

## Succinic Acid Production from Hexoses and Pentoses by Fermentation of *Actinobacillus succinogenes*

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Succinic acid is a precursor for many chemicals in the food, pharmaceutical, cosmetic, and biodegradable plastics industries. Nowadays, the biotechnological route to produce succinic acid is focused on processes based on the fermentation of natural materials characterized by high fraction of carbohydrates. *Actinobacillus succinogenes* is proposed in the literature as a promising strain for the production of succinic acid. Indeed, *A. succinogenes* may utilize a wide spectrum of carbohydrates as carbon sources. Moreover, the CO<sub>2</sub> required for the fermentation allows to include the biotechnological route among the carbon capture and conversion processes.

A key issue of fermentation processes aimed at producing succinic acid is the feedstock cost. Pre-requisites of the feedstock are: availability at high mass flow rate over all the year, and non-competitive with (human and animal) food. Lignocellulosic biomass is a potential feedstock because it fulfils the listed pre-requisites.

This contribution reports the characterization of succinic acid fermentation by *A. succinogenes* 130Z adopting as carbon source sugars representative of the hydrolysis of lignocellulosic biomass: glucose, mannose, arabinose, and xylose. Batch fermentation tests were carried out using single sugar as carbon source for a systematic characterization of the conversion process of the investigated sugars.

The conversion process was characterized in terms of concentration of biomass, sugars, and acids as well as of pH. The time-resolved data were processed to assess the sugar conversion, the succinic acid yield, and the productivity for each of the investigated sugars.

*A. succinogenes* was able to convert all investigated sugars into succinic acid using MgCO<sub>3</sub> as an indirect CO<sub>2</sub> source. Glucose was the sugar characterized by the best performance when the initial concentration was set at 40 g/L. The performances did not depend on the investigated sugars if the initial concentration was quite low (<5 g/L).

### 1. Introduction

The limited nature of fossil reserves, the increasing worldwide demand for durable consumer goods, and the increasing environmental concerns are driving forces for a net reduction of the worldwide consumption of resources. A contribution to mitigate the catastrophic effects of the listed issues may be offered by efficient biotechnological processes converting renewable resources (Willke and Vorlop, 2004).

Succinic acid (SA), a dicarboxylic acid also known as amber acid, is a well-established bio-based platform for chemicals and intermediates (Bozell and Petersen, 2010). The global market of succinic acid has been estimated to range between 30'000 and 50'000 tonnes per year in 2011. Moreover, the market is expected to grow at an annual rate of 18.7 per cent from 2011 to 2016 ([www.marketsandmarkets.com](http://www.marketsandmarkets.com), 2012). Main markets of the succinic acid are: i) surfactants, additives, foaming agent and detergent. This is the largest market; ii) the use as an ion chelator to prevent corrosion and pitting in the metal industry; iii) the food market where it is used as acidulate agent, pH regulator, anti-microbial and flavouring agent; iv) the pharmaceutical industry where it is used as an additive for the production of vitamins, antibiotics and amino acids (Zeikus et al, 1999). Moreover, the terminal carboxylic acid groups open up numerous possibilities for further processing.

Major developments include polymerisation of SA with its hydrogenated diol product (1,4-butanediol) to produce the biodegradable plastic polybutylene succinate (PBS).

The conventional process to produce SA is via the (petrol)chemical route: butane or benzene via the conversion of maleic anhydride to succinic anhydride and successive hydrolysis. SA may also be produced by the oxidation of 1,4-butanediol and by the carbonylation of ethylene glycol. Recently, the trend is the replacement of the conventional (petrol)chemical route with the biotechnological route: production of succinic acid by microbial fermentations. The biotechnological route may replace the petrochemical-based route with marked environmental benefits. Indeed, the production of succinic acid may adopt renewable resources (e.g. biomass and CO<sub>2</sub> streams) as feedstocks. Therefore, the succinic acid production via the biotechnological route contributes to reduce the request of fossil resources and mitigate the release of CO<sub>2</sub> in the atmosphere (McKinlay et al., 2005).

Several succinic acid producer gram-negative bacteria have been isolated in anaerobic environments. The most documented SA producers are wild type strains of *Actinobacillus succinogenes* (Guettler et al., 1999), *Mannheimia succiniciproducens* (Lee et al., 2002), *Anaerobiospirillum succiniciproducens* (Samuelov et al., 1991) and various recombinant strains of *Escherichia coli* (Jantama et al., 2008) and *Corynebacterium glutamicum* (Litsanov et al., 2012). *A. succinogenes* - a Gram-negative capnophilic bacterium isolated from the bovine rumen - is considered to be one of the most promising strains for industrial succinic acid production because of its ability to produce a comparatively large amount of succinic acid. Moreover, *A. succinogenes* can utilize a variety of carbohydrates as carbon sources, including glucose, lactose, xylose, arabinose, cellobiose, and other sugars (Guettler et al., 1999; Van der Werf et al., 1997). To the author knowledge, no systematic investigation has been carried out to characterize the fermentation of these sugars.

A key parameter for the success of the bioprocess on industrial scale is the cost of the fermentation substrate. Potential feedstocks for the production of succinic acid by fermentation are lignocellulosic materials as wood and agricultural residues (Akhtar et al., 2014). Lignocellulose is the most abundant renewable resource on the planet and it has great potential as substrate for fermentation because of the un-competitiveness with food resources. Its use as feedstock requires a pretreatment and hydrolysis to release the simple sugars to be fermented: glucose, mannose, xylose, and arabinose.

This contribution reports a recent activity on succinic acid production by fermentation of glucose, mannose, xylose, and arabinose. This activity is part of the national research project "Development of green technologies for production of BIOchemicals and their use in preparation and industrial application of POLImeric materials from agricultural biomasses cultivated in a sustainable way in Campania Region – BIOPOLIS" PON03PE\_00107\_1/1. The preliminary characterization of the fermentation process was carried out in batch tests. Each test was characterized in terms of succinic acid yield and maximum concentration.

## 2. Materials and Methods

### 2.1 Microorganism and media

*Actinobacillus succinogenes* DSM 22257 by DSMZ was used. Stock cultures were reactivated according to the method suggested by the supplier and stored at -80°C.

The thawed cells were inoculated into 10 mL Brain Heart Infusion Broth medium in 15 mL Hungate tubes (pre-cultures). Vials were inoculated by a syringe to ensure anaerobic conditions. The cultures were carried out for 24h under agitation (rotary shaker at 150 rpm) at 37 °C .

The composition of the fermentation medium was: 5 g/L yeast extract, 1 g/L NaCl, 0.3 g/L Na<sub>2</sub>HPO<sub>4</sub>, 1.4 g/L NaH<sub>2</sub>PO<sub>4</sub>, 1.5 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.2 g/L MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.23 g/L CaCl<sub>2</sub>·2H<sub>2</sub>O (Van Heerden and Nicol, 2013). MgCO<sub>3</sub> was also supplied in the fermentation medium at concentration of 5 or 20 g/L to act as an indirect CO<sub>2</sub> source and to buffer the pH during growth. The medium was heat sterilized (15 min at 121 °C) in anaerobic bottles sealed with a butyl rubber stopper with a nitrogen headspace (Raganati et al., 2014). MgCl<sub>2</sub>, CaCl<sub>2</sub> and sugars were prepared separately as concentrated stocks (MgCl<sub>2</sub> and CaCl<sub>2</sub> at 100 times the operating concentration; sugar at 300g/L), sterilized by filtration and supplemented aseptically to the autoclaved medium.

### 2.2 Batch fermentation tests

The experiments were carried out in 100 mL anaerobic bottles containing 75 mL of medium. The medium was inoculated with a 6.25 % (v/v) suspension of actively growing pre-cultures. The bottles were placed on a rotary shaker at 150 rpm and incubated at 37°C. Fermentation samples were taken every 2-3 h to measure optical density (OD), metabolites concentration and pH. The sugars investigated were: glucose, mannose, arabinose, and xylose. Sugar concentration was set at 5 and 40 g/L: both values were selected as representative of the typical concentration interval of lignocellulosic hydrolysate. Each test was carried out in duplicate and the mean values are reported as results.

The batch fermentations were characterized in terms of cell growth, pH, sugar conversion, and metabolites (acids and solvents) production. In particular, the measured data were worked out to assess the following parameter:

- overall sugar conversion ( $\xi_S$ ), the ratio between the sugar converted and the initial sugar  $(S_0 - S)/S_0$ ;
- sugar-to-product “i” yield coefficient ( $Y_{i/S}$ ), the ratio between the produced mass of product “i” (cells or succinic acid) and the related decrease of the substrate mass.;
- SA specific productivity ( $P_{SA}$ ), the ration between the SA concentration and the fermentation time.

### 2.3 Analytical methods

Cell density was measured as optical absorbance at 660 nm ( $OD_{660}$ ) using a spectrophotometer (Cary- 50 Varian) and converted to dry biomass concentration. pH was also measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). Dry cell mass (DCM) was determined from 10-mL samples centrifuged at 5000 rpm for 15 min at 4°C. Cell pellets were washed twice with distilled water and centrifuged between washes, then dried at 60 °C for 24 h under vacuum. The calibration procedure yielded: 1.0  $OD_{660}$  was 0.377 g/L of DCM.

The concentration of soluble species was measured in the liquid phase after sample centrifugation (13000g, 10min). Sugar and organic acid concentration was measured by high performance liquid chromatography (HPLC) using a HP1100 chromatography working station system (Agilent Technologies, USA) equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm×7.8 mm, 9  $\mu$ m; Bio-Rad Chemical Division, Richmond, CA), a UV absorbance detector (Agilent Technologies, G1315D), and a refractive index detector (Agilent Technologies, G1362A).  $H_2SO_4$  5mM was used as mobile phase at 0.6 mL/min flow rate at room temperature. The injection volume was 20  $\mu$ L.

### 3. Results

Investigations were aimed to assess the ability of *A. succinogenes* to ferment sugars typically present in the hydrolysed fraction of lignocellulosic biomass and on the effects of some operating conditions on the process performance. The tested sugars included glucose, mannose, arabinose, and xylose. Two different initial sugar concentrations were investigated: 5 g/L and 40 g/L. The time resolved concentration of sugar and metabolites was measured.

Figure 1 reports data measured during a typical batch fermentation test carried out at initial glucose concentration of 40 g/L. In the graph are reported the pH and the concentration of cells growth, pH, sugar and the SA as a function of the time. The fermentation was stopped when the concentration of the metabolites were constant for more than 24 hours.

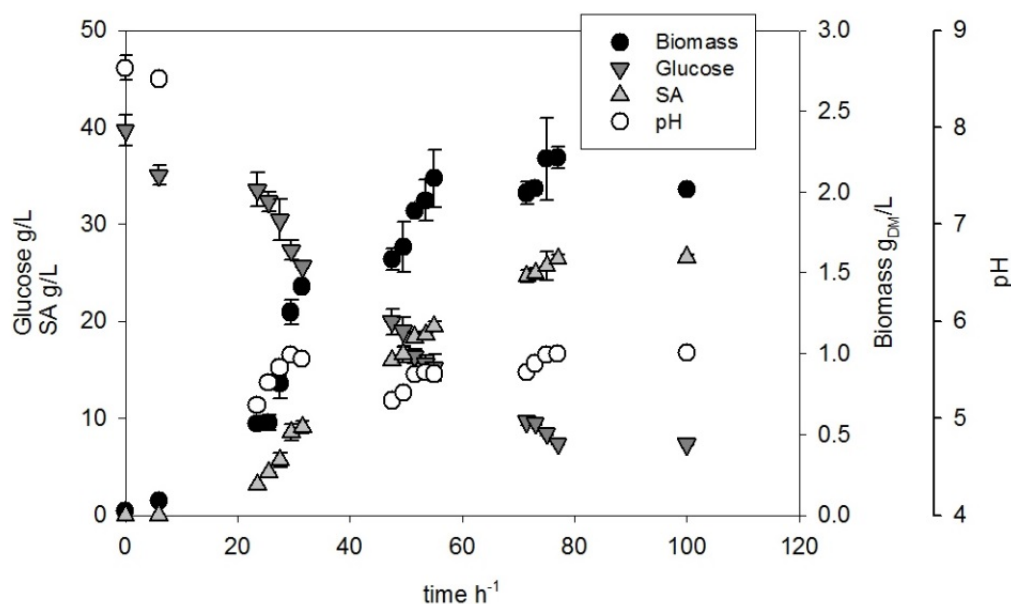


Figure 1: Time resolved concentration of biomass, glucose, SA, and pH measured during the *A. succinogenes* fermentation. Sugar: glucose. Carbone source initial concentration: 40 g/L.

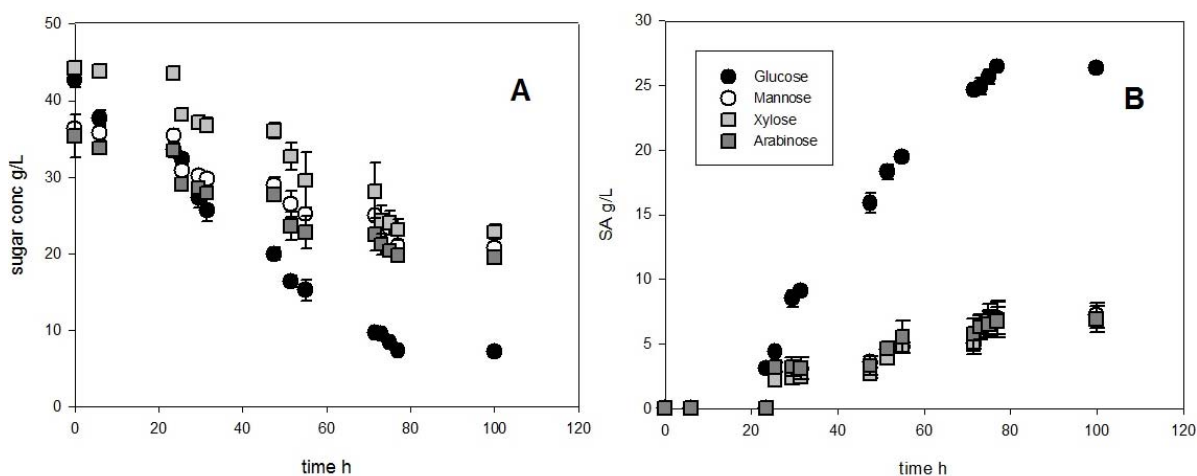
It should be mentioned that due to the presence of  $MgCO_3$ , the pH in the fermentation broth at the beginning was higher than 8. When the production of the acids started, the pH decreased to approach a constant value between 5 and 6.

Acetic acid and formic acid (data not shown) were also produced during the fermentation along with succinic acid, but at lower concentration. The final concentrations of the three acids produced are reported in Table 1.

*Table 1: Final concentration of the acids produced during the fermentation carried out at initial glucose concentration of 40g/L*

3.1.1.	3.1.2. Final concentration, g/L
3.1.3. Succinic acid	3.1.4. 26.6
3.1.5. Acetic acid	3.1.6. 6.0
3.1.7. Formic acid	3.1.8. 3.5

*A. succinogenes* had a similar growth pattern in all the fermentations and it was able to convert all sugars in acids. Figure 2 reports the time-series of sugar concentration and SA concentration measured during the fermentation tests carried out with the four investigated sugars at initial concentration set to 40 g/L. The fermentation lasted about three days. Except for xylose, no fermentation lag phase was observed. As regards xylose, about one day lag phase was observed. Figure 2A shows that: i) *A. succinogenes* was able to convert the investigated sugars; ii) the conversion degree depended on the sugar. Glucose was the sugar characterized by the best performances in terms of SA titer, SA yield, and sugar conversion degree. The performances assessed for mannose, arabinose, and xylose fermentation did not depend on the sugar and they were lower than that assessed for glucose fermentation. Results reported in Figure 2 cannot be directly compared with results reported in the literature. To author knowledge, no data are available in literature as regards batch tests with single sugar. However, the observed fermentation path was in agreement with that reported in the literature for tests carried out with sugar mixtures (Jiang et al., 2012).



*Figure 2: Time resolved concentration of sugar (A) and succinic acid (B) measured during *A. succinogenes* fermentation. Sugar initial concentration: 40 g/L*

Figure 3 reports the time-series of sugar concentration and SA concentration measured during the fermentations tests carried out with the four investigated sugars at initial concentration set at 5 g/L. The fermentation was almost completed after two days. In particular, the glucose fermentation ended for substrate depletion. No fermentation lag phase was observed for all sugars. It is worth to note that there were no significant differences in terms of sugar consumption and SA production profiles for the four sugars at this initial concentration. Glucose confirmed to be the sugar characterized by high performance even though the small differences.

Table 2 reports the results of all the fermentation tests. The SA selectivity was about constant in all the tests with values ranging between 0.50 and 0.58 g/g.

It is worth to note that for the lowest concentration investigated (5 g/L initial sugar) the performances in terms of sugar conversion degree, SA yield and productivity were comparable, indicating that there was not a strong sugar preference.

However, at initial sugar concentration set to 40 g/L, glucose was the sugar characterized by the best performances. In particular, glucose fermentation stopped when SA concentration approached a value of about 25 g/L while mannose, arabinose and xylose fermentation stopped when SA concentration approached a value of about 7 g/L, despite the sugar conversion was not complete whatever the sugar.

As it could be expected, the maximum SA productivity assessed for glucose fermentation increased with the initial concentration of the sugar as a result of the higher production rate and final SA concentration.

Moreover, the increase of the initial concentration of mannose, arabinose, and xylose from 5 to 40 g/L had only a slight effect on the maximum SA specific production. A possible explanation is that the average rate of the SA production increased as much as the SA concentration when the initial sugar concentration increased from 5 to 40 g/L.

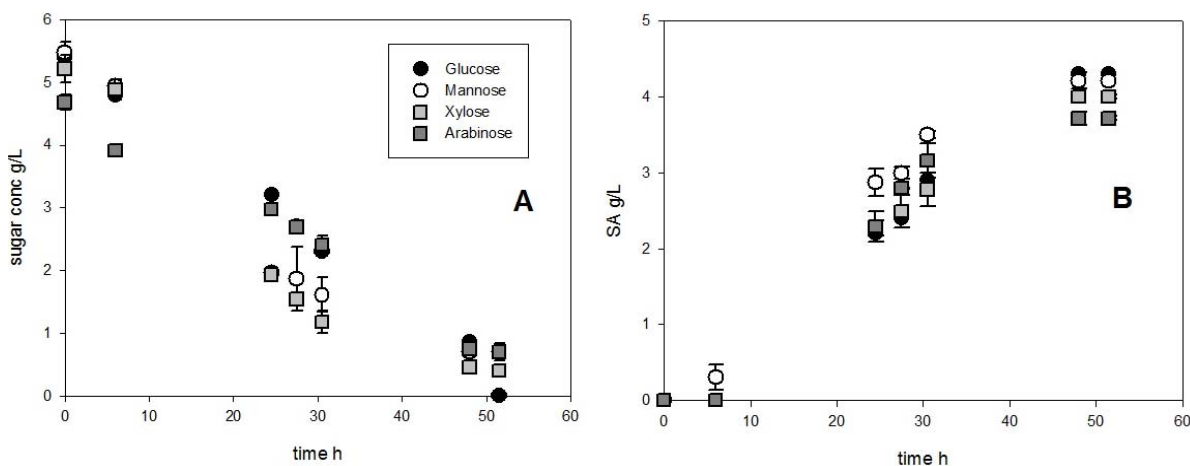


Figure 3: Time resolved concentration of sugar (A) and succinic acid (B) measured during *A. succinogenes* fermentation. Sugar initial concentration: 5 g/L.

Table 2: Main data regarding fermentation of *A. succinogenes*. The reported data were the value measured at the end of the fermentation tests. SA productivity ( $P_{SA}^{MAX}$ ) was the maximum value assessed for each sugar.

Initial sugar concentration, g/L	Sugar	Conversion degree, $\xi$	SA g/L	$Y_{SA/S}$ g <sub>SA</sub> /g <sub>S</sub>	$P_{SA}^{MAX}$ g <sub>SA</sub> /L h
5	Glucose	1	4.3	0.80	0.10
	Mannose	0.84	4.2	0.85	0.15
	Xylose	0.91	4.1	0.84	0.09
	Arabinose	0.84	4.0	0.86	0.10
40	Glucose	0.83	26.5	0.75	0.36
	Mannose	0.42	7.0	0.46	0.11
	Xylose	0.48	8.5	0.32	0.09
	Arabinose	0.44	6.8	0.43	0.13

#### 4. Conclusions

The results obtained from fermentation tests on sugars typically present in the hydrolysed of lignocellulosic biomass pointed out that *A. succinogenes* was able of utilizing products of lignocellulosic biomass hydrolysates for the production of succinic acid. Glucose was the sugar characterized by the best performance when the initial concentration is set to 40 g/L. The performances are comparable for the four sugars investigated if the initial concentration is quite low (5 g/L). Further investigation on sugar mixtures - composition miming a typical lignocellulosic biomass hydrolysate - will elucidate the synergic effects on the fermentation performances.

The kinetic characterization is also required for the design and optimization of a fermenter to produce succinic acid.

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