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Inhibitors derived from wheat straw hydrolysate can affect the production of succinic acid by *Actinobacillus succinogenes*

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ABSTRACT

Lignocellulosic biomasses are promising source of fermentative sugars for the production of succinic acid. The lignocellulosic matrix must be pretreated to make the sugars available for the fermentation, but the most tested operative conditions can generate inhibitors as acetic acid, furans, phenolic compounds. Inhibitors remained an obstacle for the implementation of succinic acid production starting from recalcitrant biomasses as wheat straw. Batch tests were performed at two starting concentrations of strain, sugars (glucose, glucose and xylose) and inhibitors (acetic acid and furfural) by comparing the fermentation in standard broth medium and hydrolysate. Notwithstanding the presence of acetic acid (52.5 mg/L) and furfural (15 mg/L), succinic acid was obtained at $9*10^{-2} \pm 7*10^{-3}$ g/L by starting from wheat straw hydrolysate that contained glucose (1.1 g/L), xylose (0.4 g/L) and without additional nitrogen source. Therefore, the study highlighted that a more concentrated inoculum was able to reduce the synergistic effect of inhibitors at their highest concentrations. The results obtained may contribute to improve succinic acid production from the biomasses that have been under-exploited but abundantly available, as wheat straw, for which solutions must be found to solve the problem of inhibitors production or to mitigate its effect on the fermentation process.

1. Introduction

The global strategy of bio-based economy evolved towards a sustainable bio-based production that is considered a priority for a low-carbon based economy and for a better future [1,2]. In the last fifteen years, one of the most interesting bio-based products has been bio-succinic acid (Bio-SA). Succinic acid was classified in the first 10 high values chemicals as a precursor for many industrial compounds and for the synthesis of biodegradable polymers such as polybutyrate succinate (PBS), polyamides and various green solvents [3,4]. Furthermore, the global demand of succinic acid are increasing and is expected a market value of over 500 million USD by 2030 [5].

Up to present day, SA has been industrially produced from n-butane through maleic anhydride by means of a petrochemical process. Although the production of succinic acid from fossil sources is still cost –effective with a price around 2.0 USD/kg, biotechnological routes have been proposed through microbial fermentation from agro-industrial residues/wastes. These processes may in fact lead noticeable environmental benefits and new opportunities of growth for the future with the reduction of more than 60 % of greenhouse gases emission when compared to the carbon footprints of petrochemical-based process [6–8].

Since 2012, four companies have launched commercial/pilot plants to produce bio-based succinic acid. Reverdia developed a direct fermentation at low pH of corn starch to produce 10,000 T/year of Bio-SA by using *Saccharomyces cerevisiae*. Myriant featured non-genetically

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modified *Escherichia coli* and sugars derived from sorghum and other commercial sugars to produce13,600 T/year [9]. Succinity reached a capacity of 10,000 T/year of Bio-SA by valorizing renewable resources as glycerol, glucose, and sucrose through *Basfia succiniciproducens* strain [10]. BioAmber-Mitsui (Canada) used the engineered yeast *Candida kruseii* (licensed from Cargill) and mainly cereal-based (corn glucose) feedstock with a production of 30,000 T, while BioAmber-France used *E. coli* and wheat glucose as feedstock for a production of 3000 T [11, 12].

Despite the fact that some companies started the industrial production of Bio-SA, there are still many technical-economic difficulties to be faced that are principally related to carbon sources, strain selection and inhibitors compounds that affect productivity and succinic acid conversion yield, as well as the downstreaming process for succinic acid recovery and overall process scalability [13].

Among the most promising carbon sources, lignocellulosic biomasses that amounted around $2*10^{11}$ T/year [14] are noticeable sources of sugars for the production of succinic acid [15]. These renewable resources can be produced as post-harvest residues from intensive cultivation of rice, wheat, maize, and sugarcane [16]. These resources contain carbohydrates polymers distributed in these fractions: cellulose 40–60 % on dry weight (dw), hemicellulose 10–40 % dw and lignin 15–30 % dw, extractives (1–2 % dw); and ashes (2–3 % dw) [17–19]. For the utilization of the carbohydrates feedstock, pre-treatments are necessary to alter the cellulose-hemicellulose-lignin matrix, to remove lignin, to make xylans and glucans available for the enzymatic attack and subsequently to convert them into fermentable sugars for microorganisms [20,21]. The pretreatments can be physical (grinding, ultrasound assisted), chemical (acidic and alkaline hydrolysis), chemical-physical (steam explosion) and biological (enzymatic treatments) [22]. The most tested pretreatments on corn, rice, wheat straw and sugarcane bagasse were dilute alkaline hydrolysis [23], which has also been tested in autoclave reactor (121 °C, 5 min) [24] and at different temperatures (H_3PO_4 5–99°C) [25], or acid hydrolysis with steam explosion [26] and autoclave reactor [27]. While, less aggressive methods were also tried with hot water on corn fiber [28], peracetic and alkaline peroxide assisted ultrasound on rice straw and sugarcane bagasse [29], alternative solvents as glycerol:water mixture [30] (Table 1, Supplementary materials S1).

Hydrolysis pretreatments (acidic and alkaline), in particular acidic conditions, at high temperatures such as steam explosion allowed to reach high sugars conversion yield but strong operative conditions generated unwanted by-products, such as organic acids (Acetic acid (AA), lactic acid (LA), formic acid (FA)), furans (5-hydroxymethyl-furaldehyde (HMF) and furfural), and phenolic compounds. Furans are produced from the dehydration of pentose and hexose sugars generated from the hydrolysis of hemicellulose, and, phenolic compounds are generated from the degradation of lignin and extractives. The most common phenols generated during the pretreatment of lignocellulosic biomass were p-hydrozybenzoic acid, vanillin, vanillic acid, cinnamic acid, benzoic acid, and syringic acid [31]. Acetic acid is produced from the hydrolysis of acetyl groups of degraded hemicellulose. Formic acid can be generated from the degradation of HMF in acidic condition or degradation of polysaccharides under alkaline conditions that also produced FA [32]. Acetic acid, formic acid and lactic acid can be also generated as by-products of metabolic pathways during the process of SA production. Formic acid can block the growth of *A. succinogenes* at concentrations between 11 and 18 g/L, acetic acid at the concentration of 33.7 g/L while lactic acid at the concentration of 55 g/L [33]. Formate and acetate have a similar mechanism of growth inhibition of the strain since they interfered on phosphate transport across the cell membrane by chemically interfering on an over requirement for ATP.

These multiple by-products were identified as inhibitors of the growth of microorganism and consequently of the productivity of the fermentative processes. Furans were able to activate different key enzymes in the microbial pathways of glycolysis and tricarboxylic acid cycle metabolism, thus reducing the sugar intake rate and target product conversion rate [34]. On the other hand, the furfurals could destroy the mitochondrial structure by inducing the accumulation of reactive oxygen (such as H_2O_2) in the cells and cytotoxicity [35]. Dessie *et al.* found that the combination of furfural and HMF at a concentrations of 3 g/L completely stopped the production of succinic acid by *A. succinogenes* [36]. Indeed, it was observed that furfurals can enhance the toxicity of other substances, such as phenols and acetic acid, to microorganisms [37]. In a recent study, Xu *et al.* [37] highlighted that the phenolic compounds, cinnamic and benzoic acid at a concentration of 12 mM had strong inhibitory effect on *A. succinogenes* by altering glucose uptake system, heath shock protein (HSP), and chemotaxis [38].

A recent study demonstrated the techno-economic feasibility of a simulated process from a sugarcane bagasse hydrolysate for the production of SA integrated in a biorefinery for the production of ethanol and electricity, can resulted in a lowering of the Bio-SA price to 2.32 USD/kg. Despite this, most of the studies on the production of SA from corn, rice and wheat straw, and sugarcane bagasse have been carried out on a laboratory scale [26–29] (Supplementary materials S1). Zheng *et al.*, investigated SA production from corn straw hydrolysate treated by alkaline hydrolysis and enzymatic treatment by obtaining 45 g/L of succinic acid at a yield of 81 % starting from 58 g/L of sugars [26]. Through simultaneous saccharification and fermentation, SA was produced at a concentration of 47.4 g/L and at a yield equal to 0.72 g/g by using corn stover [26]. Sawisit et al., 2018 obtained 48 g/l of SA at a yield of 0.84 g/g pretreated rapeseed straw after steam explosion by using *A. succinogenes* through the fermentation of liquid hydrolysate and fermentation after ethanol production [24]. Kuglarz et al., 2018 obtained from rapeseed straw treated by steam explosion and enzymatic hydrolysis 5.8 g/L of SA at a yield of 48 % without an investigation on inhibitors [27].

Lo et al., 2020 performed batch fermentation of sweet sorghum bagasse by *A. succinogenes* to obtain 17.8 g/L of SA and a yield of 0.61 $g_{SA}/g_{glucose}$ and HMF was detected around 1–2 g/100 of feedstock when acid hydrolysis was carried out at 80 °C for 60 min [25]. Jampatesh et al., 2019 produced 78 g/L of SA at a yield of 0.86 g/g by using the modified strain *E. coli* AS1600a from rice straw pretreated by acid hydrolysis and autoclaving that contained a total inhibitors concentration of 4.3 g/L of AA, FA and HMF [27]. Chen et al., 2021 produced 23 g/l of succinic acid 0.64 g/g by using sugarcane bagasse hydrolyzed by sodium hydroxide [33]. Total phenols concentration was 0.47 g/L and succinic acid production decreased from 23 g/L to 19 g/L when phenolic compounds concentration increased.

Starting from the current state of knowledge, inhibitory compounds have often been contained simultaneously at different concentration in the pretreated lignocellulosic biomass. Few works have highlighted the problematic presence of inhibitors in the lignocellulosic hydrolysate and their effect on Bio-SA production [25,27,33] also because the most recent studies tried to make the pretreatment process less impactful by using alternative methods. In this study starting from a very effective pre-treatment technology such as steam explosion for one of the less studied and more recalcitrant lignocellulosic biomasses such as wheat straw, the authors aimed to evaluate the production performance of succinic acid in the presence of multiple inhibitors by evaluating their effect on several parameters: different initial concentrations of inoculum, the mixture of sugars (glucose and xylose) at different concentrations, concentrations of inhibitors and how these can influence the resistance of the *A. succinogenes* strain to the presence of inhibitors in a rich growth medium and diluted wheat straw hydrolysate.

Succinic acid production was investigated by using standard growth medium, standard growth medium supplemented with inhibitors compounds (furfural and acetic acid), and diluted wheat straw hydrolysate. Two concentration of inoculum, sugars (glucose and mixture glucose and xylose) and two concentrations of AA and furfural were tested under batch fermentation by using *A. succinogenes* strain.

2. Material and methods

2.1. Material and microorganism

Actinobacillus succinogenes strain 130Z (CCUG-43843) was supplied by Culture Collection University of Gothenburg (CCUG, Gothenburg, Sweden) as freeze-dried pellets. The growth medium was a Tryptic Soy Broth (TSB) (22092–500 G Sigma) that contained (per liter) 17.0 g of peptone from casein, 3.0 g of peptone from soy, 5.0 g of NaCl, 2.5 g of K_2HPO_4 and 5.0 g of D-(+)-glucose. Before the use TSB was sterilized for 15 minutes at 121 °C.

2.2. Wheat straw treatment

Wheat straw was provided and treated in ENEA Research Center of Trisaia (Rotondella (MT), Italy). The raw lignocellulosic biomass was preliminarily characterized after a fine milling phase to reduce particle size at 50 mesh. The composition of wheat straw (WS) was determined as reported in Table 1 as percentage on dry weight (% w/w). Extractives quantification was performed following the methods proposed by Sluiter et al., 2015 [39]. In particular, the biomass was extracted using water and ethanol in a Soxhlet apparatus for 6 hours per cycle. The oven dry weight (dw) was measured by drying the biomass or apparatus at 105 ± 5 °C until a constant weight was achieved [39]. The characterization of lignocellulosic materials in terms of sugars, lignin, and ashes was carried out. Acid-insoluble lignin was determined gravimetrically via filtration on Whatman GFA filters following the NREL protocol proposed by Sluiter et al. in 2008 and ash content was determined using a muffle furnace at 575°C overnight [40]. Sugars analyses were performed by using ion chromatography (HPIC) with DIONEX ion chromatograph model DX300 equipped with a column Nucleogel Ion 300 OA, refractive index ED50 as detector, and H_2SO_4 0.05 M (40 °C, 0.4 mL/min) as mobile phase, and the analysis of furfural and HMF was performed using the HP 1100 system equipped with diode array UV detector [41].

Wheat straw was treated following a catalyzed acid steam explosion treatment following the optimized operational conditions for the purpose to guarantee a high hemicellulose recovery and a high hydrolysis of cellulose [42]. The biomass was minced in a blanking machine that reduced the size to measures between 1.7 and 5.5 mm. Then, 1 kg of raw biomass was immersed in 30 L of 0.5 M H_2SO_4 solution, for 10 minutes, and then transferred to the filter press to eliminate the liquid in excess. After the impregnation and the mechanical pressing, the dry matter of the wet biomass was determined around 31.2 % (w/w). After that, the steam explosion was carried out in a batch reactor (volume of 10 L) at 203 °C for 5 minutes [42].

After the steam explosion treatment, the hydrolysis tests were conducted by using the enzymatic blend Cellic Ctec2® (Danish company Novozymes A/S) adding 15FPU per gram of cellulose. The determination of enzyme activity (FPU) was carried out using the method of Ghose [43]. The hydrolysis were conducted at a solid-liquid ratio of 5 % w/v, temperature of 50°C and pH of 4.8 (obtained by adding a concentrated solution of NaOH to hemicellulose from acid pretreatment) and was stopped at 72 hours. Temperature control and agitation were performed by a shake plate incubator. The hydrolysate was filtered and sterilized. The final composition is reported in Table 2.

Table 1
Composition of wheat straw (percentage refers to dw.). Average values of three replicates.

Wheat straw composition	% (w/w)
Cellulose	38.4 ± 3.2
Xylan	16.7 ± 1.1
Organic extractives	4.3 ± 0.3
Acid insoluble lignin	20.6 ± 1.1
Ashes	6.2 ± 0.1

Table 2
D-glucose, D-(+)-xylose, acetic acid and furfural concentration in wheat straw hydrolysed (WSH).

	Concentration (g/L)			
	D-glucose	D-(+)-xylose	Acetic acid	Furfural
WSH	22.9± 1.3	11.4± 0.8	1.9±0.2	0.9± 0.1

2.3. Pre-inoculum

Before starting the batch fermentation tests on succinic acid production, *A. succinogenes* 130Z cells were grown in 150 mL sealed anaerobic bottle containing 120 mL TSB growth medium, for 6 days in a stirring incubator (New Brunswick Scientific Excella E24 Incubator Shaker Series) in the dark, at 37 °C and 180 rpm. Two starters for the inoculum were prepared at a final glucose concentration of 100 mg/L and 1250 mg/L respectively. Before that starter was transferred in fermentation bottles, a sample was taken to measure microbial growth and further analysis on the concentrations of sugars and acids organics.

2.4. Experimental set-up

Fermentation tests were performed under batch condition in sealed anaerobic bottle at a working volume of 250 mL with an inoculum of *A. succinogenes* at the ratio of 25 % (v/v) in all tests. The batch tests were performed at 37 °C and 180 rpm in the dark in a stirring incubator (New Brunswick Scientific Excella E24 Incubator Shaker Series).

The fermentation was carried out by using the standard growth medium (TSB) as control (C1) and the growth medium was prepared at two glucose concentrations (Table 3, Fig. 1): 0.4 g/L (C1-L) and 1.1 g/L (C1-H).

To evaluate the effect of several substrates as carbon sources (glucose and xylose) fermentation was carried out by using standard growth medium (TSB) supplemented with xylose, named (Control 2 C2), at two concentrations 0.2 g/L (C2-L) and 0.4 g/L (C2-H). In order to evaluate the effect of the inhibitors, acetic acid was added to standard growth medium (Test – I1) at the two concentrations of 21 mg/L (I1-L) and 52.5 mg/L (I1-H). And in a second test acetic acid at the same concentration of 21 mg/L and 52.5 mg/L, plus furfural at the two concentrations of 6 mg/L (I2-L) and 15 mg/L (I2-H) were added to the standard growth medium.

Finally, fermentation was carried by using two dilutions of wheat straw hydrolysate (WS- tests) obtained from steam explosion and enzymatic hydrolysis, that contained glucose, xylose, acetic acid and furfural at the same two concentrations tested on standard growth medium (tests C1-C2 and I1-I2) (Fig. 1a). WS-L contained as carbon sources glucose 0.4 g/L and xylose 0.2 g/L, and inhibitors acetic acid (AA) and furfural (F) at the concentration of 21 mg/L and 6 mg/L. The composition of WS-H was glucose 1.1 g/L and xylose 0.4 g/L), acetic acid (AA)

Table 3
Concentration of D-glucose, D-(+)-xylose, acetic acid (AA) and furfural (FU) at start of the fermentation experiment.

Test	Concentration g/L		Concentration mg/L	
	D-glucose	D-(+)-xylose	Acetic acid	Furfural
C1-L	0.4	0	0	0
C2-L	0.4	0.2	0	0
I1-L	0.4	0.2	21	0
I2-L	0.4	0.2	21	6
WS-L	0.4	0.2	21	6
C1-H	1.1	0	0	0
C2-H	1.1	0.4	0	0
I1-H	1.1	0.4	52.5	0
I2-H	1.1	0.4	52.5	15
WS-H	1.1	0.4	52.5	15

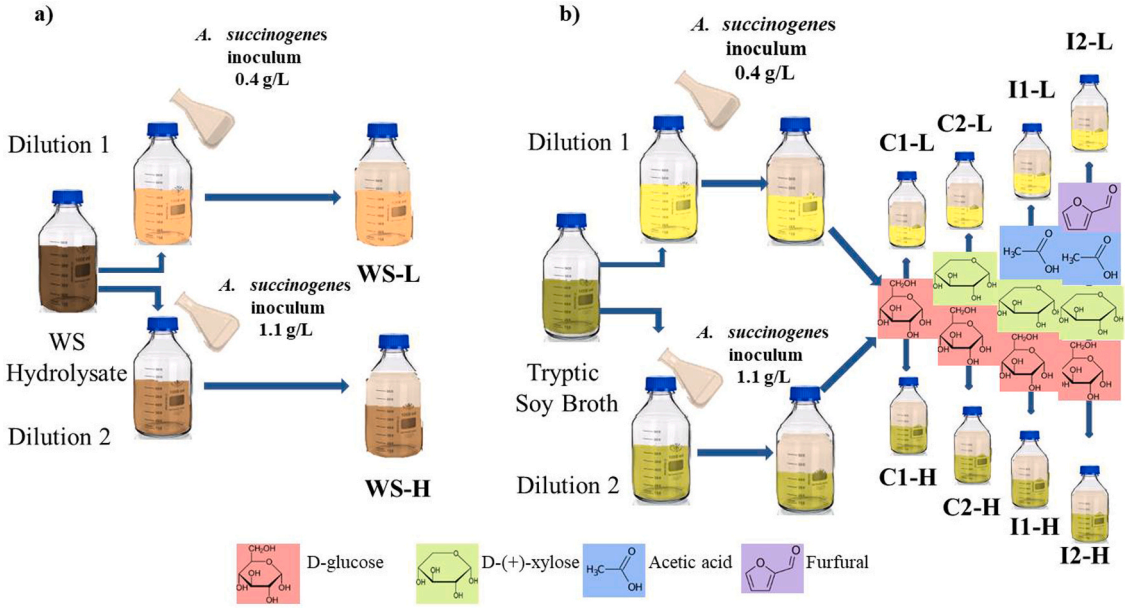

Fig. 1. Experimental design of the tests.

and furfural (F) at the concentration of 52.5 mg/L and 15 mg/L. For the fermentation by using diluted wheat straw, no additional carbon sources or nitrogen sources were used in order to evaluated the effect of a crude diluted hydrolyzed.

In addition, the effect of the different strain concentrations in the inoculum was also evaluated. For the test at low carbon sources and inhibitors the strain concentration of strain was 0.4 g/L while the strain concentration was 1.1 g/L for the test at high compounds concentrations.

The effects of the two different concentrations of carbon sources (glucose and xylose) and inhibitors, acetic acid, and together acetic acid and furfural between tests by using standard growth medium and diluted hydrolysate and the effect of two strain concentrations were evaluated on bacterial growth, sugars concentration, succinic acid production and by-products. In order to evaluate bacterial growth, monosaccharides and organic acids concentrations, samples were collected periodically (0, 24, 48, 120, and 144 hours) and prepared for further analysis and stored at −20 °C.

2.5. Analytical methods

In all tests. the bacterial growth was monitored by measuring the optical density (OD) at a wavelength of 600 nm (OD_{600}) by mean of a dual beam spectrophotometer (Perkin-Elmer Lambda 365). A calibration curve was obtained by interpolating OD values at 600 nm with known strain concentration value to calculate bacterial concentration (g/L). After OD_{600} measurement, the samples were centrifuged for 10 minutes at 4°C and 13000 rpm. Bacterial pellet was separated and the supernatant was filtered by using a nylon syringe filter, pore size 0.20 μm and stored at −20 °C for the following analysis of sugars (glucose and xylose) and organic acids (succinic acid, lactic acid, formic acid, and acetic acid).

The quantification of monosaccharides was carried out by ultra-high performance liquid chromatography, u-HPLC 1290 Infinity II (Agilent Technologies Inc., Santa Clara, USA) by using the detector ELSD (Evaporative Light Scattering Detector). InfinityLabPoroshell 120 HILIC-Z column (2.1 ×100 mm, 2.7 μm) was used to detect glucose and xylose, at a temperature of 80 °C, by using a mobile phase of ammonium acetate (0.01 M, pH 7.0) and acetonitrile (LC/MS grade) at a rate of 0.4 mL/min, with a gradient of 95–80 % for 12 minutes and 3 minutes of re-equilibration. The ELSD detector was set at a temperature of 60 °C, nitrogen flow rate was equal to 3.5 psi, signal frequency was 30 Hz [44]. Samples and analytical standards were prepared by using acetonitrile to reach acetonitrile and water 9:1 v/v ratio. Analytical standards were prepared at a different concentration in the range 12.5 mg/L −250 mg/L. Volume injection was fixed at 2.5 μL.

Carboxylic acids (SA, LA, FA, and AA) were quantified by using u-HPLC 1290 Infinity II. Diode Array Detector (DAD) was used to identify them at a wavelength of 210 nm. The Hi-Plex H Column, 7.7 ×300 mm, 8 μm (p/n 1170–6830 Agilent) was used at a temperature of 50 °C with 100 % isocratic mobile phase 0.01 M H_2SO_4 at a flow rate of 0.6 mL/min [45]. Diluted fermented broth samples and analytical standards of succinic, lactic, formic, and acetic acid (47264 Supelco) were prepared diluted by using water (LC/MS grade) and injected at a volume of 20 μL.

2.6. Statistical analysis

The inhibition rate on bacteria growth (IR) expressed as percentage was calculated following the calculation 1.

$$IR = (Bctrl - Btest)*100/ Bctrl \quad (1)$$

Where Bctrl is the average value of concentration of the strain (g/L) in control C1 and Btest is the bacterial concentration (g/L) in the other tests. The inhibition rate was calculated for control and test at the same time for each sampling time of 24, 48 and 144 hours.

Glucose consumption (Gcons) was calculated as percentage between the initial concentration (C0) and the concentration (C) at the chosen sampling time for each test (2).

$$Gcons = (C0-C)/C0*100 \quad (2)$$

Succinic acid (SA) yield was calculated as reported in (3).

$$SA\ yield = Succinic\ acid\ produced\ (g)/\ Initial\ glucose\ amount\ (g) \quad (3)$$

Succinic acid productivity was calculated as the ration between the succinic acid concentration at a fixed time and the time (hour) of the experimentation.

$$Productivity = SA\ concentration\ (mg/L)\ /time\ (hour) \quad (4)$$

The ratio SA/AA was calculated by dividing the SA concentration (mg/L) and AA concentration (mg/L) (5)

SA/AA ratio=ConcSA/ConcAA (5)

The selectivity was calculated as following (6):

Selectivity= Conc SA/ (ConcSA + ConcAA + ConcFA) (6)

Where ConcSA is the concentration of succinic acid (mg/L), ConcAA is the concentration of acetic acid (mg/L), and ConcFA is the concentration of formic acid (mg/L)

All tests were carried out in triplicate, the average values, and the standard deviation have been calculated.

Past software was used to calculated Two Way ANOVA tests that was performed following Holm-Sidak test for the comparison between control and tests. The test was also performed comparing the same experimental time between control and each test.

3. Results and discussion

3.1. Effect of inhibitors on bacterial growth

Acetic acid and furfural were the most common by-products generated after chemical (acid and alkaline hydrolysis) and thermo-chemical treatments (steam explosion) of lignocellulosic biomasses. In wheat straw hydrolysate, furfural can typically be found in concentrations between 2 and 7.1 mM [45]. Other by-products, such as vanillin, ferulic and coumaric acids can be formed at lower concentrations than furfural and acetic acid [46].

In this study the concentration of furfural was found (<9.4 mM) equivalent to 0.9± 0.1 g/L and the concentration of AA was less than 1.9 ± 0.1 g/L in wheat straw hydrolysate. For these reasons, acetic acid and furfural were chosen to compare their effect on strain growth and SA production and by-products both in hydrolysate and standard growth medium.

Two initial concentrations of *A. succinogenes* strain was considered that were 0.4 g/L for the tests at low sugars and inhibitors concentrations and 1.1 g/L for the tests at their high concentration.

The strain grew exponentially in the first 24 hours in all samples irrespective of the initial inoculum concentration and the presence of xylose, and inhibitors in the growth medium, whereas the growth trend was markedly lower when using wheat straw hydrolysate. Starting from a high inoculum concentration of 1.1 g/l in the C1-H control, a significantly higher strain concentration was reached at 24 hours than in the C1-L control. Any difference was observed on bacterial growth trend between the control and the sample in which xylose was also contained. After 24 hours, the strain reached a clear plateau phase in the presence of the inhibitors, both acetic acid and the mixture AA and furfural in the growth medium at all tested concentrations. The maximum concentration of the strain that grew in the most diluted hydrolysate (WS-L) was 4.0±0.7 g/L after 144 hours of fermentation and an initial lag phase, whereas when a more concentrated hydrolysate was used (WS-H), the strain reached a concentration of 7.0±0.5 g/L (Fig. 1, Supplementary materials, S1).

The percentage of growth inhibition was calculated between tests and the control C1 (C1-L and C1-H) (Table 4). Growth inhibition of the strain was observed in the presence of acetic acid at both concentrations of 21 mg/L and 52.5 mg/L, and together with furfural at the two concentrations tested (6 mg/L and 15 mg/L), both in the supplemented growth medium and in the wheat straw hydrolysate (WS-L and WS-H). Inhibition rates of the strain's growth were higher for the strain that grew in the diluted hydrolysates respect to the strain grown in the growth medium supplemented with inhibitors. This difference was basically due to the composition of the growth medium, which contained nitrogen sources such as casein and peptone, a source of energy for strain growth even in the presence of the inhibitors, that were absent in the hydrolysate.

Table 4
A. succinogenes concentration (g/L) and inhibition rate (IR)(%).

	A. succinogenes concentration (g/L)		Inhibition rate (IR) (%)	
TEST	0 h	24 h	48 h	144 h
C1-L	0.4	0	-	-
C2-L	0.4	0	-	-
I1-L	0.4	8	18	29
I2-L	0.4	17	24	32
WS-L	0.4	74	71	62
C1-H	1.1		-	-
C2-H	1.1		-	-
I1-H	1.1	30	31	33
I2-H	1.1	40	36	41
WS-H	1.1	66	60	59

In the growth medium supplemented with inhibitors, IR increased as the concentration of AA produced during fermentation in all the tests. Where acetic acid as unique inhibitor, had a starting concentration of 52.5 mg/L, IR was equal to 30 % at 24 h and increased weakly up to 33 % at 144 h despite the acetic acid produced being equal to 224± 3.5 mg/L but the strain concentration managed to counteract this concentration better.

In the presence of AA and furfural in the growth medium, the rate of growth inhibition was higher at 24 h than in tests with a single inhibitor, 17 % at the initial contraction of AA 21 mg/L and furfural 6 mg/L, and 40 % at the initial concentrations of AA 52.5 mg/L and furfural 15 mg/L. Inhibition tended to increase during the fermentation process for acetic acid production.

A. succinogenes growth was strongly decreased when the two diluted wheat straw hydrolysates were used. At 24 h, the IR of the strain (at initial concentration of 0.4 g/L) was higher (77 %) in the diluted hydrolysate that contained less inhibitors (WS-L), AA 21 mg/L and furfural 6 mg/L, because the strain was undergoing a latency phase. And the inhibition effect appeared to reduce slightly from 48 h to 144 h because the microorganism started to grow.

A. succinogenes better tolerated the more concentrated wheat straw hydrolysate (WS-H) starting from bacterial concentration equal to 1.1 g/L, acetic acid at 52.5 mg/L and furfural at 15 mg/L with a IR% equal to 66 % at 24 h that decreased till to 144 h. A decrease in the growth inhibition rate was observed despite the fact that acetic acid was produced by the strain and reached the concentration of 215± 2.7 mg/L.

Compared with previous studies in which higher concentrations of these inhibitors were tested, lower concentrations were tested to maintain a ratio of bacterial to glucose concentration around 1:1 as demonstrated in a previous work [47]. Li *et al.* (2010) tested different concentrations of AA from 10 g/L to 80 g/L and an initial *A. succinogenes* concentration of 0.03 g/L showing that the strain can tolerated up to 10 g/L of acetic acid. The authors reached a bacterial concentration of 0.06 g/L but the growth was slow over 12 hours and an inhibition rate of 86 % was recorded. [48]. On the other hand, *A. succinogenes* can tolerate furfural up to the maximum concentration of 0.28 g/L [49]. Nevertheless, a different inhibition trend was observed in this work between the strain growth in the medium (simulated) and the hydrolysate due to the production of AA in the simulated that increased the inhibition rate. Among the organic acids, formic acid was also produced that can blocked the growth of the strain at the critical concentration of 18 g/L [50]. Specifically, inhibition rates in samples in which growth medium was supplemented with acetic and furfural acid at both low and high concentrations were similar from 48 h to 144 h for acetic and formic acid production trends. In the hydrolysate, on the other hand, inhibition rates were higher but decreased slightly in response to strain growth, and AA and FA concentrations were still lower than those produced in the simulated as shown in the following paragraphs (3.3 and 3.4).

3.2. Glucose and mixed sugars consumption

Glucose and xylose were the main fermentable sugars released after the pre-treatments of a lignocellulosic biomass that accounted for around 90 % of the total sugars. Even if glucose was the monosaccharide principally investigated for the production of succinic acid, xylose also

enabled its production as demonstrated by using a hydrolysate of corn stover (xylose concentration 57.0 g/L), with a conversion equal to 80 % and SA production of 32.5 g/L in a continuous fermentation process by a biofilm reactor [51]. Even though, *A. succinogenes* was also able to use till to 60 g/L pure xylose to produce around 6 g/L of succinic acid in shake flask fermentation [52], SA production can be affected by the co-utilization of glucose and xylose since their ratio can affect the intracellular ATP production by interfering with metabolic pathways [53]. Zheng *et al.*, tested several glucose-xylose ratio for a total sugar concentration of 60 g/L and the results showed an increase in succinic acid production when glucose was higher in the ratio of the two sugars [54]. A similar result was observed on the production of SA from pure glucose and the mixture with sucrose, fructose, and xylose, where better performance of succinic acid production was observed in the presence of glucose as the unique sugar source compared to the glucose:xylose ratio 0.9:0.1 [47].

In this study, since the glucose:xylose ratio was found equal to 0.7:0.3 in the wheat straw hydrolysate, this ratio has been repeated in the control tests with TSB growth medium (C2) and in the simulated hydrolysate samples. A predominant consumption of glucose was observed in all tests irrespective of the concentration and the presence of xylose, which did not significantly influence glucose consumption trends. Xylose was poorly consumed (3.5 % xylose consumption) in control from an initial concentration of 0.2 g/L while, in the control (C2-H) at a high concentration of sugars (glucose 1.1 g/L and xylose 0.4 mg/L), 6.1 % xylose was consumed. The consumption decreased from 6.1 % to 4.2 % and to 5.4 % where the growth medium (TSB) contained acetic acid and acetic acid and furfural respectively. When wheat straw hydrolysate was used 3.7 % xylose was consumed.

The Fig. 2 showed the percentage of the consumed glucose during the fermentation. In the control tests, the strain consumed up to 32 ± 2 % of glucose from an initial concentration of 0.4 g/L (C1-L, Fig. 2A) and 51 ± 2 % glucose from 1.1 g/L (C1-H, Fig. 2B) after 144 hours. The results showed that the consumption of glucose decreased in the presence of inhibitory compounds as evidenced by the decreased growth of the strain as discussed in the previous paragraph. When AA was contained in the supplemented growth medium as a single inhibitor, at the two initial concentrations 21 mg/L (I1-L) and 52.5 mg/L (I1-H), glucose was consumed up to 24 ± 3 % and 25 ± 3 % respectively, without significant differences ($p>0.05$). The highest concentration of acetic acid (52.5 mg/L in I1-H) significantly affected glucose consumption, which decreased by almost 50 % compared to glucose consumption in the control (C1-H).

The greatest evidence of the reduction in glucose consumption in presence of both inhibitors (AA and furfural) was observed at their highest concentration (I2-H) (AA 52.5 mg/l of and furfural 15 mg/L) in the supplemented growth medium. In the hydrolysate, the highest amount of glucose consumed was observed at 144 h that was around 19 % ± 3 when a more concentrated wheat straw hydrolysate was used (WS-H). The greatest difference in glucose consumption between the two wheat straw dilutions was observed at 144 hours. As the consumption rates were very similar for the dilutions at 24 hours (6 % ± 1 and 7 % ± 1), the differences observed on the glucose were due to the growth of the strain that started higher in the more concentrated hydrolysate.

3.3. Effect of inhibitors on succinic acid production

The concentration of succinic acid produced by *A. succinogenes* is shown for the samples at low concentrations of strain, sugars and inhibitors (Fig. 3a) and those at high concentrations (Fig. 3b). The highest concentration of SA was recorded when inhibitors were absent and without significant differences in the presence of xylose, while SA decreased significantly in all tests in which unwanted compounds were present both at low and high concentrations than the control tests ($p<0.05$). At an initial glucose concentration of 0.4 g/L the SA maximum concentration was 200 ± 10 mg/L. Respect to control, the highest decrease of SA concentration in supplemented growth medium was observed when AA and furfural was added at the initial concentration of 21 mg/L and 6 mg/L (Fig. 3a). In this case, SA production amounted to 66 ± 3 mg/L compared to 200 ± 10 mg/L in the control, with a statistically significant difference ($p <0.017$). The presence of AA at a concentration of 21 mg/L, on the other hand, yielded up to 95 ± 5 mg/L of succinic acid. The concentration of SA obtained by using the less concentrated wheat straw hydrolysate (WS-L) was recorded as less representative and amounted to 10 ± 7 mg/L (Fig. 3a).

The highest production of SA was equal to 520 ± 16 mg/L and it was observed in the control at starting glucose concentration of 1.1 g/L (Fig. 3b). Any important difference was observed on SA concentration when xylose was used. The combined effect of acetic acid and furfural was also observed in this case and succinic acid concentration reached 196 ± 9 mg/L. The concentration of succinic acid produced by using the more concentrated wheat straw hydrolysate was 90 ± 7 mg/L (WS-H), which was significantly 9 times higher than the achieved by using the more diluted wheat straw hydrolysate (WS-L test: 10 ± 7 mg/L). The SA concentration 90 ± 7 mg/L (WS-H) was also approximately 2.2 times

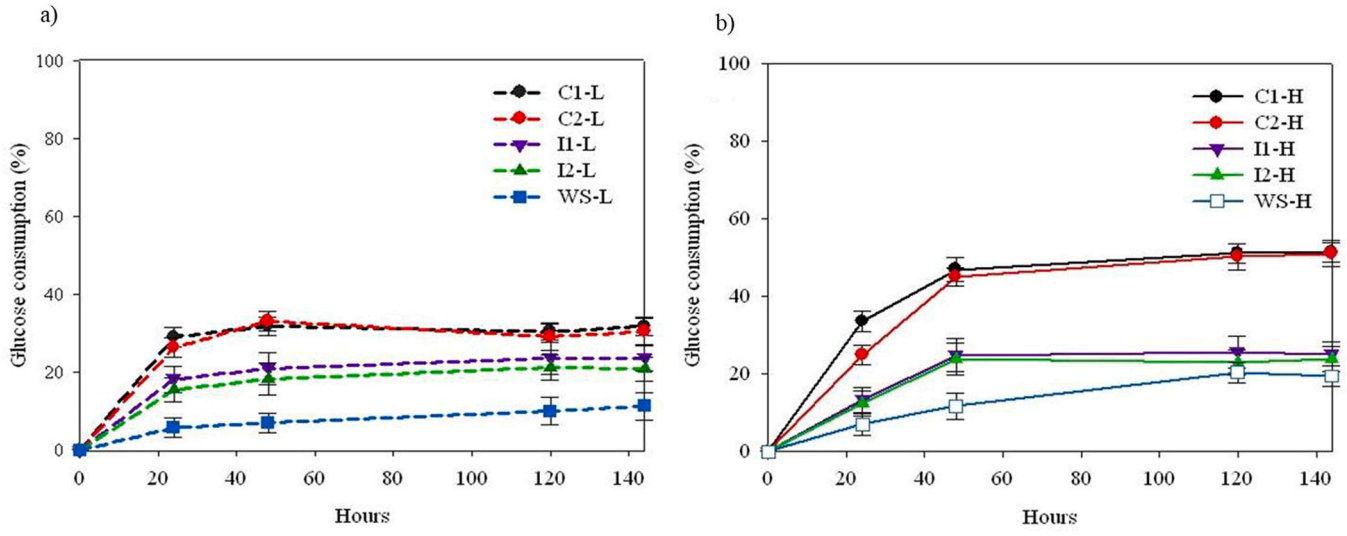

Fig. 2. Glucose consumption (%) in test L (low concentration – dashed lines) and H (high concentration – continuous lines). Circles represent Control 1 and control 2; triangles represent tests with inhibitors compounds; square represents wheat straw sample. Error bars indicated standard deviation.

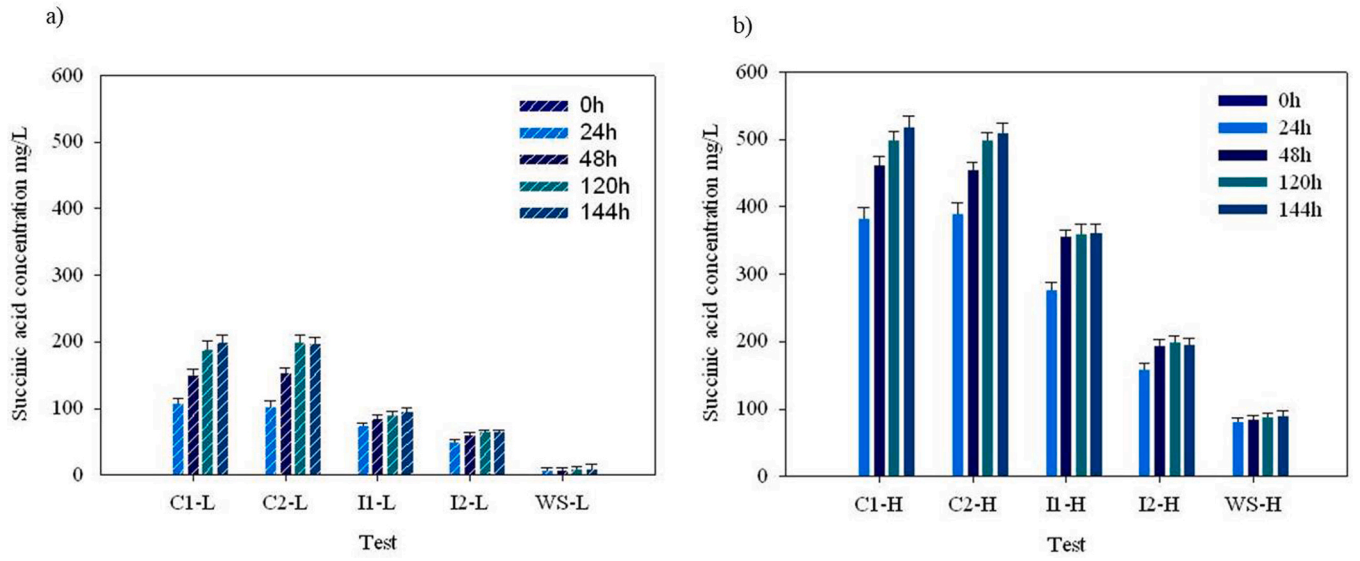

Fig. 3. Succinic acid concentration (mg/L) in test L (low concentration – dashed vertical bars) and test H (high concentration – full vertical bars). Control 1 (C1), control 2 (C2), test with acetic acid (I1), test with acetic acid and furfural (I2), wheat straw sample (WS). Error bars indicated standard deviation.

lower than the corresponding simulated in the growth medium with acetic acid and furfural (I2-H, AA 52.5 mg/L and furfural 15 mg/L) at the high concentration.

An overview of the final concentration and percentage reduction of succinic acid is reported in Table 5 for all tests compared to control (C1). The percentages of SA reduction in the tests of standard medium supplemented with AA were 53 % at the low initial concentration of AA (21 mg/L) and 30 % at 52.5 mg/L. The reduction rate 53 % of SA was not due to acetic acid concentration but to the less growth of the strain compared with the corresponding test at higher AA concentration.

The inhibition effect of the acetic acid and furfural mixture seemed to be not dependent on their two concentrations with a reduction in succinic acid concentration of 67 % and 62 % at their low and high concentrations respectively. The similarity between the two inhibition rates was also due to the effect of FA produced in greater amounts than acetic acid in the I2-L test.

Table 5
Inhibitors effect on succinic acid concentration respect to controls at low and high concentration of unwanted compounds.

	Succinic production mg/L and reduction (%)			
Carbon source	NO inhibitors	AA 21 mg/L	AA 21 mg/L + Furfural 6 mg/L	AA 21 mg/L + Furfural 6 mg/L in wheat straw
Glucose 367 mg/L	200 ± 10 (100 %)	95± 5* (53 %)	66± 3* (67 %)	10± 7* (95 %)
Glucose 367 mg/L + Xylose 171 mg/L	198± 9 (100)			
A. succinogenes Final concentration g/L	10.5/10.7	7.4	7.1	4.0
Carbon source	NO inhibitors	AA 52.5 mg/L	AA 52.5 mg/L + Furfural 15 mg/L	AA 52.5 mg/L+ Furfural 15 mg/L in wheat straw
Glucose 1130 mg/L	520±16 (100 %)	362±13* (30 %)	196±9* (62 %)	90±7* (83 %)
Glucose 1130 mg/L + Xylose 428 mg/L	510±14 (100 %)			
A. succinogenes Final concentration g/L	17/17.2	11.3	10	7.02

* $p < 0.050$ (Two WAY Anova)

In contrast, the inhibition effect of the compounds contained in the wheat straw hydrolysate was greatest at their lowest concentrations with a 95 % reduction in the concentration of SA, while at the highest concentrated hydrolysate the reduction was fewer (83 %).

The effects on the succinic acid production of the main inhibitory compounds generated by the pre-treatment of lignocellulosic biomass as furans, acids and phenolic compounds were already partially investigated as reported in Table 6. Dessie et al., demonstrated that at the same concentration of HMF and furfural, *A. succinogenes* tolerated more HMF [36]. In a batch fermentation process starting from 30 g/L of glucose, if furfural concentration was 3 g/L the SA production reduced of 84 % while when both furfural and HMF were present SA concentration decreased of 93.2 % despite the total concentration of inhibitors was less than 3 g/L [36]. This phenomena was due to a synergistic effect of the inhibitors. In this study, only furfural was detected in hydrolysate so the synergistic effect of the two furans was not observed.

Salvachúa *et al.*, observed the synergistic effect of furans together with acetic acid by using a diluted corn stover hydrolysate in which the concentrations of AA was 5.8 g/L, HMF 1.4 g/L, and furfural 0.17 g/L that decreased SA concentration to 95 % [58]. In the same study, acetic acid, at the concentration of 5.8 g/L, but as the only inhibitor, affected SA concentration only for the 30 % [58]. This synergetic behaviour of acetic acid and furans was also observed in this study, in addition at this effect authors highlighted the strong effect of formic acid respect to acetic acid on the reduction of SA equal to 67 % (Tables 5 and 6). In addition to the synergistic effect with furans, it was also observed that acetic acid, when present as a single inhibitor, can cause a delaying effect on succinic acid production at 24 hours [58].

The effect of the inhibitors was also tested on other microorganisms such as *Basfia succiniciproducens* using a hydrolysate of *Arunndo donax* at different dilutions [55]. The more concentrated hydrolysate (90 % hydrolysate) (containing AA 8 g/L, HMF 63 mg/L and furfural 45 mg/L) totally inhibited SA production up to 20 hours. After this inhibition, at 48 hours, succinic acid concentration reached the same value (5 g/L) obtained when a more diluted hydrolysate (20 % hydrolysate) at the concentration of acetic acid equal to 2 g/L was used.

Xu *et al.* (2015) tested other compounds, such as vanillin and syringaldehyde, in combination with furfural and HMF, at different concentrations, starting from a corn cob hydrolysate on an engineered

Table 6
The main results on the effect of inhibitors on succinic acid production.

Inhibitors	Inhibitors concentration (g/L)	Strain	SA reduction (%) and concentration (g/L)	References
Furfural	3	*Actinobacillus succinogenes*	84 % (3.5)	[37]
HMF	3		62 % (8.4)	
Furfural	3		83.3 % (2.7)	
HMF	3		75 % (4.0)	
Furfural + HMF	2 (F) + 2 (HMF)		93.2 % (1.1)	
Acetic Acid	5.8	*Actinobacillus succinogenes*	30 % (5)	[55]
Furfural + HMF + Acetic acid	1.4 (F) + 0.17 (HMF) + 5.8 (AA)		95 % (5)	
Furfural + HMF + Acetic acid	1.4 (F) + 0.17 (HMF) + 2.3 (AA)		25 % (30)	
Acetic acid	2	*Basfia succiniciproducens*	75 % (5)	[56]
Acetic acid + HMF + furfural	8 (AA) + 0.063 (HMF) + 0.045 (FU)		0 % 24 h 75 % (5) 48 h	[56]
Acetic acid	>10	*Corynebacterium glutamicum*	92 %	[57]
furfural	0–2		20 % (1.5)	
5-HMF	0–2		30 % (1.5)	
vanillin	0–2		60 % (1.5 & 2.0)	
syringaldehyde	0–2		50 % (1.5 and 2.0)	
Inhibitors mixtures	0–2		60 %	
Acetic acid	0.021 (AA) 0.052.5 (AA)		53 % (0.095± 0.05) 30 % (0.362±0.13)	In this study
Acetic acid + furfural	0.021 (AA) + 0.006 (FU)	*Actinobacillus succinogenes*	67 % (0.066± 0.03)	In this study
Acetic acid + furfural	0.052.5 (AA) + 0.015 (FU)		62 %(0.196±0.9)	In this study

C. glutamicum strain [56]. Syringaldehyde inhibited more SA production than furfural and HMF. The corn cobs hydrolysate was tested with and without detoxification treatment, and the hydrolysate without detoxification reduced SA production by 50 % compared to the detoxified hydrolysate [56].

3.4. Effect of inhibitors on by-products

The decrease of by-products such as acetic, formic, and lactic acid is one of the most challenging topic to improve the production of succinic acid. In this study, the concentration of the acetic and formic acids produced during the fermentation in all the tests were evaluated and the effect of the inhibitors on their production was also observed (Figs. 4 and 5). An interesting decrease of the acetic acid was recorded when xylose was used as feedstock with glucose, both at the concentrations of 0.2 g/L (C2-L) and 0.4 g/L and (C2-H) (Fig. 4a-b). In particular, when xylose was 0.2 g/L, the concentration of AA was 2.6 times lower respect to control (C1-L) when only glucose was used.

When acetic acid was added at the initial concentration of 21 mg/L (I1-L) in the standard growth medium alone and with furfural (I2-L) the AA concentration slightly increased without significant difference in the two tests ($p>0.05$). In the diluted hydrolysate, its concentration slightly increased till to 70 mg/l.

The effect of glucose-xylose mixture was also observed on AA at their high concentration (Glucose 1.1 g/L and xylose 0.4 g/L) (C2-H). In this case, the final concentration of acetic acid was only 1.3 times lower than control (C1-H). Starting at acetic acid concentration of 52.5 mg/L, its concentration increased in supplemented growth medium and hydrolysate but the production of AA was reduced respect to control (C1-H).

AA reached the concentration of 224± 3.5 mg/L and 215± 2.7 mg/L without statistically difference ($p>0.05$) in the supplement standard growth medium, respectively in I1-H and I2-H. A slight, but non-significant, decrease of AA production was observed in supplemented TSB with acetic acid and furfural (I2-H) ($p>0.05$).

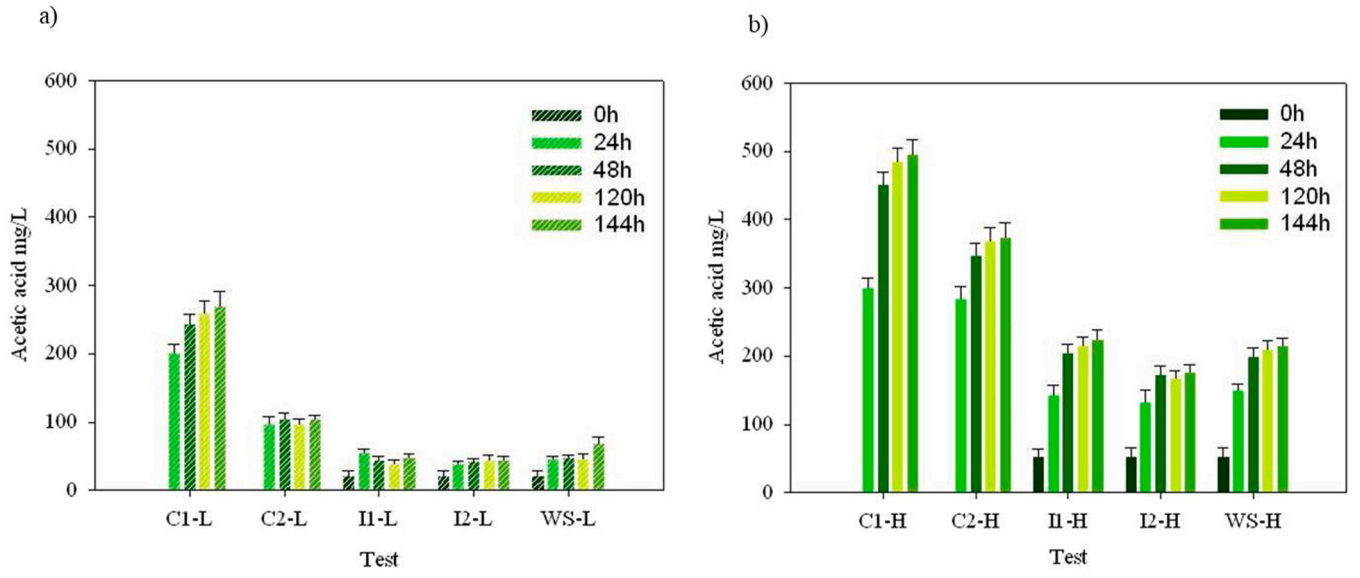

Fig. 4. Acetic acid concentration (mg/L) in test L (low concentration – dashed vertical bars) and test H (high concentration – full vertical bars). Control 1 (C1), control 2 (C2), test with acetic acid (I1), test with acetic acid and furfural (I2), wheat straw sample (WS). Error bars indicated standard deviation.

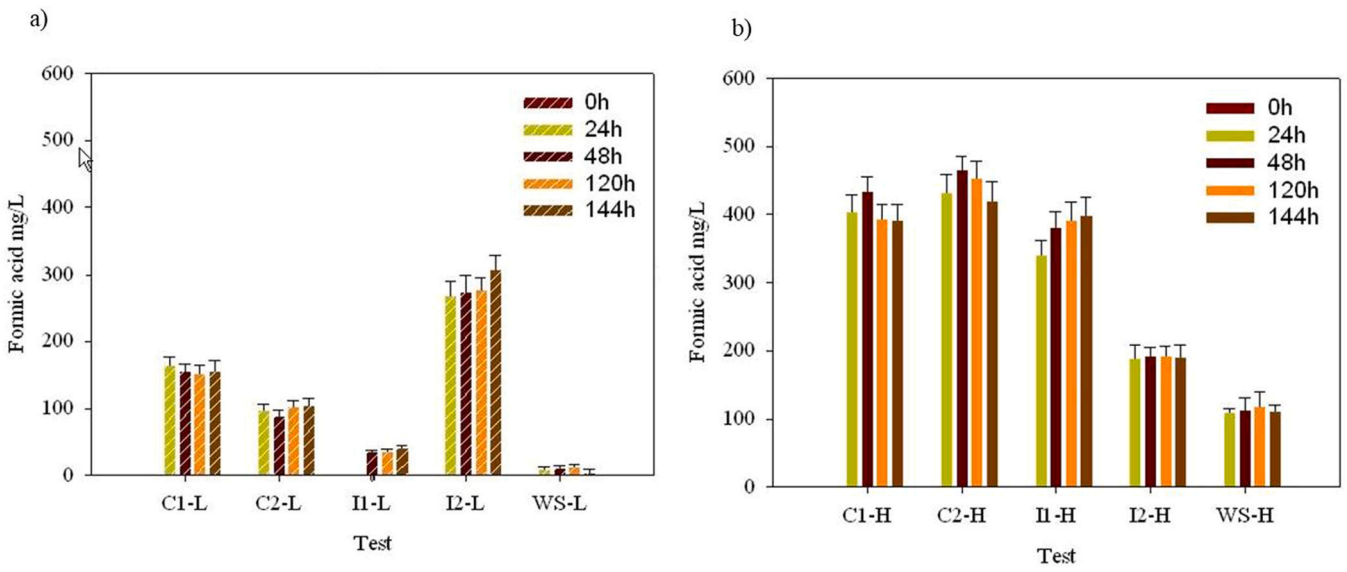

Fig. 5. Formic acid concentration (mg/L) in test L (low concentration – dashed vertical bars) and test H (high concentration – full vertical bars). Control 1 (C1), control 2 (C2), test with acetic acid (I1), test with acetic acid and furfural (I2), wheat straw sample (WS). Error bars indicated standard deviation.

In addition the concentration of formic acid was also evaluated (Fig. 5). An unexpected high production of formic acid was observed in the supplemented growth medium with acetic acid at 21 mg/L and furfural at 6 mg/L (I2-L) respect to the others group tests (Fig. 5a).

Formic acid production did not differ significantly between control C1-H, supplemented growth medium with the mixture of sugars (glucose 1.1 g/L and xylose 0.4 g/L), and added AA 52.5 mg/L (Fig. 5b) ($p>0.05$). In the co-presence of AA and furfural (15 mg/L), formic acid decreased considerably in both the TBS growth medium (I2-H) and in the hydrolysate (WS-H).

3.5. Succinic acid production performance and strategies

The results indicated that the growth of the strain, glucose consumption and succinic acid production and the others by-products were strongly influenced by the presence of the inhibitors both under growth conditions in a rich medium, such as TSB, and under wheat straw hydrolysate at the two tested dilutions. But the different initial concentration of inoculum equal to 0.4 g/L and 1.1 g/L demonstrated that the more concentrated inoculum was able to grow more, to consume more glucose, to produce more SA when using a rich growth medium. The more concentrated inoculum was able to also grow and counteract the effects of using dilute raw hydrolysed. The negative effect of acetic acid production was recorded but less pronounced than the synergistic effect of AA and furfural on succinic acid. Yield ($gr_{SA}/gr_{Glucose}$), the 24-hour productivity, and the ratio between SA and AA, and the selectivity were evaluated (Table 7) to better understand the performance of the succinic acid production.

Table 7
Process performance of succinic acid production.

TEST	Succinic acid concentration (mg/L)	Yield (gr_{SA}/gr_{Glu})	Productivity (mg/(L*h))-24	Ratio SA/AA	Selectivity ($g_{SA}/g_{SA}+g_{AA}+g_{FA}$)
C1-L	200 ±10	55.6 %	4.5	0.7	0.3
C2-L	198 ±9	53.5 %	4.3	1.9	0.5
I1-L	95 ±5	25.7 %	3.1	2.0	0.5
I2-L	66 ±3	17.8 %	2.1	1.5	0.2
WS-L	10 ±7	2.7 %	0.3	0.1	0.1
C1-H	520 ±16	47.3 %	16.0	1.05	0.4
C2-H	510 ±14	46.4 %	16.3	1.4	0.4
I1-H	362 ±13	32.9 %	11.5	1.6	0.4
I2-H	196 ±9	17.8 %	6.7	1.1	0.3
WS-H	90 ±7	8.2 %	3.4	0.4	0.2

In this study, under conditions of the absence of inhibitor stress *A. succinogenes* produced succinic acid at the concentration equal to 520 ± 16 mg/L and at a yield of 47.3 %. When less glucose and less inoculum were used succinic acid concentration was 200 ± 10 mg/L at a yield of 55.6 %.

To better understand the potentiality of diluted wheat straw hydrolysate as unique feedstock of carbon sources (glucose and xylose) for the production of SA, sugars concentrations and organic acids were reported in Fig. 6, where the data obtained from the use of the more concentrated hydrolysate were reported compared to control.

In absence of inhibitors (Fig. 6a) *A. succinogenes* grew in a rich growth medium and quickly consumed about 50 % (51.3 ± 2.4 %) of glucose at 48 hours and produced at the end of the fermentation SA 520 ±16 mg/l (144 h) at a yield of 47.3 % respect to the initial amount of glucose. Respect to the others organic acids, the production of SA increased till the end of the fermentation without significant difference respect to the produced AA that was explained with a ratio SA/AA equal to 1.0. While FA concentration decreased from 48 h (Fig. 6a).

In Fig. 6b, xylose concentration was not reported due to the low consumption that was around 6 % respect to the starting concentration of 0.4 g/L. Under the co-presence of two carbon sources, the strain preferred to use glucose to produce a similar concentration of SA around 510 ±14 mg/L at 144 h at a yield of 46.4 %. In the presence of glucose alone or glucose and xylose, no change was found on the growth trend of the strain. Although *A. succinogenes* can utilize several monosaccharides and others disaccharides and reducing sugars [57], it was abundantly demonstrated that the strain preferred to use glucose that can tolerate up to the concentration of 158 g/L [59]. By using glucose as carbon source, *A. succinogenes* can produce relatively large quantities of SA till to 70 g/L at a yield of 0.6 $g_{SA}/g_{glucose}$, and a productivity equal to 1.4 g/L/h [60].

Although xylose was partially consumed by the strain at the two concentrations, it was observed that the SA/AA ratio increased to 1.4 and 1.9 respectively when the initial xylose concentrations were 0.2 g/L and 0.4 g/L. The increase in the value of SA/AA ratio was due to the decrease in AA production in both tests but the concentration of formic acid was recorded higher. In addition, when glucose and xylose were at the initial concentrations equal to 0.4 g/L and 0.2 g/L, and the concentration of SA was 198±9 mg/L, the selectivity was also improved ($0.5\ g_{SA}/g_{SA}+g_{AA}+g_{FA}$) (Table 7) as the concentration of both AA and

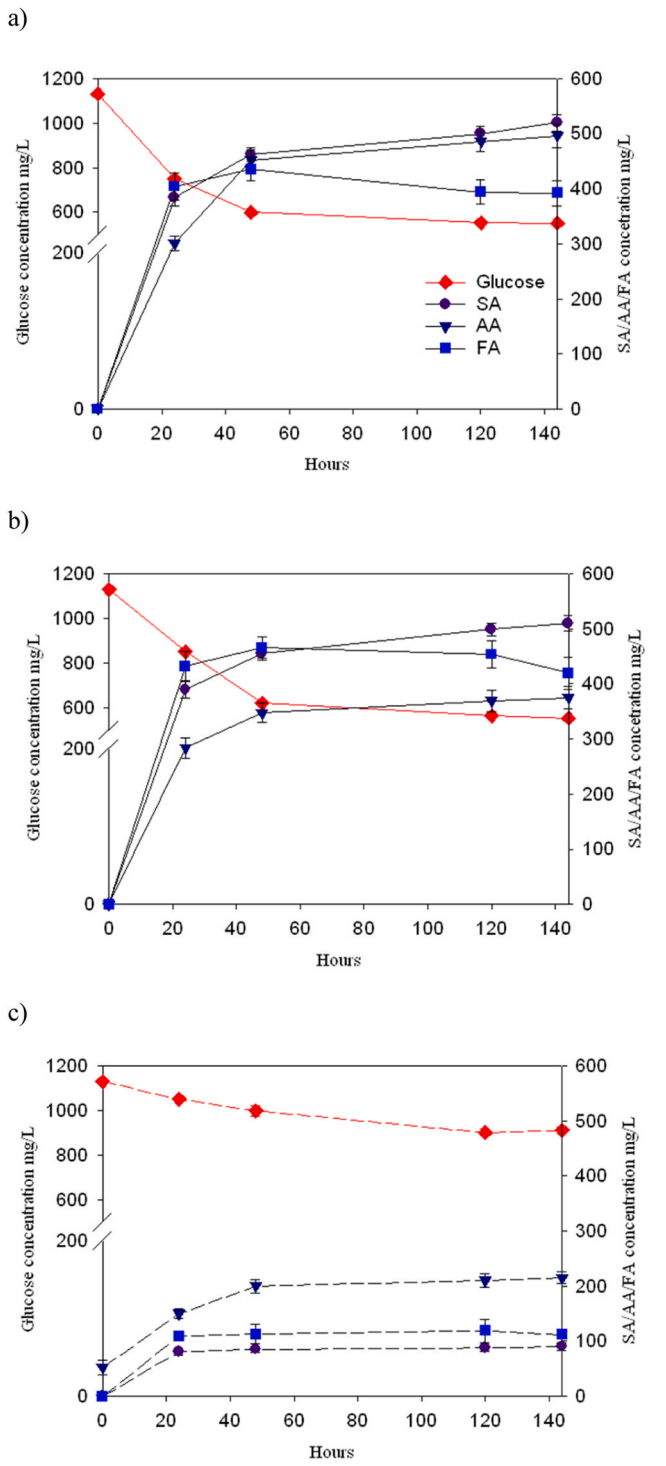

Fig. 6. Glucose and organic acid concentration (Succinic acid SA, Acetic acid AA, Formic acid FA) in: a) standard growth medium supplemented with glucose 1.1 g/l, b) standard growth medium supplemented with glucose 1,1 g/l and xylose 0.4 g/l, c) wheat straw hydrolysate (high concentration of sugars and inhibitors). Error bars indicated standard deviation.

FA decreased (Figs. 4–5).

This selectivity value was also calculated in the presence of acetic acid at the initial concentration of 21 mg/L given the higher production of succinic acid compared to the other byproducts (acetic and formic acid) (Table 7).When the hydrolysate was used (Fig. 6c), the strain underwent a reduction in growth capacity with an observable initial latent phase (66 % inhibition rate) demonstrated by the trend of glucose concentration reaching only at the end of the experiment a concentration of 910 g/L when the growth inhibition rate dropped to 59 %. At the end of fermentation the SA produced was 90±0.7 mg/L with a predominance of production of the other organic acids especially AA with a concentration of 215 ± 2.7 mg/L. In fact, the SA/AA ratio decreased to 0.42 and the selectivity reached 0.2 $g_{SA}/g_{SA} + g_{AA} + g_{FA}$. The best yield of SA was obtained at 24 h with the 8.2 % $g_{SA}/g_{glucose}$ respect to the initial glucose amount.

A slower initial phase of glucose consumption when using a dilute lignocellulosic biomass hydrolysate, and a reduction in succinic acid production were also observed by the authors Salvachúa et al., [58]. In this study, a corn stover hydrolysate obtained by acid hydrolysis at 120 °C was tested. Glucose was firstly utilized by the *A. succinogenes* strain but whose consumption started as well as for xylose after 48 h when the strain grew in hydrolysates at furfural concentration 1.7 g/l, HMF 0.17 g/L and acetic acid at 2.3 g/L and 5.8 g/L. SA production also started with latent phase after 48 h, and in the hydrolysate with higher acetic acid concentration, SA concentration was 4 times lower than in the control with productivity almost 10 times lower (0.7 g/L/h) at the time when productivity was highest in the control (6.36 g/L/h) [58].

The results obtained in this work on SA production by using the two diluted wheat straw hydrolysates were certainly lower than the results already obtained using a wheat straw hydrolysate after alkali treatment obtaining SA production of 18.9 g/L with a yield of 74 % from 19.4 g/L glucose with 57 % sugar consumption [54]. It should be noted, however, that in the work of Zheng et al. the lowest succinic acid production performance was precisely observed when wheat straw was used compared to hydrolysate of other biomasses such as corn straw and core, and rice straw. The difficulty to be degraded by microorganisms was related to the complex, multi-layered structure of wheat straw composed of the crystalline cellulose with a density of 1.59 g/cm^3 higher than the amorphous hemicelluloses and lignin, the hemicelluloses, lignin, and the complex formed between lignin and carbohydrates [51]. For this reason, this challenging feedstock was chosen in this study for a preliminary assessment of succinic acid production by using wheat straw hydrolysate as unique source and to assess the effect of the inhibitors (acetic acid and furfural) produced after its pre-treatment by steam explosion and enzymatic treatment. Cimini et al., tested several dilution of *Arundo donax* hydrolysate obtained from acid hydrolysis (acetate buffer), sterilization and enzymatic hydrolysis for SA production by the strain *Basfia succiniciproducens*. The authors observed as SA yield decreased as the concentration of the hydrolysate increased till to 90 % that contained the highest inhibitors concentrations (AA 8 g/L, HMF 63 mg/L, furfural 45 mg/L). The increase of inhibitor concentrations caused a decrease in glucose and xylose consumption and an onset of succinic acid production after 24 h with a productivity of 0.09 g/L/h [55]. The trends of decrease of SA production when using wheat straw hydrolysate were common problems highlighted in most of the papers where lignocellulosic biomass were used as feedstock. It should also be pointed out that in works in which lignocellulosic biomass such as sweet sorghum bagasse or rapeseed straw was pre-treated by acid hydrolysis or steam explosion and used as a substrate for *A. succinogenes* strain, SA yield was quite lower such as 61 % or 48 % [24,25] compared to studies in which modified strains (E. coli AS1600a) were used, which was able to produce SA at the yield of 86 % in the presence of inhibitors at the total concentration of 4.3 g/L [27]. Recently, in order to implement the utilisation of wheat straw for succinic acid production, the fungus *Aspergillus niger* was engineered by using the RNP-based CRISPR-Cas9 system to obtain an increase in succinic acid production up to 15 g/L from 100 g/L glucose at a yield of 15 % $g_{SA}/g_{glucose}$ compared to the wild type, that did not show SA production under the same conditions [61]. The same engineered strain was tested for succinic acid production by using sugarcane molasses and wheat straw hydrolysate filtered and supplied to micronutrients (biotin), calcium carbonate and ammonium nitrate, and other minerals salts. The authors (Yang *et al.*) obtained higher succinic acid production by using molasses (20 g/L) from

110 g/L sucrose, 55 g/L glucose and fructose respectively at a yield of 20 % g_{SA}/g_{sugars}. When wheat straw hydrolysate was tested the authors observed a delayed phase and the production of succinic acid started after 48 hours when was used, and a less final concentration of SA equal to 9 g/L was obtained after 144 hours at a yield of 9 % g_{SA}/g_{sugars} from an initial stock of sugars of 45 g/L xylose and 65 g/L glucose [61].

In addition, the obtained results on SA production by using a glucose rich medium were in line with the results obtained by other authors as Zhou *et al.*, that performed batch tests without the use of any fermentation strategies, without resorting to direct and indirect sources of CO_2 or pH modifiers, that might be essential in improving SA production [62].

The yield obtained in this study (8.2 $g_{SA}/g_{glucose}$) where *A. succinogenes* was used, and the achieved SA production by using a wheat straw hydrolysate (90±0.7 mg/L) as the unique carbon source without the supplementation of nitrogen source and the lag phase observed in the 24 hours are in line with the results obtained by Yang et al. that tested the engineered fungus *A. niger* for the production of SA from wheat straw hydrolysate.

In this study, a concomitant cause of the presence of the inhibitors in wheat straw hydrolysate and resulting in low SA production was the deficiency of a nitrogen source such as yeast extract. Indeed, Putri *et al.* showed that as the concentration of yeast extract increased in the process for the production of succinic acid from rice straw, the yield and concentration of SA increased too [29].

The results presented in literature showed that the use of wheat straw is quite challenging for succinic acid production. The findings achieved in this study from these preliminary batch fermentation tests emphasized the ability of the strain *A. succinogenes* to utilize the glucose feedstock contained in the wheat straw hydrolysate, despite the synergistic action of the inhibitors and the absence of nitrogen sources, the starting more concentrated inoculum seemed a great strategy to increase resistance against inhibitors normally present in wheat straw hydrolysate.

4. Conclusion

The wheat straw hydrolysate was used as unique carbon source without nitrogen supplement as low-cost feedstock for succinic acid production. The wheat straw hydrolysate contained inhibitors as acetic acid and furfural generated from steam-explosion pre-treatment. This study evidenced as different inhibitory effects was observed in relation to the growth of the strain, and succinic acid production performance in supplemented rich growth medium and in the crude hydrolysate. The presence of more carbon sources (glucose and xylose) in the hydrolysate decreased the production of acetic acid respect to succinic acid. And the use of a more concentrated inoculum seemed to be a great strategy to overcome the inhibition effects of unwanted compounds in wheat straw hydrolysate. The results obtained can surely improve the knowledge towards a better valorization of wheat straw residues for succinic acid production. Certainly, the use of wheat straw hydrolysate needs to be further investigated to develop targeted strategies for pre-treatment and fermentation to improve succinic acid production.

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CRediT authorship contribution statement

Patrizia Casella: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Raffaele Loffredo:** Methodology, Investigation, Data curation. **Maria Rao:** Writing – original draft, Validation, Supervision, Methodology. **Roberto Balducchi:** Supervision, Project administration, Funding acquisition. **Federico Liuzzi:** Methodology, Investigation. **Isabella De Bari:** Methodology, Investigation. **Antonio Molino:** Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.procbio.2024.08.017.

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