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Preliminary Assessment on *Actinobacillus Succinogenes* Growth and Succinic Acid Production for Bioplastics

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Severe plastics pollution derived from the wide use and the past incorrect dispersion of petroleum-based plastics. The development of bioplastics/biopolymers with the same chemical/physical characteristics of conventional plastics can be a solution to the problems of their degradability. Polybutylene succinate was among the most investigated biopolymers for these characteristics that can be produced from bio-based succinic acid and butan-1,4-diol. Succinic acid can be produced through fermentation process using microorganisms as *Actinobacillus succinogenes* that were able to utilise glucose from several sources as lignocellulosic biomasses. Lignocellulosic biomass can be ideal candidates for glucose supply but the processes to release fermentable sugars can produce inhibitors (acids and furans) of the biological processes for succinic acid production. The aim of this paper was to evaluate the growth of *A. succinogenes* for the production of succinic acid under acetic acid and furfural at different concentration on inoculum with pre-adaptation and without adaptation. The results highlighted that pre-inoculum adaptation was essential for the growth of the strain and succinic acid production that decreased strongly under the synergistic effect of acetic acid and furfural in the broth (63% inhibition rate of growth) respect to inoculum with pre-inoculum adaptation (29% inhibition rate of growth).

1. Introduction

The petroleum-based plastics have been one of the most widely used materials for their low price and the durability. In 2016 from 9 to 23 million metric tons of plastics polluted lakes, rivers and oceans, and 13 to 25 million metric tons contaminated the terrestrial environment (Borrelle et al., 2020). The disposal of plastics has been a critical issue. since the plastics were designed to be durable and persist in the environment for long time. Furthermore, the life cycle of plastic resulted to impact strongly on CO_2 emissions (1.7 Gt CO_2/year) respect to other sector as global aviation industry (0.4 Gt CO_2/year) (Jiao et al., 2024).
To reduce the environmental impact of fossil-based plastics, biodegradable and compostable polymers have recently been tested and developed as polyhydroxyalkanoate (PHAs), polylactic acid (PLA), polybutylene succinate (PBS). Economic interest in biopolymers as substitutes for fossil plastics was growing exponentially, in fact global bioplastics production was set to increase significantly from around 2.18 million tons in 2023 to approximately 7.43 million tons in 2028 (European bioplastics, 2023).
Among biopolymers, PBS was considered one of the most interesting for his mechanical endurance, ductility, and toughness (Barletta et al., 2022). PBS was principally produced from condensation polymerization of succinic acid and butan-1,4-diol. Since, succinic acid (SA) has been one of the principal chemical building blocks to produce PBS, biotechnological routes were developed to produce SA by using microbial fermentation. The microorganisms can use most sugars (principally glucose) derived also from renewable

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sources as agro-industrial by-products. The anaerobic facultative strain *Actinobacillus succinogenes* was one of the most tested microorganisms for the capability of using different sugars sources (monosaccharides and disaccharides) and to produce large quantities of succinic acid (Yang et al., 2020).
In this context, the possibility of utilizing a lignocellulosic biomass as feedstock was interesting, because these raw materials were rich in glucose and xylose, and they were available in large quantities. However, the complex matrix of lignocellulosic biomass needed pre-treatments to convert polysaccharides into fermentable sugars. The non-specificity of the pre-treatment processes and the harsh operational conditions of some treatments as steam explosion (such as the high temperatures and pressure) leaded to the formation of unwanted compounds, capable of inhibiting the growth and consequently the productivity of the microorganisms. These inhibitors can be divided into three main groups: organic acids (acetic acid, lactic acid, formic acid, levulinic acid), furan derivates (5-hydroxymethylfurfural and furfurals), and phenolic compounds (Basak et al., 2020).
In this paper, the growth of *A. succinogenes* and the production of succinic acid were assessed, under optimal conditions and in presence of acetic acid and the mixture acetic acid and furfural, two of the most representative inhibitors that were formed during pre-treatment processes of lignocellulosic biomasses. Furthermore, the effect of these inhibitors was evaluated on growth of the strain and succinic acid production on the adapted and not-adapted strain.

2. Material and methods

The freeze-dried pellets of the strain *Actinobacillus succinogenes* 130Z (CCUG-43843) was supplied by Culture Collection University of Gothenburg (CCUG, Gothenburg, Sweden). The strain was grown in Tryptic Soy Broth (TSB) (22092-500G Sigma) at 37 °C and 180 rpm in a stirring incubator (New Brunswick Scientific Excella E24 Incubator Shaker Series) in the dark (Molino et al*.,* 2020).
The effect of sugars and potential inhibitors were directly tested on *A. succinogenes* without pre-inoculum adaptation and in tests after inoculum adaptation. The growth of *A. succinogenes* without adaptation was tested at a concentration of glucose equal to 229 mg/l, glucose and xylose (229 mg/l and 114 mg/l), and acetic acid and furfural equal to 14 mg/l and 6 mg/l (Table 1).

Table 1: -(+)-glucose, D-(+)-xylose, acetic acid and furfural concentrations at the start of fermentation experiment without pre-inoculum adaptation

	Concentration (mg/l)			
	CTL-G	CTL-G/X	IN-AA	IN-AA/F
A. succinogenes	300	300	300	300
D-(+)-Glucose	229	229	229	229
D-(+)-Xylose	0	114	114	114
Acetic acid	0	0	14	14
Furfural	0	0	0	6

Inoculum adaptation was performed in two subcultures (Sub1 and Sub2) that were grown at the same conditions, in 150 mL sealed anaerobic bottle containing 120 mL medium, at 37°C, 180 rpm in the dark, at two different glucose concentrations of 100 mg/l and 1250 mg/l respectively. In table 2, the concentrations of strain, sugars and potential inhibitors after inoculum adaptation were reported. A control (CTL) was prepared to compare the growth of the strain under favorable conditions respect to the presence of potential inhibitors, acetic acid (AA) and AA with furfural at low (L) and high concentration (H). *A. succinogenes* was inoculated in each test at the ratio 25% (v/v).

Table 2: D-(+)-glucose, D-(+)-xylose, acetic acid and furfural concentrations at the start of fermentation experiment.

	Concentration (mg/l)							
	CTL-G-L	CTL-G/X-L	IN-AA-L	IN-AA/F-L	CTL-G-H	CTL-G/X-H	IN-AA-H	IN-AA/F-H
A. succinogenes	370	370	370	370	1100	1100	1100	1100
D-(+)-Glucose	367	367	367	367	1130	1130	1130	1130
D-(+)-Xylose	0	171	171	171	0	428	428	428
Acetic acid	0	0	21	21	0	0	52.5	52.5
Furfural	0	0	0	6	0	0	0	15

The bacterial growth was monitored by measuring the optical density (OD) at a wavelength of 600 nm by mean of a dual beam spectrophotometer (Perkin-Elmer Lambda 365). A calibration curve was built to estimate the concentration of the strain (equation 1), by using OD values at known concentrations of the strain:

Actinobacillus succinogenes concentration (g/l) = 12.54*OD 660 value – 0.9708 (1)

After the spectrophotometric analysis, the samples were centrifuged for 10 minutes at 4 °C and 13000 rpm to separate the bacteria form the broth. The broth was filtered by a syringe filter of nylon (0.20 µm of porosity) and then it was stored at -20 °C for the following analysis.
The quantification of SA was carried out by ultra-high performance liquid chromatography, u-HPLC 1290 Infinity II (Agilent Technologies Inc., Santa Clara, USA) using aa Diode Array Detector (DAD) at a wavelength of 210 nm. The Hi-Plex H Column, 7.7 x 300 mm, 8 µm (p/n 1170-6830 Agilent) was used to separate organic acids following the chromatographic conditions reported in the method Agilent 5991-8984EN. Diluted analytical standards of organic acids (47264 Supelco) were prepared for identification and quantification.
All tests were carried out in triplicate, the Data Analysis ToolPack (Microsoft Excel 365, Microsoft Corp., USA) was used to calculate the standard deviation. IBM SPSS Statistics v26® was used for ANOVA (One way analysis of variance) statistical analysis.

3. Results and discussion

The tests were designed to evaluate the effects of two of the most common inhibitors derived from the pretreatments of the lignocellulosic biomass: acetic acid and furfural. Their effects were evaluated firstly on *A. succinogenes* strain without a pre-adaptation (Figure 1). *A. succinogenes* without a pre-adaptation reached the highest concentration (3.0 ± 0.2 g/l) after 72 hours starting from the initial strain concentration of 0.3 g/l without any difference between the test with glucose (229 mg/l), and with glucose and xylose (229 mg/l and 114 mg/l) ($p>0.05$) (Figure 1). The effect of inhibitors was more evident after 48 hours for AA in IN-AA and the mixture AA and furfural (IN-AA/F) when the strain concentrations reached the values equal to 1.83 ± 0.1 g/l and 2.24 ± 0.3 g/l respectively.

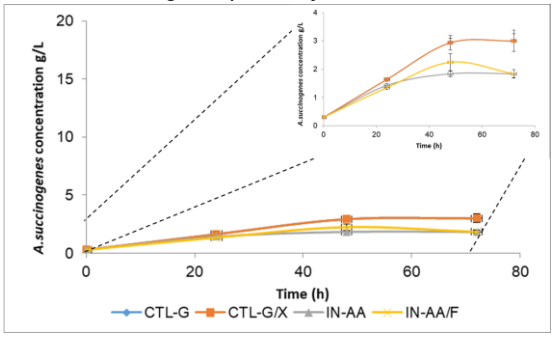

Figure 1: growth curve of A. succinogenes (g/l) in tests without pre-inoculum adaptation. Error bars indicates the standard deviation.

For tests with inoculum pre-adaptation, the same initial concentration of strain equal to 0.17 g/l was tested at two glucose concentrations 0.10 g/l (Sub1) and 1.25 g/l (Sub2) for 144 hours. At 24 hours, the strain concentration was similar in the two tests (1.35 ± 0.01 g/l (Sub1) vs 1.31 ± 0.04 g/l (Sub2) respectively). At the final growth state (144 h), *A. succinogenes* reached the highest concentration of 4.85 ± 0.02 g/l in Sub2 when the starting concentration of glucose was 1.25 g/l. In Sub 1 the strain reached the concentration equal to 1.90 ± 0.03 g/l.
After inoculum adaptation, the tests on the effect of inhibitors were started at an initial concentration of *A. succinogenes* equal to 0.37 g/l (Test L) and 1.1 g/l (Tests H) (Figure 2). Given the response of the strain without an adaptation phase, it was decided to increase the concentrations of all compounds including AA and furfural and to test them at a low and a high concentration (Figure 2) on inoculum after per-adaptation. In all the analyzed samples, the best concentration value was recorded after 144 hours of fermentation.
In the tests with low concentrations of sugars (367 mg/l glucose and 171 mg/l xylose) (figure 2A), the growth of the strain was similar in the tests with glucose and with the mixture of glucose-xylose: 10.5 ± 0.3 (CTL-G-L) and 10.7 ± 0.0 g/l (CTL-G/X-L), respectively.

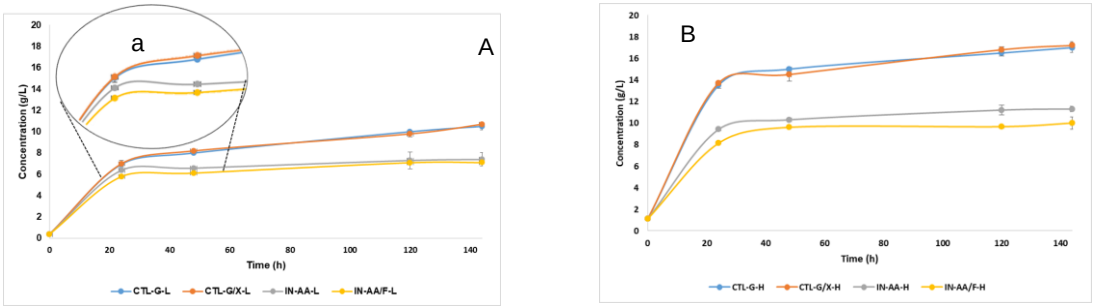

Figure 2: growth curve of A. succinogenes (g/l) in tests with low (A) and high (B) concentrations of sugars and inhibitors. Error bars indicates the standard deviation.

When acetic acid (AA) and the mixture of AA (21 mg/l) and furfural (6 mg/l) were used, a similar growth trend was observed, and the concentration of the strain decreased till to 7.4 ± 0.6 (CTL-AA-L) and 7.1 ± 0.1 g/l (CTL-AA/F-L) at the end of experimentation (144h). Under the effect of the AA and the mixture AA and furfural, the growth of *A. succinogenes* started to decrease after 24 hours respect to the control (CTL-G-L) (figure 2a).
In the tests with high concentrations of sugars and inhibitors (Figure 2b), *A. succinogenes* reached the concentration of 17.0 ± 0.4 g/l (CTL-G-H) when glucose concentration was 1130 mg/l, and 17.2 ± 0.3 g/l (CTL-G/X-H) when glucose (1130 mg/l) and xylose (428 mg/l) were used. The effect of the mixture AA and furfural was already detected at 24 hours and stronger than the effect of AA. For a better evaluation of the effect of acetic acid and furfural on *A. succinogenes* strain, the inhibition rate was calculated for all tests (table 3). Strain growth decreased compared to the control in the presence of acetic acid and the acetic acid and furfural mixture at all concentrations tested for both the pre-adapted strain and the strain without pre-adaptation. Although the concentration of acetic acid was at a low concentration (14 mg/l) in the test of the strain without an adaptation phase (table 3a), the highest inhibition rate was detected (38.7% at 72 hours). While the inhibition rate was equal 33.5% at 144 hours on inoculum after pre-adaptation when 52.5 mg/l of acetic acid was used.

Table 3: percentage of growth inhibition of A. succinogenes (%): A) test without inoculum adaptation, B) test with inoculum adaptation. These values were calculated compared to the control.

Inhibition growth (%) (A)		
Time (h)	IN-AA	IN-AA/F
24	13.0	20.6
48	37.4	37.4
72	38.7	63.1
144	-	-

Inhibition growth (%) (B)				
Time (h)	IN-AA-L	IN-AA/F-L	IN-AA-H	IN-AA/F-H
24	8.1	16.6	30.4	39.8
48	17.8	23.6	31.3	36.0
120	27	29.1	32.1	41.5
144	29.5	32.4	33.5	41.2

It was noted that the inhibition effect of acetic acid alone was slightly lower than the mixture with furfural on the strain without adaptation. In co-presence of acetic acid and furfural at the concentration of 14 mg/l and 6 mg/l, the inhibition rate was equal to 63.1 % on *A. succinogenes* without adaptation. After strain adaptation, the most evident difference between the effect of acetic acid and the mixture was observed at 24 hours in the test at the low concentrations (8.1% IN-AA-L *vs* 16.6% IN-AA/F-L). The highest effect of the mixture AA and furfural was observed at 120 hours (41.5 % inhibition rate) when the high concentration of the inhibitors was used on the adapted strain. The differences found on growth between the strain without adaptation and with adaptation were in line with the findings of Escanciano et al. (2022). The authors (Escansiano et al., 2022) observed that the inoculum without adaptation grew 1.4 times less than the inoculum with adaptation in favorable conditions and in the absence of inhibitors, while in this work the concentration of the strain without adaptation was approximately 3.3 times lower than the inoculum with adaptation. The effect of furfural on *A. succinogenes* growth was also detected by Xu et al. (2015). The authors detected a decrease of OD600 value of 50% respect to control at a furfural concentration of 2 g/l. The inhibitory effects of furans derivates and weak acids (acetic acid) was also investigated by Van der Maas et al. (2021) on the growth of *Bacillus subtilis*, that found a reduction of 29% of strain concentration at the concentration of acetic acid equal to 2 g/l, with. Furthermore, in our study a strong inhibitory effect of the mixture of furfural and acetic acid was observed at both low and high concentrations. This finding was like the data detected by Franden et al. (2013), that

observed an increased growth inhibition (31%) on *Zymomonas mobilis* when acetic acid was in combination with furan than the inhibition caused by acetic acid alone (23%).

a) b)

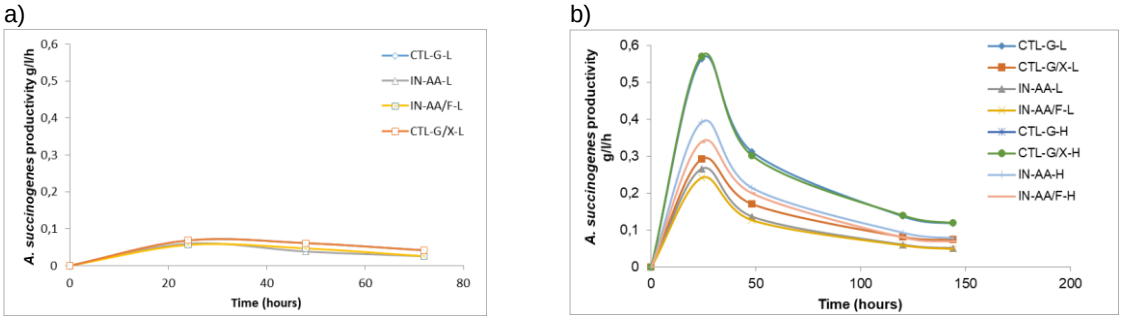

Figure 3: A. succinogenes productivity (g/l/h): a) strain without adaptation; b) strain with adaptation

Strain productivity was markedly lower in the case of the inoculum without adaptation (0.07 g/l/h at 24 hours)(Figure 3a), and decreased to 0.06 g/l/h in the presence of the inhibitors. In the tests with the adapted inoculum, the highest productivity was obtained in the CTL-G-H and CTL-G/X-H tests with a value of 0.6 g/l/h (10 times higher than the value of the strain without adaptation). At the high concentrations of the inhibitors, it could be observed that the productivity of the strain decreased to 0.4 g/l/h (IN-AA-H) and 0.3 g/l/h (IN-AA/F-H).

*Table 3: SA concentration (mg/l) after 24h in all tests (*without adaptation).*

				Succinic acid concentration mg/l							
CTL G*	CTL G/X*	IN AA*	IN AA/F*	CTL G-L	CTL G/X-L	IN AA-L	IN AA/F-L	CTL G-H	CTL G/X-H	IN AAH	IN AA/F-H
62	78	58	41	108	102	74	50	384	390	277	160

The maximum succinic acid concentration was reached when the high concentration of sugars was used 390 ± 7 mg/l (CTL-G/X-H) (Table 3). SA concentration dropped in the samples with inhibitory compounds, mostly under acetic acid and furfural effects (50 ± 3 mg/l in IN-AA/F-L and 160 ± 9 mg/l in IN-AA/F-H), thus highlighting a combined toxicity effect for the strain both at low and high concentrations of AA and furfural. In addition, despite inoculum without adaptation achieved low productivity values showing a slight response to the conditions tested, the strain was able to produce in 24 h in the absence of inhibitors up to 62 mg/l which was reduced to 41 mg/l in the presence of acetic acid and furfural.

So, the effect of acetic acid was observed at each tested concentration on inoculum without adaptation and with adaptation. The synergistic inhibitory effect of acetic acid and furfural was strongly observed in all tests, but the strain demonstrated the capacity to tolerate them. The highest resistance of the strain against the inhibitors was observed at the highest initial strain concentration (1.1 g/l) after a pre-inoculum adaptation. In line with these findings, Dessie et al. (2019) observed that hydroxymethylfurfural and furfural, when together were contained in the fermentation broth, strongly inhibited (93.4%) the succinic acid production by *A. succinogenes*, even at the concentrations of both equal to 2 g/l.

4. Conclusion

These results highlighted that *Actinobacillus succinogenes* suffered without a pre-adaptation cultivation under the presence and the absence of inhibitors respect to pre-adapted inoculum. Unfavorable conditions were due to the presence of inhibitors compounds as acetic acid and the mixture of acetic acid and furfural that caused a decrease of *A. succinogenes* growth. The inhibition effects of these compounds were strongly evident on the inoculum without pre-adaptation. The strain after the pre-adaptation phase was better able to tolerate acetic acid and suffered more both acetic acid and furfural when they were together at low and high concentrations. Therefore, this preliminary study can provide the basis for further insights into the effects of fermentation inhibitory compounds on *A. succinogenes*. These early results highlighted how further research and strategies will be necessary to mitigate the effects of potential inhibitors generated using lignocellulosic biomass to produce succinic acid. Some strategies could be developed by using less aggressive pre-treatment processes or detoxification strategies for the production of succinic acid. Also, a better understanding of critical parameters of fermentation can be fundamental to optimize the process and therefore the production of succinic acid.

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