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Preliminary Batch Tests for Succinic Acid Production and Co-Products through Fermentation by *Actinobacillus Succinogenes*

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Succinic acid is one of the most used building blocks in different production chains. Conventionally, this carboxylic acid is produced through petrochemical routes, but can be also produced via biological routes. Many microorganisms, such as *Actinobacillus succinogenes*, synthesized succinic acid as an intermediate of many biochemical pathways, as the Krebs cycle. Nowadays, the industrial production of bio-succinic acid has been limited due to different critical issues, like the low productivity and yield and downstreaming processes. To improve the bio-based production, the operative conditions of the fermentation process needed to be optimized especially when agro-industrial residues were used as sugars feedstock. To this aim, in this study fermentation batch tests were performed by using different concentrations of *A. succinogenes* at a given glucose concentration. The results suggested that glucose consumption and succinic acid production were not directly influenced by the initial bacterial concentration respect to glucose concentration. But the best results were obtained at the ratio of bacteria:glucose equal to 1:1 and further tests will be necessary to implement succinic acid yield and productivity.

1. Introduction

Sustainable bioprocesses and green chemicals will be necessary for the transition to bio-based production. One of the most interesting green chemicals is succinic acid (SA) for its great potential in bio-based economy as precursor of bioplastics/biopolymers. Although SA can be produced through bio-based processes it is still produced by a petrochemical route by using fossil fuel sources at a price of 2 $/kg while bio-based production resulted at uncompetitive price of 2.6 – 4.5 $/kg (Morales et al. 2016) with consequence on economy and on environmental problems (Mancini et al., 2022).
Bio-based succinic acid can be produced by many microorganisms as intermediate and end-product of fermentative biochemical pathways, including the Krebs cycle. The bacteria *Actinobacillus succinogenes* was one of the most promising microorganisms to produce bio-based succinic acid; in fact, it could utilize a wide range of sugars, in particular glucose till to 158 g/l (Lin et al., 2008), and this characteristic made it interesting for use on a commercial scale (Yang et al., 2020). The succinic acid production by the strain *Actinobacillus succinogenes* occurred mainly under anaerobic conditions at 37 °C by using principally glucose or fermentative sugars from biomass and agro-industrial residues. This strain is an anaerobic facultative so it can grow under oxidative conditions, but in that conditions unwanted by-products as lactic acid can be produced that decreased succinic acid concentration.
Some works were published on different fermentation strategies, batch tests on succinic acid production starting from single and mixed sugars (Molino et al., 2020), fed batch tests (Hoefel et al., 2012) that were the most common approaches; furthermore, continuous succinic acid production by immobilized cells was developed (Ercole et al., 2021). Bio-based succinic acid production has been also developed at industrial scale but there are still critical issues such as the low productivity and yield, and the high costs for the

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recovery and purification to obtain a high purity bio-succinic acid. One of the most critical issues on succinic acid production have been addressed when agro-industrial wastes were used, especially lignocellulosic biomass residues as feedstock. Fermentative processes can be influenced by several parameters (like pH, temperature, inoculum and starting sugars concentration) but in particular the composition of hydrolysates obtained from lignocellulosic biomasses can induce the presence of unwanted compounds that adversely affect the process.
Furthermore, the concentration between inoculum and sugars is also one of the important parameters in the fermentation process as
the microorganism's growth can be subject to substrate inhibition due to amounts of glucose that can be too high relative to the strain concentration.
In this study, batch tests on succinic acid production were performed at different concentrations of bacterial strain *Actinobacillus succinogenes* at a given glucose concentration to evaluate the effect of inoculum size on glucose consumption, succinic acid production and by-products., Multiple samples were collected at different times to assess the microbial growth by means of a spectrophotometer at 600 nm for optical density measurements and organic acids concentration by uHPLC.

2. Materials and methods

2.1 Materials and chemicals

The strain *Actinobacillus succinogenes* 130 Z was bought from the CCUG (Culture Collection University Of Gothenburg) as active freeze-dried pellets. The Tryptic Soy Broth (TBS) was used as growth medium. TBS was composed of casein peptone (8.5 g/l), soia peptone (1.5 g/l), NaCl (2.5 g/l), K_2HPO_4 (1.25 g/l) and glucose (1.25 g/l). The broth was sterilized at 121 °C for 10 min before the use. For the carbohydrates and organic acids quantification uHPLC/LC-MS grade reagents were used. Analytical standards (monosaccharides and organic acids kit) were used for analytical quantification.

2.2 Batch tests on *succinic acid production*

A. succinogenes freeze-dried pellet was weighted and transferred into the TBS medium and gently shaken to allow uniform distribution. The experimentation was carried out on batch tests at initial glucose concentration of 1.25 g/l and at different concentrations of bacterial strain *Actinobacillus succinogenes* (test 1: 0.38 g/l) and (test 2: 1.36 g/l) (Figure 1). Growth of the strain, glucose consumption and organic acids concentration were monitored.

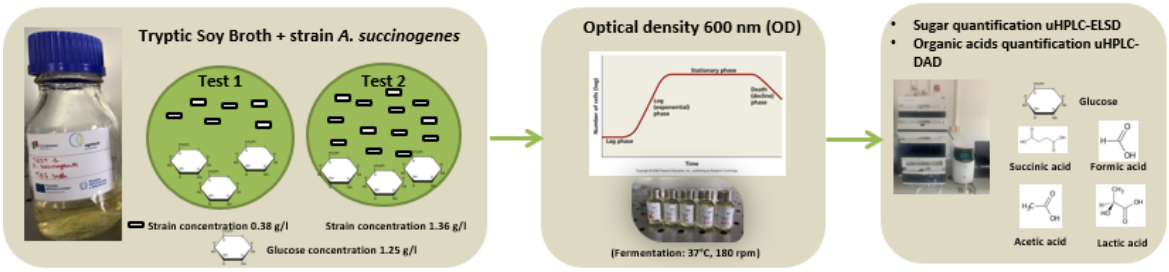

Figure 1: Experimental set-up

Each sample was transferred in a temperature-controlled shaker at 37 °C, stirring at 180 rpm, in the dark under not strictly anaerobic conditions for the growth of *A. succinogenes*. The growth of the strain was monitored by measuring the absorbance at 600 nm by a dual beam spectrophotometer. Strain concentration (g/l) was calculated by the following equation that was elaborated at OD value at 600 nm of specific quantity of the strain (dry weight):

A. succinogenes strain concentration (g/l) = 0.079x+0.078

2.3 Quali-quantitative analysis of carbohydrates and organic acids

For sugar analysis, the uHPLC 1290 Infinity II instrument with ELSD (Evaporative Light Scattering, model 1260 Infinity II) detector was used following the method Agilent 5991-8984EN. The chromatographic column HILIC-Z (2.1 x 100 mm, 2.7 µm) was used at 80 °C to quantify glucose. Ammonium Acetate (0.1 M) and acetonitrile were used as mobile phase at a gradient of 95-80% in 12 minutes at flow rate of 0.4 ml/min. The

ELSD detector was used for the identification of sugars at the following parameters: evaporation 60 °C, nebulization 30 °C, 3.6 bar (1.5 SLM), 30 Hz (signal frequency).
For the analysis of carboxylic acids, the uHPLC 1290 Infinity II instrument, was used following the method Agilent 5989-5672EN. Samples and standards were injected at a volume of 20 µl by using a Hi-Plex-H column (7.7 x 300 mm, 8 µm) at 50 °C. Isocratic gradient of 100 % H_2SO_4 0.01 M was used at a flow rate of 0.6 ml/min. Detection of carboxylic acids was done at the wavelength of 210 nm by diode array detector (DAD).

2.4 Succinic acid performance

Glucose consumption was calculated as:

$$\text{Glucose consumption (\%)} = (C0 - Ct)/C0 * 100$$

Where C0: Initial concentration of glucose, Ct was the concentration of glucose at a fixed time.

Succinic acid yield was calculated as:

$$\text{SA yield} = g\ (SA)/\ g\ (\text{glucose initial concentration})$$

Succinic Selectivity was calculated as following:

$$\text{Selectivity g/g} = g\ SA/(g\ SA)+(g\ AC+ g\ AF)$$

3. Results and discussion

In this work two different concentrations of A. succinogenes were tested on batch conditions to evaluate the effect of different initial strain concentration on succinic acid production performance. To this aim, a synthetic growth medium, the tryptic soy broth (TBS), was used at fixed glucose concentration (1.25 g/l). The choice of this culture medium was due to its composition which provided all essential nutrients for the growth of the strain and the fact that it was also used in previous studies in which *A. succinogenes* was tested to produce succinic acid (Lin et al., 2008; Van Heerden & Nicol, 2013). This preliminary step turned out to be fundamental to better understand the optimal initial sugar-strain concentration ratio before to move towards the use of more complex starting feedstocks such as agro-industrial and/or lignocellulosic matrices.
Bacterial growth was monitored till to 48 hours, but the maximum concentration of the strain was obtained at 24 hours at the low strain concentration (test 1: 0.38 g/l) and at 8 hours at the high strain concentration (test 2: 1.36 g/l). In table 1 the final concentration (48 h) and the maximum concentration of strain were reported.

Table 1: A. succinogenes concentrations (mg/l) Test No 1 and Test No 2

	Test 1	Test 2
Initial Concentration (C0) strain (mg/l)	380	1360
Final concentration strain (mg/l)	1800 (48 h)	2290 (48 h)
Max. concentration growth strain (mg/l)	3000 (24 h)	4710 (8 h)

The strain of test 1 reached at 24 hours the highest concentration equal to 3000 mg/l while at the end of the growth the concentration decreased to 1800 mg/l. Test 2 reached the highest concentration 4710 mg/l at 8 hours *(figure 2)* and the concentration decreased at 24 h and 48 h by reaching 3650 mg/l and 2290 mg/l respectively. The results obtained showed that the greatest growth occurred in the first 8-24 hours reaching a plateau or decrease in strain concentration thereafter for the two concentrations tested. The trend of the growth resulted slightly difference between test 1 and test 2. Although the strain reached the highest concentration in test 2, a better growth rate was observed in test 1 in which the highest concentration at 24 hours was 7.8 times higher than the initial concentration while in test 2 the highest concentration at 8 hours was 3.5 times higher than the initial concentration. The different progress of biomass growth was mainly due to the relationship between inoculum concentration and glucose concentration. In test 1 the ratio between inoculum and glucose was 0.3 while in test 2 the ratio was 1.1. In test 2, the biomass concentration reached high point at 8 hours and rapidly decreases until 48 hours. In contrast, this behavior was less evident in test 1 where the strain concentration decreased after 24 hours. This growth trend can be explained as the limiting effect of the glucose concentration in relation to the initial strain concentration as reported in the kinetics study of Corona-González et al., 2008. Corona-González et al., 2008 demonstrated that high glucose concentration

(> 20.4 g/l) affected biomass concentration that reached the maximum concentration (4.9 g/l) at 20 hours but after that time of growth the *A. succinogenes* concentration rapidly decreased.
In figure 2, glucose concentration and organic acids concentration were shown together with strain concentration.

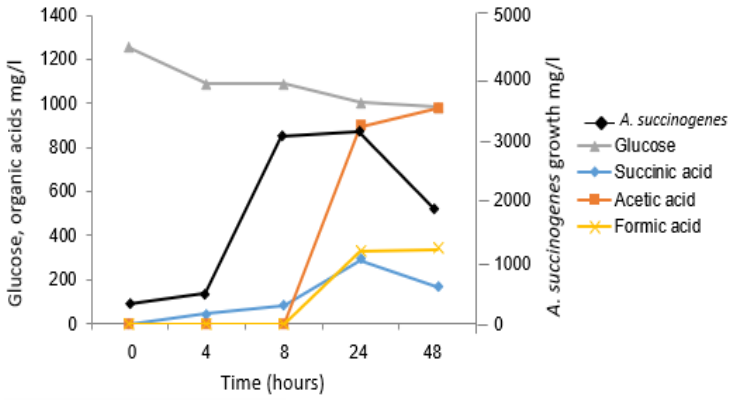

Figure 2: TEST 1: 0.38 g/l strain concentration: Actinobacillus succinogenes growth curves (line black), glucose concentration (grey line), succinic acid concentration (blue line), formic acid concentration (yellow line), acetic acid (orange line).

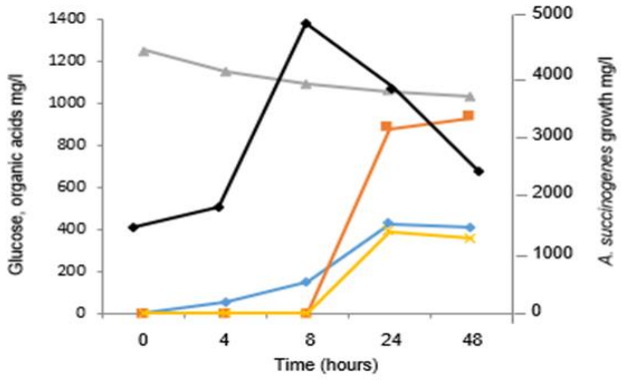

Figure 3: TEST 2: 1.36 g/l strain concentration: Actinobacillus succinogenes growth curves (line black), glucose concentration (grey line), succinic acid concentration (blue line), formic acid concentration (yellow line), acetic acid (orange line).

The glucose concentration decreased to 986 mg/l in test 1 and 1032 mg/l in test 2 with a final consumption at 48 hours of 21.1 % in test 1 and 17.4 % in test 2. Glucose consumption was observed around 19.7% after 24 hours in test1, while in test 2 the glucose consumption was 15.7% at 24 hours. On average, 20% glucose consumption was found with no significant differences between the two tests ($p>0.05$).
As shown in figures 1 and 2, the peak of organic acids concentration was observed at 24 hours for all tests. The concentration of succinic acid reached 288.6 mg/l and 428.3 mg/l respectively in test 1 and 2. The production of by-products acetic and formic acids was higher than succinic acid. Acetic acid was the most abundant by-product, and its concentration was equal to 894.8 mg/l at 24 hour (Test 1) and 879.1 mg/l at 24 hour (test 2). Selectivity calculation demonstrated for both tests that the concentration of by-products was higher than succinic acid, but the ratio between succinic acid and by-products become better in test 2. Also, succinic acid yield was slightly increased in test 2 (0.24 g/g) respect to test 1 (0.19 g/g). The productivity of succinic acid resulted lower in test 1 (12 mg/l/h) than the value in test 2 (18 mg/l/h).

Table 2: TEST 1-2-Glucose consumption, SA, AA and FA concentration (mg/l), SA yield (g/g) and selectivity (g/g)

Test	N°1		N°2	
	24 h	48h	24h	48h
Glucose consumption 24 h (%)	19.7	21.1	15.7	17.4
SA Concentration (mg/l)	288.6	169.7	428.3	408.9
AA Concentration (mg/l)	894.8	978.6	879.1	931.1
FA Concentration (mg/l)	331.4	339.1	389.1	358.4
SA yield (g/g)	0.23	-	0.34	-
SA productivity (mg/l/h)	12		18	
Selectivity SA/AA (g/g)*	0.19	0.11	0.25	0.24

The results on the low productivity of succinic acid were in line with those obtained by the authors Salma et al., 2021, that reached succinic acid productivity <12 mg/l/h in tests under micro-aeration conditions, highlighting how maintaining anaerobic conditions was an essential factor to improve the performance of the succinic acid production process.

The results obtained in this study were achieved under near optimal conditions, such as utilizing a synthetic broth with a standard composition suitable for bacterial growth. However, bio-based production of succinic acid presents some several issues that need to be fixed before to start industrial-scale production. Firstly, the choice of lignocellulosic biomasses as abundance, low cost and high carbohydrates content feedstock has been one of the most critical due to their complex structure and high mechanical strength. In lignocellulosic biomasses, sugars were not directly available for the microorganisms, and pre-treatments were needed to make them available to the fermentation process. But these preliminary pre-treatments processes could be expensive and could generate some inhibitory compounds for microorganisms, such as furans, phenolic compounds and acids (Xu et al., 2022). Furan derivatives appeared to have a strong toxic effect on *A. succinogenes*. In fact, Dessie et al. (2019) observed that, even if present in low concentrations (3 g/l), hydroxymethylfurfural and furfural completely inhibited the growth of the strain. Among the acids, the most common was the acetic acid, that could also be produced by the strain as end-product during fermentation process. In this study, a high acetic acid concentration was produced (978.6 and 931.1 mg/l in test 1 and test 2, respectively, at 48h) than succinic acid under the conditions tested, which did not differ from the initial inoculum concentration but was certainly due to the operating conditions in micro-aeration. According to the study of Li et al. (2010), the strain *A. succinogenes* at a fixed initial concentration (0.03 g/l) could tolerate up to 10 g/l of acetic acid, with a slowdown in growth over 12 hours and an inhibition rate of 86% was observed than control without acetic acid. In fact, when the concentration of acetic might be critical, a toxic effect was revealed such as the block of the functions of many key enzymes. Therefore, using a more complex medium that better represents the composition of agro-industrial residues or lignocellulosic biomass could present various critical issues, such as observing an inhibitory effect on the growth of the strain and consequently low yield of succinic acid. Another critical aspect of the production of bio-succinic acid was the downstream process for the recovery and purification of the final product. Many techniques were tested, however, some issues still need to be addressed such as the use of large number of chemicals to fulfil the process, that was the biggest problem in reactive extraction. The precipitation, that was one of the most widely used methods to recovery succinic acid, resulted in the production of large quantities of solid waste, such as gypsum. Even more advanced techniques that used membranes, such as electrodialysis, was not yet applicable on an industrial scale for fouling's troubles. The effects of these issues were low yields and purity of the final product, and high production costs, which limited industrial scale application of these methods. The techno-economic analysis performed by Li & Mupondwa (2021) confirmed that the choice of starting biomass and the downstream process were two critical aspects. In fact, the simulation of the industrial scale production of bio-succinic acid demonstrated that the cost of the raw material accounted for 31.5% of the operating costs of which only the initial feedstock (corn syrup) was equal to 31.2 % of the total operating costs. Instead, the downstream purification process, made up 83% of utility cost.

4. Conclusions

The results obtained in this paper demonstrated that the bacterial and sugar concentrations tested did not affect the overall performance of the process because comparable glucose consumption was achieved among the different initial strain concentrations. However, after these preliminary results further tests will be necessary to better investigate other processes parameters before to study the potential use of more complex feedstock as lignocellulosic biomasses.

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