

Article

Production of High Added-Value Chemicals in *Basfia succiniciproducens*: Role of Medium Composition

Hunor Bartos ^{1,*} , Márta Balázs ¹, Ildikó Hajnalka Kuzman ², Szabolcs Lányi ² and Ildikó Miklóssy ² 

¹ Faculty of Science, University of Pécs, Ifjúság 6, H-7624 Pécs, Hungary; balazsmarta@uni.sapientia.ro

² Department of Bioengineering, Sapientia Hungarian University of Transylvania, Piata Libertatii 1, 530104 Miercurea Ciuc, Romania; kuzman.hajnalka@gmail.com (I.H.K.); lanyiszabolcs@uni.sapientia.ro (S.L.); miklossyildiko@uni.sapientia.ro (I.M.)

* Correspondence: bartoshunor@uni.sapientia.ro

Abstract: Succinic acid production through biological fermentation led to new pathways in the integration of renewable feedstock from different industries into biosynthesis. In this article, we investigate the population growth dynamics and succinic acid production potential of the recently isolated natural succinic acid producer, *Basfia succiniciproducens*, using in silico constraint-based metabolic models as well as in vitro experiments. Our work focuses on the influence of different renewable substrates and added yeast extract on fermentation dynamics, and the produced metabolites of the strain cultured in mineral (minimal) medium. According to our experiments, which were carried out as small-scale fermentations and in bioreactor conditions, glucose is the preferred carbon source, while the addition of 1% yeast extract has a significant positive effect on biomass formation. In the case of *B. succiniciproducens* cultured in minimal salt medium, a production potential as high as 47.09 mM succinic acid was obtained in these conditions. Industrial applications related to this bacterial strain could contribute to new possibilities for the re-use of byproducts by using fermentation processes, leading to high added-value compounds.

Keywords: *Basfia succiniciproducens*; succinic acid; yeast extract effect; bacterial growth; batch cultivation



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1. Introduction

Worldwide, bio-based succinic acid annual production adds up to 36,600 tons using strains of *B. succiniciproducens* [1], *Escherichia coli* and yeasts (possibly *Candida krusei*) on biomass feedstock like glycerol, sorghum or corn [2]. The projected potential market size of succinic acid is expected to reach 700.000 tons/year by 2020 [3], while biosynthesis based on renewable resources is viewed as a sustainable alternative to replace the petrochemical based succinic acid production. As Nghiem et al. reports, an LCA (Life-cycle assessment) study was conducted at Myriant's Louisiana plant. Sorghum grains were used as feedstock in comparison with petrochemical-based succinic acid production. The costs were half as much in the case of bio-based succinic acid production; USD 1.17/kg compared to USD 2.89/kg (in the case of petrochemical-based synthesis). Furthermore, global warming potential (GWP) and non-renewable fossil cumulative energy demand (non-ren CED) in the case of petrochemical routes was 3.85 and 10.44 times higher, respectively, than the base case, where glucose was used. Based on information in the study, it is indicated that biosynthetic production should be used [4]. Use of sustainable raw materials instead of petrochemicals is of great importance, in order to reduce the negative health, social and economic impact of air pollution, as well as to contribute to the development of circular economy [5–15]. Ferone et al. reports that high-sugar-content beverages (HSCBs) can be used for succinic acid production [16]. Whey or bakery waste also can be used as Louasté et al. and Zhang et al. present [17,18]. Glucose can be derived from many renewable sources, including the linear glucose chain which builds cellulose as Ramesh et al. explain [19]. As Kuenz et al. report, after several steps (hot water extraction, concentration,

ultrafiltration, recovery of monomeric/oligomeric carbohydrates) xylose can be derived from birch wood [20]. The transesterification of waste frying oil results in a step by step method to achieve glycerol [21].

In recent years, bio-based chemical precursor production have become a strongly growing industry, especially for biopolymer and bioplastic compounds [22–24]. Various bacterial strains, such as *E. coli* and *Mannheimia succiniciproducens* (further shown in Table 1) are used for the production of different platform molecules [23–30].

Several biotechnology companies (BioAmber, Succinity, Myriant, Reverdia) produce thousands of tons of succinic acid with microorganisms such as *E. coli*, *M. succiniciproducens* and other microbial strains mentioned in Table 1 [31], mainly from renewable carbon sources. Succinic acid or amber acid is a naturally occurring compound in various forms of esters [4], due to its wide range of applications in the pharmaceutical, chemical and food industry. It is included in the list of the top 12 platform chemicals by the US Department of Energy.

For the successful bio-based production of a target molecule, several criteria have to be reached, mainly cheap substrates and fermentative conditions on one hand, and high titers, production rates, and yield on the other. In order to address these issues, different microorganisms in different conditions have been tested over the last decade to optimize the production potential. Some of the potential industrial hosts are presented in “Table 1”.

Table 1. Potential microbial candidate strains for succinic acid production through fermentation.

Bacterial Strain	Reached Titer (g·L ^{−1})	Substrate	Reference
<i>Anaerobiospirillum succiniciproducens</i>	100	Sorbitol, glycerol	[32]
<i>Actinobacillus succinogenes</i>	50	Corn stover	[33]
<i>A. succinogenes</i>	109	Glucose	[4]
<i>Corynebacterium glutamicum</i>	113	Glucose	[34]
<i>B. succiniciproducens</i>	17	<i>Arundo donax</i> hydrolysate	[3]
<i>B. succiniciproducens</i>	5.21	Crude glycerol	[35]
<i>B. succiniciproducens</i>	26	Xylose	[36]

Cimini et al. [22] reports that *Arundo donax* was used as feedstock for biosynthesis of succinic acid after preliminary hydrolysis of the raw material. Spent yeast cells as a nitrogen source and corn fiber as a carbon source were used by the research group of Chen [37]. In these conditions, a succinic acid yield of 67.7% from 70.3 g·L^{−1} total sugars was reached with an *A. succinogenes* strain. Later on, Liu et al. used pretreated sugarcane bagasse as substrate to obtain 18.88 g·L^{−1} succinic acid with *E. coli* BA204 strain under anaerobic fermentation [38]. These raw materials are theoretically sustainable and cost advantageous. Their limitation, however, consists in the presence of different inhibitors due to pretreatment such as furfural, acetate or 5-hydroxymethylfurfural (HMF), which can have a remarkable impact on cell growth [3,39].

B. succiniciproducens is a member of the *Pasteurellaceae* family, which was first isolated from bovine rumen juice in 2008 and described in detail by the German chemical company BASF in Ludwigshafen, Germany [40]. The presented microorganism is a prominent succinic acid producer due to its facultative anaerobic metabolism and broad substrate utilization spectrum [41].

Currently, metabolic reconstructions are widely used to simulate and analyze the metabolic potential of an organism under different environmental and genetic conditions [42,43]. Here, we utilize a systems biology approach to determine the metabolic flux

distribution and growth rate of *B. succiniciproducens*, using flux balance analysis (FBA) mathematical modeling to quantitatively predict microbial metabolism under steady-state conditions.

The goal of this work was to monitor the population growth dynamics and metabolic profile of the *B. succiniciproducens* bacterial strain under different conditions, namely different carbon sources and concentration of yeast extract. Several substrate concentrations were tested and their effect on the fermentation was assessed. Bioreactor experiments were conducted after several preliminary smaller scale tests in mineral media.

2. Materials and Methods

2.1. In Silico Simulations

In our simulations, we used the most recent metabolic reconstruction of *B. succiniciproducens*, representing the base model, which accounts for more than 60 reactions and metabolites [44]. Simulations were carried out using MATLAB (Mathworks Inc., Natick, MA, USA) and COBRA Toolbox software packages with Gurobi Optimizer (Gurobi Inc., Ann Arbor, MI, USA) [45,46]. During simulations, the substrate uptake rates were fixed as follows: glucose, xylose, glycerol to 7.7/9.24/15.06 mM gDW⁻¹ h⁻¹, respectively, based on literature data [44]. For maintaining the carbon number in the case of each substrate (glucose—6 carbon atoms, xylose—5 carbon atoms, glycerol—3 carbon atoms), well calculated fluxes were used. Two types of flux analyses were performed; firstly, substrate and oxygen uptake rate were fixed, as described above. In the second case, theoretical maximum predictions were simulated, which meant that fixing the quantity of biomass could be produced (this value was set to 0.1 h⁻¹) near the above-mentioned constraints. Regarding the environmental conditions, the oxygen uptake rate was set to zero to create anaerobic conditions. Simulations were carried out by solving a linear optimization problem (FBA, (1)) with a biologically relevant objective function, namely biomass formation:

$$\begin{aligned} \max Z &= c^T v \\ \text{subject to } S v &= 0 \\ v_{lb} &< v < v_{ub} \end{aligned} \quad (1)$$

where Z is the objective function for maximization or minimization, c is a vector of weights, presenting how each reaction marked with v contributes to the objective function, S is the stoichiometric matrix, and v_{lb} and v_{ub} mean the lower and upper bounds/limits of the fluxes. For example, the objective function can be the maximum production of biomass or a given product (organic acid) [43].

2.2. Strain

The examined strain *B. succiniciproducens* (DSM-22022) was obtained from DSMZ-German Collection of Microorganisms and Cell Cultures [1]. Cells were rehydrated in TSB (Tryptic soy broth) media, containing 17 g·L⁻¹, peptone from soy meal 3 g·L⁻¹, D (+)-glucose 2.5 g·L⁻¹, NaCl 5 g·L⁻¹, K₂HPO₄ 2.5 g·L⁻¹, pH was set to 7 (chemicals were purchased from VWR and Sigma-Aldrich, Taufkirchen, Germany). General culture maintenance was carried out at 37 °C and 130 rpm in a shaking incubator (Sartorius CERTOMAT®BS-T, Yumpu, Switzerland) for 8 h.

2.3. Microplate Experiments

Small volume population growth dynamics studies were carried out in a 96-well microplate (BRAND plates®, Taufkirchen, Germany) and set-up and absorbance was monitored on $\lambda = 595$ nm by a FLUOStar Optima (BMG Labtech GmbH, Ortenberg, Germany) microplate reader. 100 μ L total volume of media per well on microplate set-up were inoculated with the same cell density (initial OD₅₉₅ of 0.3) and population growth dynamics parameters were followed by an in situ measurement of optical density at 595 nm, without changing the total volume. Applied culture conditions were as follows: incubation at pH = 6.8 incubation temperature 37 °C, optical density at 595 nm, 40 cycles of 1800 s cycle time, 90 s shaking with 150 rpm before each measurement. Population growth dynamics

were examined on the tested three substrates: glucose, xylose and glycerol in 5-15-30-50 and 70 g·L⁻¹ concentration. Throughout the fermentations, minimal media was used with the following composition: 0.1/1 g·L⁻¹ yeast extract, 1 g·L⁻¹ NaCl, 0.2 g·L⁻¹ MgCl₂·6H₂O, 0.2 g·L⁻¹ CaCl₂·2H₂O, 3 g·L⁻¹ K₂HPO₄, 5 g·L⁻¹ (NH₄)₂SO₄ (chemicals from VWR and Sigma-Aldrich, USA).

2.4. Bioreactor Fermentation and Metabolic Profile Analysis

Conditions in the bioreactor in all experiments were 37 °C, 50 cm³·min⁻¹ CO₂ flow rate, pH = 7, and agitation speed was controlled at 150 rpm (radial impeller), using the above-described minimal medium with 50 g L⁻¹ substrate. During the fermentation carried out in the bioreactor, the pH was monitored by continuous measurements and was regulated with 1 M NaOH and 1 M HCl. In this work for fermentations, a performant Sartorius Biostat[®] (Taufkirchen, Germany) A Plus system with BioPAT[®] (Goettingen, Germany) MFCS/DA monitoring and controlling unit was used with 1 L total volume reactor vessel and 0.5 L working volume. During fermentation, the samples (2 mL) were taken every two hours for monitoring key parameters and the produced organic acids (succinic acid, acetic acid, formic acid, lactic acid) quantity through the fermentation time. The preparation of samples for high pressure liquid chromatography analysis was the following: culture samples were centrifuged for 10 min at 14,000 rpm and filtered over 0.45 µm Whatman[®] (Taufkirchen, Germany) sterile filters. Analysis of organic acids and carbohydrates was carried out using the Agilent Infinity 1260 HPLC system, equipped with diode array detection (DAD) and refractive index detector (RID), respectively. In auto-sampling mode, 20 µL samples of the filtered culture supernatants were analyzed, separated on a Coregel 87H3 column. Measurements were carried out using the following parameters: 50 °C column temperature, mobile phase was 0.008 N H₂SO₄ with 600 mL·min⁻¹ flowrate.

3. Results and Discussion

By using sustainable feedstocks, as well as minimal mediums (containing ideally inorganic salts), the production of value-added components (e.g., succinic acid) redounds to a cost-efficient solution. While this article shows the usability of different substrates, the literature reports that the origins of these can be explained. In order to contribute to the understanding of target metabolite production, we proposed the investigation of population growth dynamics and target product forming potential in different substrate conditions of the recently isolated natural succinic acid producer *B. succiniciproducens*, using in silico constraint-based metabolic models and in vitro experiments.

Constraint-based simulations were carried out for the strain under the specified conditions—glucose uptake rate set to the closest to that observed experimentally, anaerobic conditions—and the results show that, on glucose, the optimal growth rate for this strain is 0.3 h⁻¹. Throughout predictive simulations along the target reaction (biomass), there are several metabolites which are produced such as succinic acid, acetic acid, lactic acid and formic acid, the concentrations of which are determined in mM gDW⁻¹ h⁻¹. Using flux balance analysis and theoretical maximum predictions, we can see the succinic acid production differences between undetermined and fixed biomass (undetermined vs. fixed). In the case of each substrate, the predictions show a doubled succinic acid flux. Glucose and xylose reach 5.57 and 5.79 mM gDW⁻¹ h⁻¹ fluxes, respectively, compared with glycerol, which presents a lower flux with a 4.52 mM gDW⁻¹ h⁻¹ value in normal conditions (undetermined biomass flux). By setting the constraint on biomass to 0.1 value, we can see an increased succinic acid flux in each case. If pathways to biomass creation are limited, carbon fluxes are headed to organic acids that can be produced. Results are presented in Figure 1.

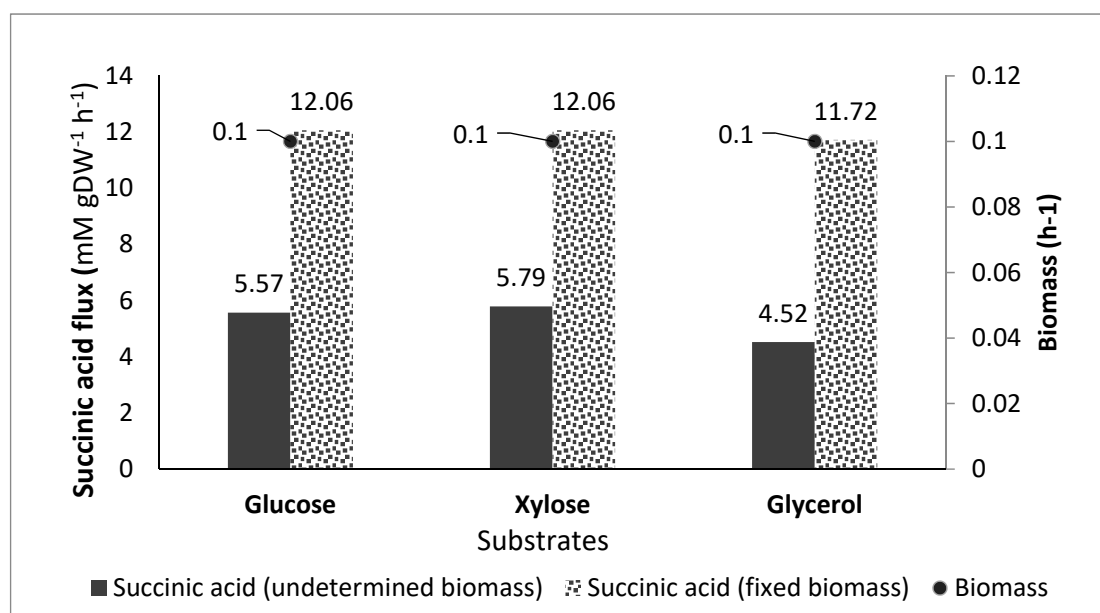


Figure 1. Succinic acid fluxes using flux balance analysis and theoretical maximum predictions (*in silico* experiments).

Thus, our goal in this phase was to assess, in small-scale cultures, the effect of different renewable substrates (glucose, xylose and glycerol) on population growth dynamics of the strain in mineral media, as well as to what extent the essential vitamin and amino acid containing yeast extract would contribute to biomass formation. Cultivation condition in this set up can be considered microaerobic due to the volume of the cultures ($100 \mu\text{L}$ culture/well) and reduced shaking during fermentation. A first set of experiments was carried out using minimal medium with different substrate ($5\text{--}70 \text{ g}\cdot\text{L}^{-1}$) and yeast extract concentrations (0.1 and $1 \text{ g}\cdot\text{L}^{-1}$). Utilization of minimal media was based on the theory that metabolic modelling is based on mathematic exchange reactions. These reactions are described in minimal conditions, where media composition has the least impact on cell activity. The impact of yeast extract and substrate concentration on cellular growth under the specified environmental conditions was assessed using a microplate reader, evaluating, in real time, the potential substrate inhibition on cell growth. Results describing microplate experiments are shown in “Figure 2”, where we can observe that *B. succiniciproducens* is able to grow even on high substrate concentrations ($70 \text{ g}\cdot\text{L}^{-1}$). The highest optical density in microtiter plate experiments was found in the case of xylose, reaching a maximum OD of 1.33, for an initial substrate concentration of $30 \text{ g}\cdot\text{L}^{-1}$ supplemented with $1 \text{ g}\cdot\text{L}^{-1}$ yeast extract. Regarding the differences between carbon sources, xylose can be considered the most effective substrate from the tested range, as cultures grown on this substrate presented the highest OD values under every condition tested. Moreover, in the case of this substrate, our culture presented the shortest adaptation period/phase under this experimental setting. Considering our results from microplate experiments, it seems that, in *B. succiniciproducens*, the initial substrate concentration did not significantly influence the population growth dynamics, but rather was controlled by the concentration of yeast extract. In the case of glycerol, in every condition tested, we observed a reduced growth potential of the strain on this hardly assimilable substrate compared to other examined substrates (glucose, xylose). In the case of this substrate, the growth-promoting effect of yeast extract is obvious; our cultures showed a maximal OD_{595} value of around 1.2, regardless of the concentration of substrate, in the case of the addition of $1 \text{ g}\cdot\text{L}^{-1}$ yeast extract. The critical inhibitory concentration for glycerol was also evaluated in microtiter plates and found to be $50 \text{ g}\cdot\text{L}^{-1}$ in our experimental setting.

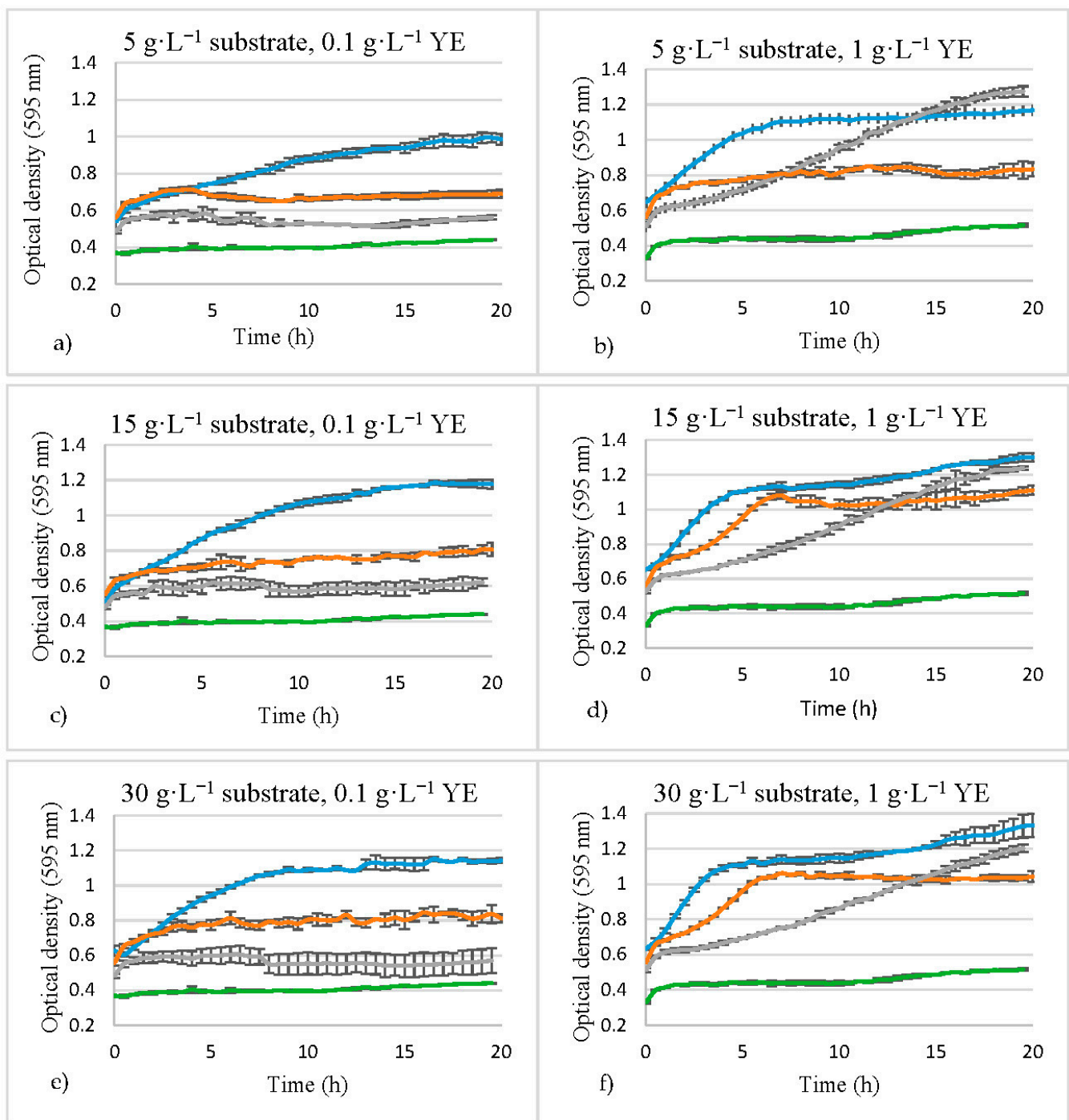


Figure 2. Cont.

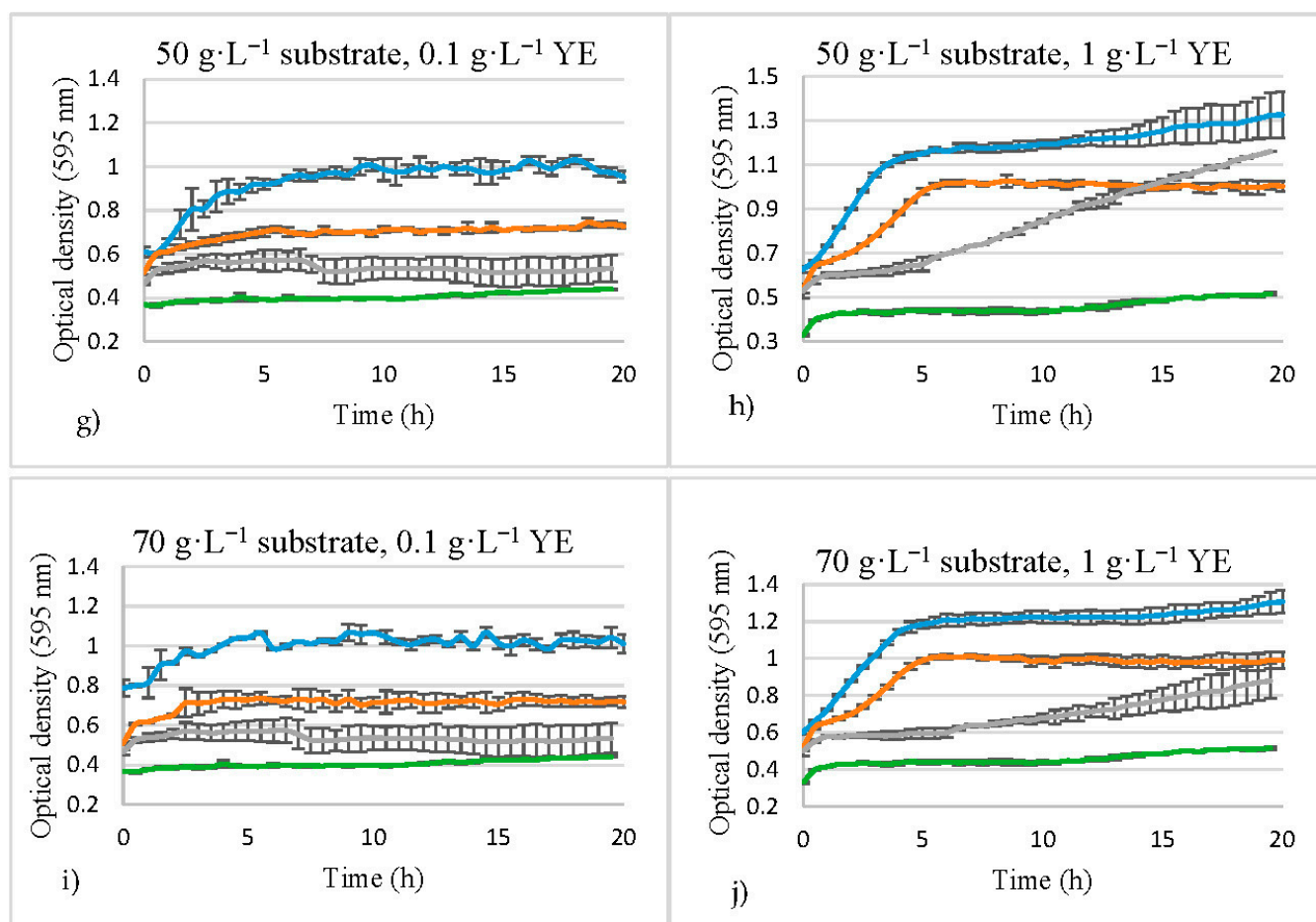


Figure 2. Effect of initial substrate and yeast extract (YE) concentration on *B. succiniciproducens* population growth dynamics in microplate experiments. Legend: orange line—glucose, blue line—xylose, grey line—glycerol, green line—control (without added bacteria). (a,c,e,g,i) shows the optical density over time with concentrations 5, 15, 30, 50, 70 g·L⁻¹ respectively on glucose, glycerol and xylose with supplemented 0.1 g·L⁻¹ YE. Subfigures (b,d,f,h,j) presents the optical density over time with concentrations 5, 15, 30, 50, 70 g·L⁻¹ respectively on glucose, glycerol and xylose with supplemented 1 g·L⁻¹ YE.

In the following sets of experiments, we proposed to examine the population growth dynamics and metabolic profile of our strain, in a scaled-up and controlled environment, under the conditions identified in the previous step of our work. Briefly, the following parameters were used for bioreactor fermentations: 50 g·L⁻¹ substrate (xylose, glucose, glycerol), 1 g·L⁻¹ yeast extract in mineral medium.

Due to the fact that *B. succiniciproducens* is capnophilic bacterium, CO₂ can be metabolized and may play a key role in the fermentation process [1]. To address this issue, fermentation experiments were performed in a bioreactor (1 L total volume) with a working volume of 0.5 L, with CO₂ sparging at a rate of 50 cm³·min⁻¹. Growth profiles of *B. succiniciproducens* in bioreactor cultures on three different substrates are presented in “Figure 3”.

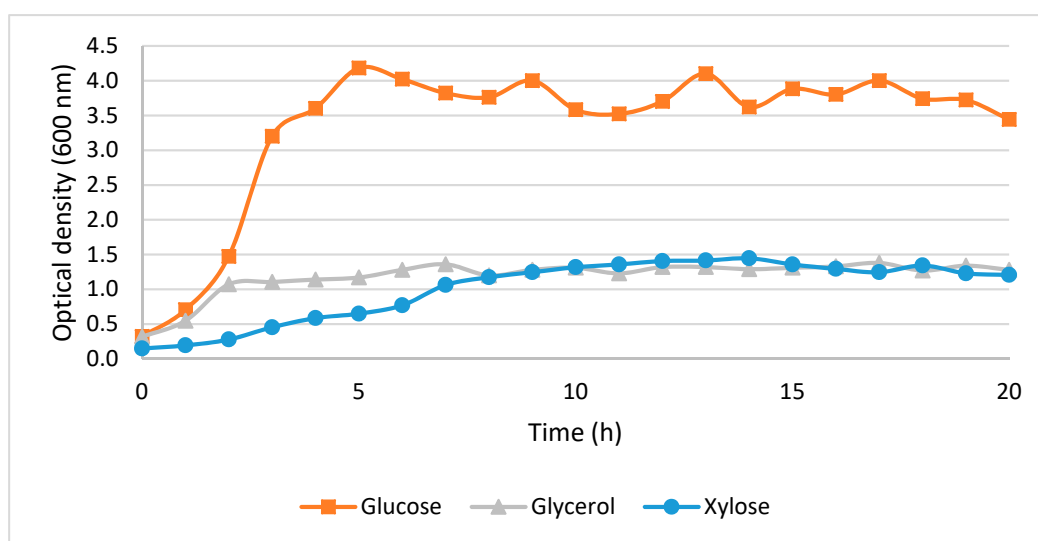


Figure 3. Population growth dynamics of *B. succiniciproducens* (strain DSMZ—22022) in bioreactor experiments, CO₂ purging in headspace (50 cm³·min^{−1}) flowrate with substrates (glucose, xylose, glycerol) in 50 g·L^{−1} concentration.

Significant differences were observed in the bioreactor setting between glucose and the other substrates, mainly faster growth and short adaptation for glucose and the reached optical density being four times higher compared to xylose or glycerol. Glycerol and xylose consumption was slower, and the highest optical density during fermentation was only 1.2 for both substrates after 20 h of fermentation. Although the two substrates might present the same effect on the culture in terms of biomass production at the end of the fermentation period, there is a significant difference in the adaptation period for the two cultures: the culture grown on xylose needed a three-times longer adaptation period compared to the glycerol-fed cultures until reaching the plateau phase of growth. A higher reduction grade in the case of glycerol, which can cause significant redox imbalance in the cell, can result in reduced growth rate in the case of this substrate, which could explain the lower biomass production through fermentation [47]. In the case of xylose, however, we observed a different behavior of our strain in the bioreactor setting compared to our small-volume experiments, the growth profile in the bioreactor culture being closer to the less assimilated glycerol-fed culture. One possible explanation could be the CO₂ atmosphere with reduced O₂ inlet, which could limit biomass formation by inhibition of NAD⁺ regeneration via the oxygen-dependent NADH oxidase step [48].

As previously reported, the wild type of the studied microorganism can naturally produce relatively high amounts of succinic acid from different carbon sources. To assess the metabolic potential of our cultures grown on different carbon sources in mineral medium, the produced metabolites (succinic acid, acetic acid, formic acid and lactic acid) were analyzed at the end of the fermentation (“Figure 4”).

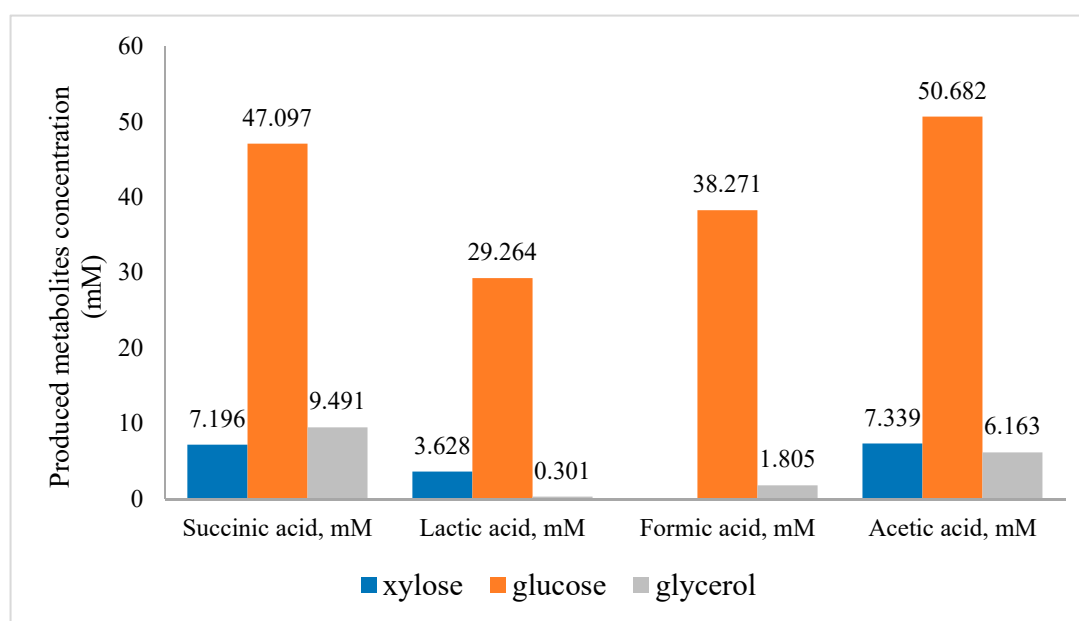


Figure 4. Batch fermentation profiles of *B. succiniciproducens* for organic acid production (succinic acid, lactic acid, formic acid, acetic acid) obtained in bioreactor for xylose, glucose and glycerol after 20 h of fermentation.

Based on the obtained data, we can conclude that the highest amount of succinic acid, 47.097 mM, was reached after 20 h of fermentation in the case of glucose, and acetic, formic and lactic acid were the major organic acid by-products. In the case of glycerol, around 79% lower succinic acid production was observed at the same time point, with a 9.491 mM concentration. In the case of xylose, the highest measured succinic acid concentration was 7.196 mM. In order to increase the production of succinic acid, it is necessary to optimize the fermentation conditions and to reduce or eliminate the by-product formation, e.g., by a metabolic engineering approach and long-term adaptation experiments.

4. Conclusions

Mathematical models describing metabolic systems are powerful tools to analyze the metabolic potential of different organisms under different environmental conditions and to decipher the possible modification necessary to design industrially important strains. The *in silico* simulation results revealed the metabolic reconstruction of *B. succiniciproducens*, even if it is not a genome-scale reconstruction, can be used to analyze the most important metabolic functions and make predictions regarding the growth and production rate of organic acids, including succinic acid as well. Bacterial behavior, in the case of the natural succinic acid producer *B. succiniciproducens* as a result of different environmental effects, was investigated based on fermentation data. The present study provides valuable information about the strain growth potential on different renewable carbon sources (glucose, xylose and glycerol), as well as the effect of additives on population growth dynamics. Our goal was to show the effect of yeast extract concentration on cellular growth, while the obtained data shows a clear difference between the two studied yeast extract concentrations.

According to the literature, *B. succiniciproducens* is a robust succinic acid producer with a wide substrate utilization spectrum, while in-depth physiological description of the species is scarce. Our results revealed the effect of substrate concentration on the population growth dynamics of the strain. Our data suggest that the strain can produce biomass over a wide range of substrate concentrations, for example, it can reach OD₅₉₅ 1.3 value on xylose (70 g·L⁻¹ substrate concentration). According to our findings, in the case of glycerol, we determined a substrate inhibition concentration of 50 g·L⁻¹ that affects growth and biomass formation of the strain. The addition of 1 g·L⁻¹ yeast extract,

compared to $0.1 \text{ g}\cdot\text{L}^{-1}$ yeast extract, had a higher positive impact over bacterial growth in all examined conditions.

According to our studies conducted in the bioreactor set up under CO_2 atmosphere, glucose was the most suitable substrate. The metabolite profile of fermentation showed the presence of metabolic by-products (acetic acid, formic acid and lactic acid) and the target product, succinic acid, was formed. Our findings suggest that lignocellulosic feedstocks (containing glucose, xylose) can be utilized as industrial substrates, and would be a cost efficient solution for the production of high added-value components, such as succinic acid [49]. The highest succinic acid concentration, $5.55 \text{ g}\cdot\text{L}^{-1}$ (47.097 mM), reached is comparable to those presented in the literature: $4.6\text{--}6.4 \text{ g}\cdot\text{L}^{-1}$ with glycerol [35], $26 \text{ g}\cdot\text{L}^{-1}$ using xylose [36], and $30 \text{ g}\cdot\text{L}^{-1}$ on lignocellulosic hydrolysate, used as main carbon sources [50].

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Ethics Approval and Consent to Participate: The manuscript it is not submitted to more than one journal for consideration. This work is original and it is not published elsewhere in any form or language. In the manuscript experiments no human subject was included for whom the participate consent should be declared.

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