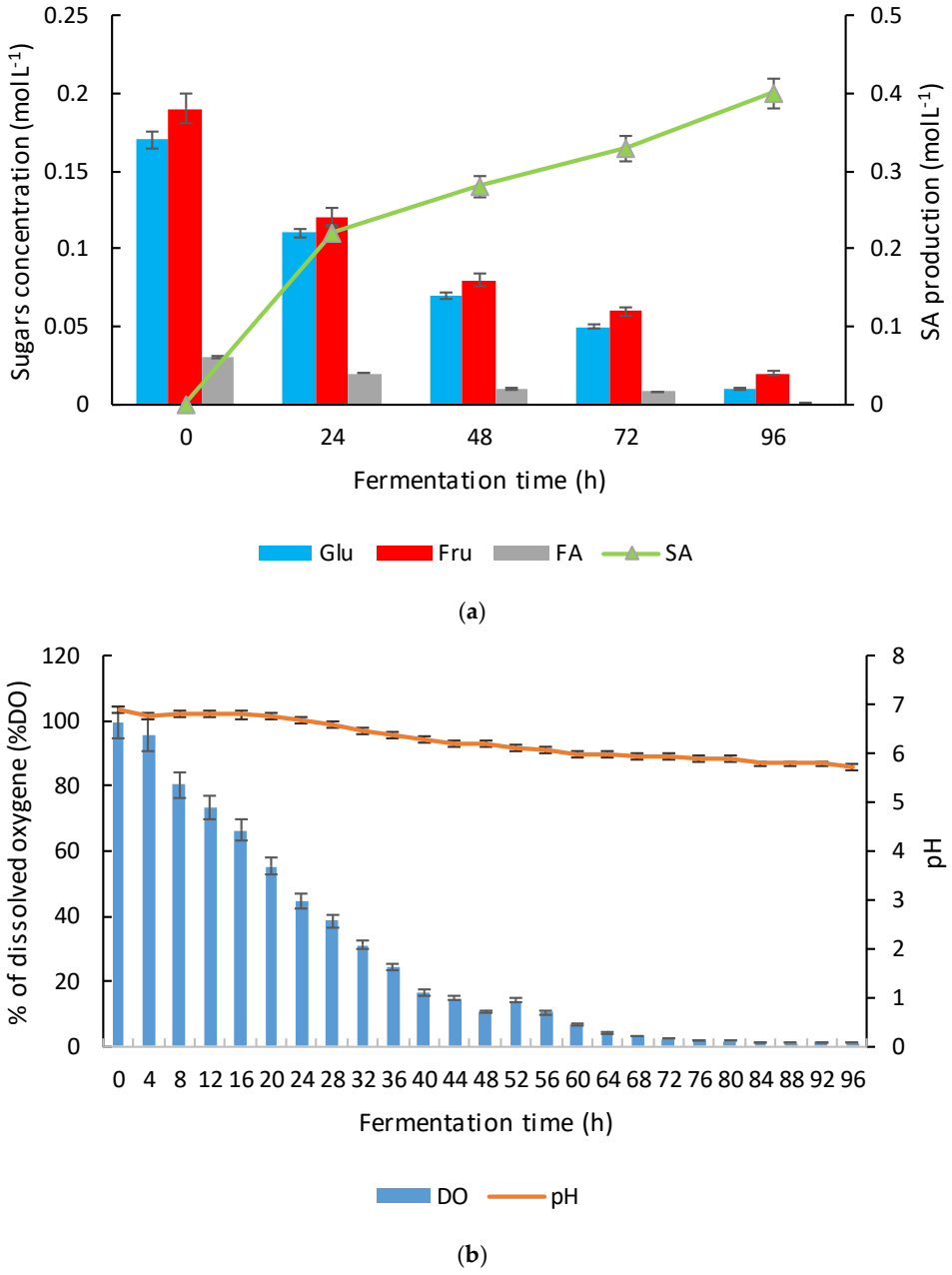


Figure 6. (**a**) sugars consumption vs. succinic acid concentration, (**b**) pH and dissolved oxygen evolution during fermentation time in 3 L bioreactor performed in anaerobic conditions at 37 °C.

SA production of (0.3 mol L^{-1}) using glucose as the sole carbon substrate in the reactor was lower when compared to the mixture of sugars (data not shown). When a reactor is run in batch mode, important process variables, (e.g., cell mass, pH, dissolved oxygen (DO), substrate concentration) may vary significantly [57]. If the dissolved oxygen level is kept constant, the aeration efficiency can be used as an indicator of the biological activity and it may also be beneficial for process supervision. For instance, elevated oxygen levels are unfriendly with SA production and therefore the dissolved oxygen variation should be monitored. *A. succinogenes* can grew-up in aerobic and anaerobic conditions by modifying its physiology and metabolic pathways to adapt the climate change [58,59]. SA production is controlled by regulatory systems that detect oxygen levels and transmit a signal to modify gene expression accordingly [60]. As a result, the concentration of SA increased in anaerobic conditions when compared to aerobic ones. The results given above throw light on the relation between cultivation modes (the dissolved oxygen environment) and

SA production and in the light of this speculation, fermentation of *A. succinogenes* established in microaerobic conditions can be shifted to anaerobic environment when O_2 is completely consumed without nitrogen sparging. The accumulation of SA in the broth might however affect biomass formation, as already described in the literature [61]. It is known that non-dissociated organic acids can penetrate the lipidic membrane of bacteria cells and be dissociated at intracellular pH (6.8), decreasing intracellular pH. As a consequence, energy (ATP) will be required to adjust the intracellular pH and anaerobic microorganisms such as *A. succinogenes* will use more energy to expel protons instead of using energy for biosynthesis and growth [62].

3.7.1. pH Regulation

The pH culture is a crucial factor in fermentation. In bioreactor, NaOH was used as buffering agent for SA production, along with $MgCO_3$. Although the use of NaOH could promote a significant accumulation of Na^+ ions in the broth in addition to NaH_2PO_4 and Na_2HPO_4 present in the media used to neutralize pH and organic acids synthesis. Na^+ ions are known to be involved in intracellular pH regulation [31], their accumulation resulting in a hyperosmotic environment and subsequently damage of the cell morphology. The effect might be so severe that it could actually affect cells even at an early stage of fermentation, reducing the growth rates. Without pH regulation, the pH value in the culture decreased from 6.8 to 5.7 at the end fermentation due to the accumulation of succinic acid. For this reason, it was essential to study the significance of pH on *A. succinogenes* production performances in a bench-top reactor. The effect of pH on SA accumulation was examined by setting-up the pH at 6.8 during the entire fermentation process. When the pH was adjusted to 6.8, no significant effect was shown on SA biosynthesis at the end of the fermentation and the neutral environment appeared favorable to an accumulation of SA (Table 6). When pH was controlled, SA yields and productivity were increased if compared to experiments carried out in 250 mL anaerobic bottles (0.88 mol C-SA/mol-C sugars and 0.5 g L^{-1} h^{-1}) respectively, in addition to SA final concentration (0.41 mol L^{-1}). Maintaining the pH fermentation within suitable range for the microorganism and the selection of an appropriated base to neutralize the produced acid, had a significant effect on the overall SA production costs [63]. Cell density and sugars consumption were therefore almost similar during pH controlled and non-controlled fermentation.

Table 6. Succinic acid production from 0.2 mol L^{-1} equal mixture of glucose and fructose with 0.2 mol L^{-1} of $MgCO_3$ at the end of fermentation time (96 h) in 3 L benchtop reactor with a volume of 1800 mL.

Runs	Controlled pH	Non-Controlled pH
Total sugars concentration (mol L^{-1})	0.4	0.4
Consumed glucose (%)	96.83	93.30
Consumed fructose (%)	98.26	91.39
Consumed fumarate (%)	96.49	96.07
dP/dt (g L^{-1} h^{-1})	0.50	0.49
Yp/s (mol-C/mol-C)	0.88	0.87
SA (mol L^{-1})	0.41	0.40

3.7.2. Fermentation Studies in Different Sizes of Bioreactors

In this study, fermentation techniques are formulated using bottles and small lab scale fermenters. The surrounding environment of *A. succinogenes* may change with the change in scale of production. Different parameters can be observed in number during lab scale fermentation setups. The parameters obtained for the various volumes examined and the impact on the SA production are shown in Table 7. An increase in the scale of fermentation (volume) without compromising yield or productivity was seen in 250 and 3000 mL. Results show that the data were representative, when scaled up, similar values of SA concentration and productivity were obtained, as compared to those in 3000 mL. However, in the fermentation done in one liter, results were different, for this reason, identify the factors that contribute to decrease and to rectify it is necessary. This might be due to a lack of scale-up considerations. The speculations that must be considered while developing the process of higher fermentation productions are the same in terms of inoculum development, sterilization, pH, temperature, environmental parameters and agitation

Table 7. Comparison of fermentation parameters in different sizes of bioreactors.

Volume (mL)	**250**	**1000**	**3000**
Working volume (mL)	200	800	1800
SA (mol L^{-1})	0.40	0.28	0.41
dP/dt (g L^{-1} h^{-1})	0.49	0.26	0.50
Yp/s (mol-C/mol-C)	0.81	0.57	0.88

Although, previous studies carried out in anaerobic bottles proved that the decrease of cell density and/or biomass could not be correlated to high sugar concentrations regardless of the sugar nature. Instead, it might be related to the accumulation of SA in the broth, as mentioned in the study of Lin and colleagues [61]. As cell growth stops, flux towards C_3 pathway also decreases as there is no further need to maintain such NADH demand via acetate and formate production [64]. Thus, the available carbon is directed to produce SA and then SA yield increased. The maximum SA concentration is not associated with total sugar consumption, while the production of SA begins to slow down at 52 h. An inhibitory effect of SA on bacterial growth and its production can be suggested. From the investigated experiments, the highest concentration of SA obtained by *A. succinogenes* was identical to the 250 mL anaerobic bottles experiments, exhibiting a value of 0.28 mol L^{-1} with a sugars consumption and SA production rate of 1.2 g L^{-1} h^{-1} and 0.76 g SA g L^{-1} h^{-1} after 48 h, respectively (Figure 5a). The results obtained in these experiments were compared with the results reported in previous work in Table 8 for succinic acid production from different substrates; it is similar to the other cited substrates. Succinic acid production from *A. succinogenes* CGMCC1593 was reported at 0.50 mol L^{-1} which was an increase of 55.5% in comparison to the batch fermentation of 0.32 mol L^{-1} [65]. *Mannheimia succiniciproducens* LPK7 was employed to produce succinic acid from glucose in batch run and increase by a 2.9-fold in succinic acid concentration when compared to fed-batch fermentation. For the fed-batch system, the succinic acid concentration and productivity were 0.4 mol L^{-1}, while the values for batch fermentation were 0.1 mol L^{-1} respectively [66]. Cassava roots were employed for SA production using *A. Succinogenes* and this resulted in concentration, yield and productivity of 0.78 mol L^{-1}, 1.3 and 1.87 g L^{-1} h^{-1}, in batch mode and 1.27 mol L^{-1}, 2.4 and 3.22 g L^{-1} h^{-1}, in fed-batch mode respectively [67]. Corn straw was investigated for succinic acid fermentation by *A. succinogenes* recording a SA value of 0.38 mol L^{-1}, 0.44 mol L^{-1} corresponding to a productivity of 0.95 g L^{-1} h^{-1},1.21 g L^{-1} h^{-1} in batch and fed-batch fermentation, respectively.

Table 8. Comparison of succinic acid production by fermentation with previous studies.

Micro-Organisms	Substrate	Fermentation Strategy	Succinic Acid Production			Ref.
			Concentration	Yield	Productivity	
A. succinogenes CGMCC1593	Glucose	Batch	0.32	1.25	1.00	[56,65]
		Fed-batch	0.50	1.2	1.30	
M. succinio-producens LPK7	Glucose	Batch	0.11	0.97	1.22	[66]
		Fed-batch	0.44	1.16	1.80	
A. succinogenes DSMZ 22257	Glucose, Fructose	Batch	0.50	0.88	0.48	This study
A. Succinogenes 130 Z	whey	Batch	0.11	1.67	0.61	[68]
A. succinogenes NJ113	Sweetsorghum bagasse	Batch	0.15	0.93	0.98	[69]
A. succinogenes 130 Z	Rapeseed meal	Batch	0.13	0.20	0.22	[70]
		Fed-batch	0.19	0.20	0.33	
A.succinogenes 130 Z	Raw carob pods	Batch	0.07	0.55	1.32	[24]
A. succinogenes ATCC55618	Cassava roots	Batch	0.78	1.3	1.87	[67]
		Fed-batch	1.27	2.4	3.22	
A. succinogenes CGMCC1593	Corn straw	Batch	0.38	1.36	0.95	[71]
		Fed-batch	0.44	1.4	1.21	

3.8. Effect of SA on Bacterial Growth

SA can be produced in relatively high yields from the C_6 sugars, glucose and fructose using *A. succinogenes* [43]; yet, maximum SA concentration was 0.4 mol L^{-1} during fermentation. Until now, no study was done on the tolerance of *A. succinogenes* against SA. Consequently, to examine this point, time-courses of SA consumption at different initial concentrations of SA (SA_0) are shown in Figure 7. It can be clearly seen that SA consumption remained constant during the course of culture.

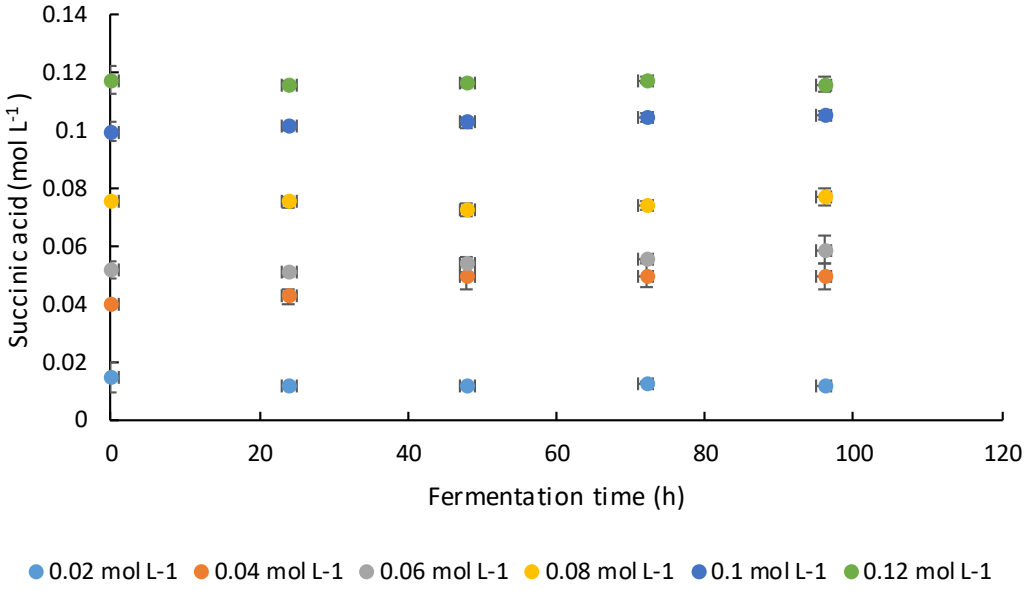


Figure 7. consumption of different concentration of succinic acid in mol L^{-1} during the course of the fermentation in 250 mL anaerobic bottles with a volume of 200 mL.

Therefore, the aim was to obtain more detailed information about the inhibitive forms of the substrate/product as well as about the concentrations that cause inhibition. The product inhibition was tested in batch tests and on Petri dish using TSA media. Each experiment was carried out using a set of six Petri dishes. One of them served as negative control, while the five others contained increased concentrations of succinic acid, in the range 0.1 to 0.5 mol L^{-1}. After 24 h of incubation, the strain was developed on TSA containing 0, 0.1, 0.2 and 0.3 mol L^{-1} of SA, while no development was observed on TSA containing 0.4 and 0.5 mol L^{-1} of SA (Figure 8). Results displayed the initial critical concentration of SA for *A. succinogenes* biological activity was around 0.4 mol L^{-1} and at this SA_0 no bacterial growth was observed. In fact, succinate had an important inhibitory effect on cell growth as well as on SA production; to our knowledge, this observation was not widely reported. Cells growth was suitable for inhibition detection caused by SA production [72]. The results obtained from inhibition tests carried out on different Petri dishes showed negative growth starting from 0.4 mol L^{-1} of SA concentration. This value correlate fairly well with that obtained by Lin et al., [61] who tested the effect of different concentrations of substrate and product on *A. succinogenes* growth and figure out SA critical concentration of above the bacteria cease to grew up for 0.38 mol L^{-1} of SA and further support the concept of growth inhibition of *A. succinogenes* by the product in batch fermentations. Although our results differ to some extent from those of [73], who quantified the inhibitory phenomena and critical inhibitory concentration of mixed acids in batch fermentation which was observed at 0.18 mol L^{-1} for SA using *A. succinogenes*. Even though this value differs from our results obtained and from previous studies, they are consistent with the concept that the accumulation of fermentation products such as weak acids can act as inhibitory factors and decrease considerably the cell growth [74,75].

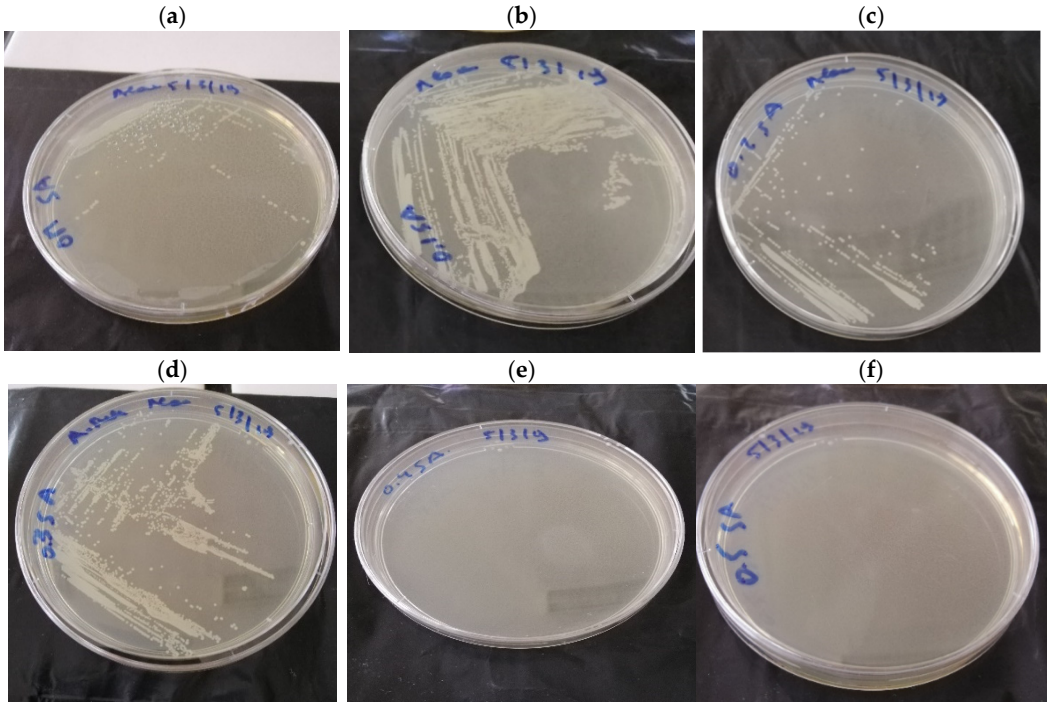


Figure 8. Effect of increased concentration of succinic acid on *A. succinogenes* growth (**a**) without succinic acid, (**b**) 0.1 mol L^{-1} SA, (**c**) 0.2 mol L^{-1}.SA, (**d**) 0.3 mol L^{-1} SA, (**e**) 0.4 mol L^{-1} SA and (**f**) 0.5 mol L^{-1} SA.

4. Conclusions

Among the media used for *A. succinogenes*, synthetic fermentation media containing fumarate gave the highest SA concentration and productivity, 0.49 mol L^{-1} and 0.48 g L^{-1}

h^{-1}, respectively. Interestingly, the addition of a mediator during the anaerobic fermentation process favored the production of SA with a conversion yield of about 96%. The yields and concentrations obtained 0.88 (molC SA/molC sugars consumed anaerobically) with 0.5 mol L^{-1} SA, respectively, were of the same order of magnitude as those reported in the literature. The experimental results pointed out the inhibitory effect of SA on *A. succinogenes* growth. SA fermentation performance could be improved by shifting the process from batch mode into continuous mode. Therefore, from a sustainability perspective and industrial side-streams, it is interesting to valorize agricultural by-products, food residues and effluents into building block chemical. To complete this work, scaling up the process from laboratory scale to pilot plant bioreactors in batch and continuous mode could be carried out to confirm the promising experimental results obtained.

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Consent to Participate: All authors consented to participation in this research.

Appendix A

Glycolysis	Pentose phosphate
$Glu + ATP \leftrightarrow G6P$	$G6P \leftrightarrow 2NADH + RU5P + CO2$
$G6P \leftrightarrow F6P$	$RU5P \leftrightarrow R5P$
$F6P + ATP \rightarrow F1{,}6P$	$R5P \leftrightarrow S7P$
$F1{,}6P \leftrightarrow DHAP + GA3P$	$GA3P + S7P \rightarrow E4P + F6P$
$GA3P \leftrightarrow DHAP$	TCA Cycle
$GA3P \leftrightarrow 1{,}3PG + NADH + ATP$	$PEP + CO2 \leftrightarrow OAA + ATP$
$1{,}3PG \leftrightarrow 3PG + ATP$	$OAA \rightarrow PYR + CO2$
$3PG \leftrightarrow 2PG$	$OAA + NADH \leftrightarrow MAL$
$2PG \leftrightarrow PEP$	$MAL \leftrightarrow PYR + NADPH + CO2$
$PEP \leftrightarrow PYR + ATP$	$MAL \leftrightarrow FUM$
Fructose catabolism	$FUM + NADH + 2/3ATP \rightarrow SUCC$
$FRU + ATP \rightarrow F1P$	Transhydrogenation
$F1P + ATP \leftrightarrow F1{,}6P$	$NADPH \leftrightarrow NADH$
Biomass formation equation	By-products
Not Available	Not detetcted

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