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Succinic Acid Production as Main Player of the Green Chemistry Industry by using Actinobacillussuccinogens

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The growing consciousness for environmental issues have stimulated the search for more sustainable biochemical processes. One of the most attracting chemicals with enormous potential in a biobased economy is succinic acid (SA, butanedioic acid or 1,2-ethanedicarboxylic acid). This compound represents the chemical building block for various high value-added substances which find applications in the detergent/surfactant, food, ion chelator and pharmaceutical markets. Nowadays, the SA market is estimated to be about 20000–30000 ton per year worldwide and its industrial manufacture is mainly based on the catalytic hydrogenation of petrochemically derived maleic acid or maleic anhydride. However, since SA is fermentative product and an intermediate of several biochemical pathways, including the tricarboxylic acid cycle, it can be produced by many microorganisms. The biological SA production at commercial scale and potentially for the commodity chemical market is still a challenging target and requires the development and the optimization of microorganisms-based processes able to guarantee high product concentrations to justify economically feasible recovery.
The aim of this paper is the conversion of a mixture of sugars, mainly containing glucose, into SA by SA-producing strain *Actinobacillussuccinogens*, that was isolated from the bovine rumen. It is a facultative anaerobic, pleomorphic, Gram-negative rod. Experimental tests were carried out by using *Actinobacillussuccinogens* bought by Culture Collection of Goteborg and by using TBS (Tryptic Soy broth) by changing the concentration of glucose as well as that of other sugars as fructose, xylose, sucrose with the main focus to evaluate the effect of the fermentation for the *Actinobacillussuccinogens* with the single sugars in a concentration range from 1 to 15 g/l. All the experiments were carried out in anaerobic conditions at 37°C and in a pH range 7-8 for several days. For each test, carboxylic acids and sugars were analyzed by using u-HPLC while the growth of the selected microorganism was monitored by means of a spectrophotometer at 600nm for OD tests.

1. Introduction

SA (1,4-butanedioic acidor 1,2-ethanedicarboxylic acid) is a four-carbon dicarboxylic acid, called also as amber acid and butanedioic acid. This compound belongs to the group of organic acids with potential industrial applications in pharmaceutical, agricultural and food sectors. SA naturally occurs in plant, animaltissue and microorganisms because it is an intermediate of a fundamental metabolic pathway, i.e. the tricarboxylic acid cycle (TCA). A very huge applicationisrecognised for SA as surfactants, detergents, foaming agent, corrosive inhibitors in metal industry, flavouring agent and pH regulator in food industry (Akhtar et al., 2014). SA is also a prominent building blocks recognized as one of top high valueadded bio based compounds that could substitute products from petroleum based feedstocks (Werpy& Petersen 2004). SAis

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employed for the production of important chemical products as 1,4 butanediol (BDO), tetrahydrofurane (THF), gammabutyrolactone (GBL) and derivative polymer as polybutylene succinate for bio-plastics'production.
Until 2013 the production of SA was mainly dominated by fossil fuels with a production greater than 50000 metric tons/year while in the following years there was a change towards bio-based production until to 150000 metric tons/year (Pinazo et al., 2015). In addition, the production of bio-based SA is expected to reach 600 kilo tonnes by 2020 with a market value of around $530 million. (Taylor et al., 2015). At the moment, the price of chemically produced SA is still lower (2.6-2.8 $/kg SA) than that produced biologically (2.9 $/kg SA). However, the trend towards processes that have less impact on the environment and that can exploit renewable resources calls for a reduction in the price of bio-based SA. This can be reached, for example, through the possibility of exploiting different available renewable resources feedstocks from agro-industrial, food and textiles sector (Li et al., 2019). SA is chemically produced through the oxidation of n-butane, or aldehydes into maleic anhydride, and subsequent hydration to maleic acid followed by hydrogenation (Joeri et al., 2010) while the bio-based pocess is principally carried out by microbial fermentation of rich-sugars biomasses (Nhuan et al., 2017).
Actinobacillus succinogenes is one of the most promising succinic acid producing strains. It is an anaerobic facultativestrain, pleomorphic, Gram-negative rod first isolated from bovine rumen (Guettler et al., 1999). *A. succinogenes* can utilize a wide range of sugars and it can tolerate high glucose concentration until to 143 g/L producing up to around 70 g/l of SA (Lin et al., 2008). Different studies have investigated the production of SA by *A. succinogenes* at different concentrations of glucose or other sugars such as xylose and sucrose. Bradfiled et al. 2015 tested the production of SA from the fermentation of a xylose rich hydrolysate in a biofilm reactor obtaining a production of 32.5 g/l of SA using 57 g/l of xylose while, Jiang et al., 2014 demonstrated a production of 57 g/l of SA by using a concentration of sucrose equal to 100 g/l.
In comparison to the state of art, the production of SA by *Actinobacillus succinogenes* by using different concentrations of glucose and mixtures of sugars such as xylose, fructose and sucrose, was tested in this work. Bacterial growth was measured by optical density and correlated to sugars and organic acids (succinic, formic and acetic acids) concentration, measured by uHPLC-DAD-ELSD.

2. Materials and Methods

Actinobacillus succinogenes 130Z (CCUG-43843) strain was purchased as freeze-dried pellets from the Culture Collection University of Gothenburg (CCUG, Gothenburg, Sweden). The strain was inoculated into Tryptic soy broth (TSB) (22092-500G SIGMA) containing 17.0 g/l peptone from casein, 3.0 g/l peptone from soy, 5.0 g/l NaCl, 2.5 g/l K_2HPO_4, 5.0 g/l D-(+) glucose. The medium was sterilized for 15 minutes at 121 °C before using it. Growth occurred at 37 °C according to the supplier's information, pH7.8, under anaerobic conditions and agitation at 120 rpm. The growth and the production of organic acidsweres tested both on single monosaccharides and on mixtures of monosaccharides according to experimental set-up in table 1.

Table 1: D-(+) glucose, fructose, sucrose and D-(+)xylose tested concentration

	D-(+)Glucose Conc.	Fructose Conc.	Sucrose Conc.	D-(+)Xylose Conc.
	g/l	g/l	g/l	g/l
TEST-1	4.9	-	0	0
TEST-2	11.4	-	0	0
TEST-3	5.1	0.45	0	0
TEST-4	4.9	-	1.0	0
TEST-5	5.1	-	0	0.65

The bacterial growth was monitored at specific intervals by measuring the optical density at a wavelength of 600 nm. After spectrophotometric measurement of the OD, the sample was centrifuged for 5-10 min at 4°C and 4000 rpm. The supernatant was filtered bya nylon syringe filter (porosity 0.22 µm) and stored at -20 °C for the subsequent quantification analysis of monosaccharides and organic acids. The concentration of individual monosaccharides and organic acids in the fermentation broth was detected by u-HPLC 1290 Infinity II (Agilent) using ELSD (Evaporative Light Scattering) and DAD (Diode Array Detector) detectors, respectively. Monosaccharide quantification was performed using the InfinityLabPoroshell 120 HILIC-Z column, 2.1 × 100 mm, 2.7 µm (p/n 685775-924 Agilent) at a temperature of 80 °C, by using a mobile phase of ammonium acetate (100 mM, pH 7.0) and acetonitrile 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 to a temperature of 60 °C, nitrogen flow rate equal to 3.5 psi, 30Hz signal frequency. The chromatographic conditions were fixed by followingthe Agilent 5991-

8984EN application note. Prior performing the analysis, analytical standards D-(+)Glucose, Fructose, D-(+)Xylose (47267,Supelco), Sucrose (47289, Supelco) and samples were added toacetonitrile and water (9:1 v/v) and then injected at a volume of 2.5 µl. Organic acids (succinic, acetic, formic acid) weresmeasured by using the Hi-Plex H column, 7.7 x 300 mm, 8 µm (p/n PL1170-6830 Agilent) 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 (Agilent 5991-8984EN). Fermented broth samples and analytical standards of succinic, acetic, formic acid (47264, Supelco) were injected at a volume of 20 µl. Organic acids were identified at a wavelength of 210 nm.

3. Results and Discussion

The firsts two experimental tests were carried out at two glucose concentrations, i.e. 4.9g/l and 11.4g/l by fixing the yeast extract concentration at 156ppm. Results are showed in the figure below (Figure 1).

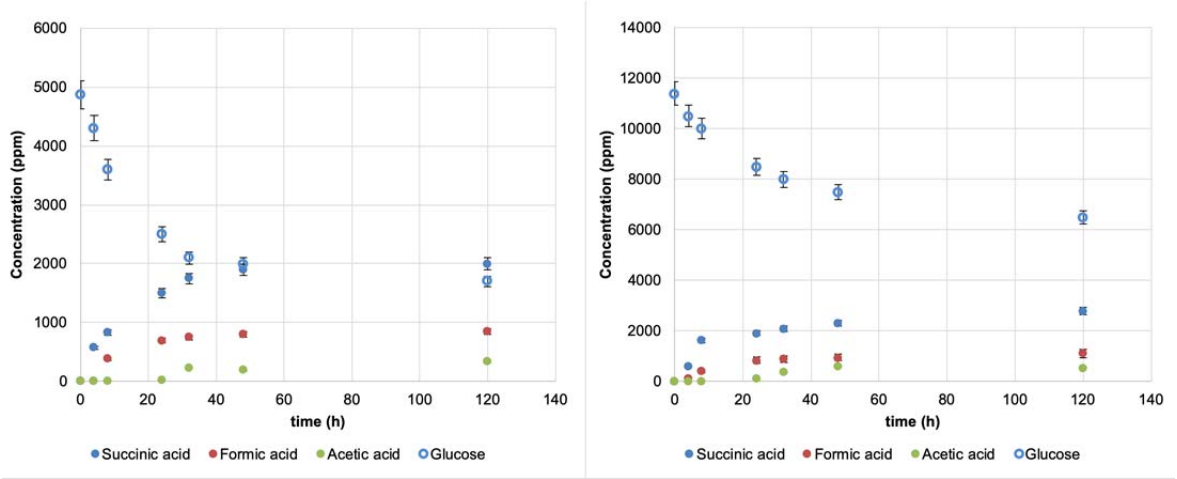

Figure 1: Glucose, SA, Formic acid, Acetic acid and Glucose concentration versus time. On the left, starting from 4.9g/l of glucose and on the right with 11.4g/l of glucose

Figure 1 shows asin both the analyzed cases glucose concentration achievesa steady state after 48h with glucose concentration that decreasetill to around 3000ppm. At the same time, SA concentration achieves its maximum value of 2.0g/l in the test carried out with glucose concentration lower than 2.8g/l obtained for the test carried out with 11.4g/l of initial glucose concentration.
The main difference of these two tests is related to the SA productivity at fixed time.
Defining SA productivity as:

$$SA\ productivity = \frac{SA\ concentration}{fermentation\ time}$$

and SA yield as:

$$SA\ yield = \frac{SA\ concentration}{Sugars\ concentration}$$

It is possible to evaluate these parameters for test-1 and test-2 as reported in the table 2.

Table 2: max SA concentration (g/l), SA yield and SA productivity at three time: 8h, 24h and 48h

			SA Productivity at: (g/l/h)		
	max SA conc. (g/l)	SA Yield (g/g)	8h	24h	48h
TEST-1	2.0	0.41	0.06	0.06	0.02
TEST-2	2.8	0.24	0.08	0.07	0.02

Table 2 evidences that also if the productivity value at 48h for both tests are the same, the main difference is related to the productivity at 8h as well as 24h. This effect can be explained by seeing the OD_{660} for cells as reported in table 3.

Table 3: Optical Density at 660nm for TEST-1 and TEST-2

	OD_{660} 4h	OD_{660} 8h	OD_{660} 24h	OD_{660} 32h	OD_{660} 48h	OD_{660} 120h
TEST-1	0.26	0.80	1.09	1.08	1.10	1.10
TEST-2	0.50	0.91	1.22	1.25	1.29	1.25

Table 3 highlights that during the fermentation stage with higher glucose concentration (11.4g/l), the OD at 660nm is higher than the test with glucose at 4.9g/l and therefore the yeast extract concentration is influenced by initial glucose concentration also if at the steady state the OD value in both cases is like the same.This effect was investigated by other research group which report similar results. Zheng studied the effect of glucose:xylose mixture obtaining lower SA concentration than the only glucose feed. In both cases, experimental tests started with a low yeast extract respect to the necessarycontent to avoid overgrowth of the yeast extract respect to that required for the fermentation. Further tests will start with a better glucose/yeast ratio for the next development of the experimentation.After these preliminary tests, fixing the glucose concentration at a value of about 5g/l, three tests were carried out by adding fructose, sucrose and xylose at initial concentration of 0.45, 1.0, 0,65 g/l respectively. Results are shown in Figure 2.

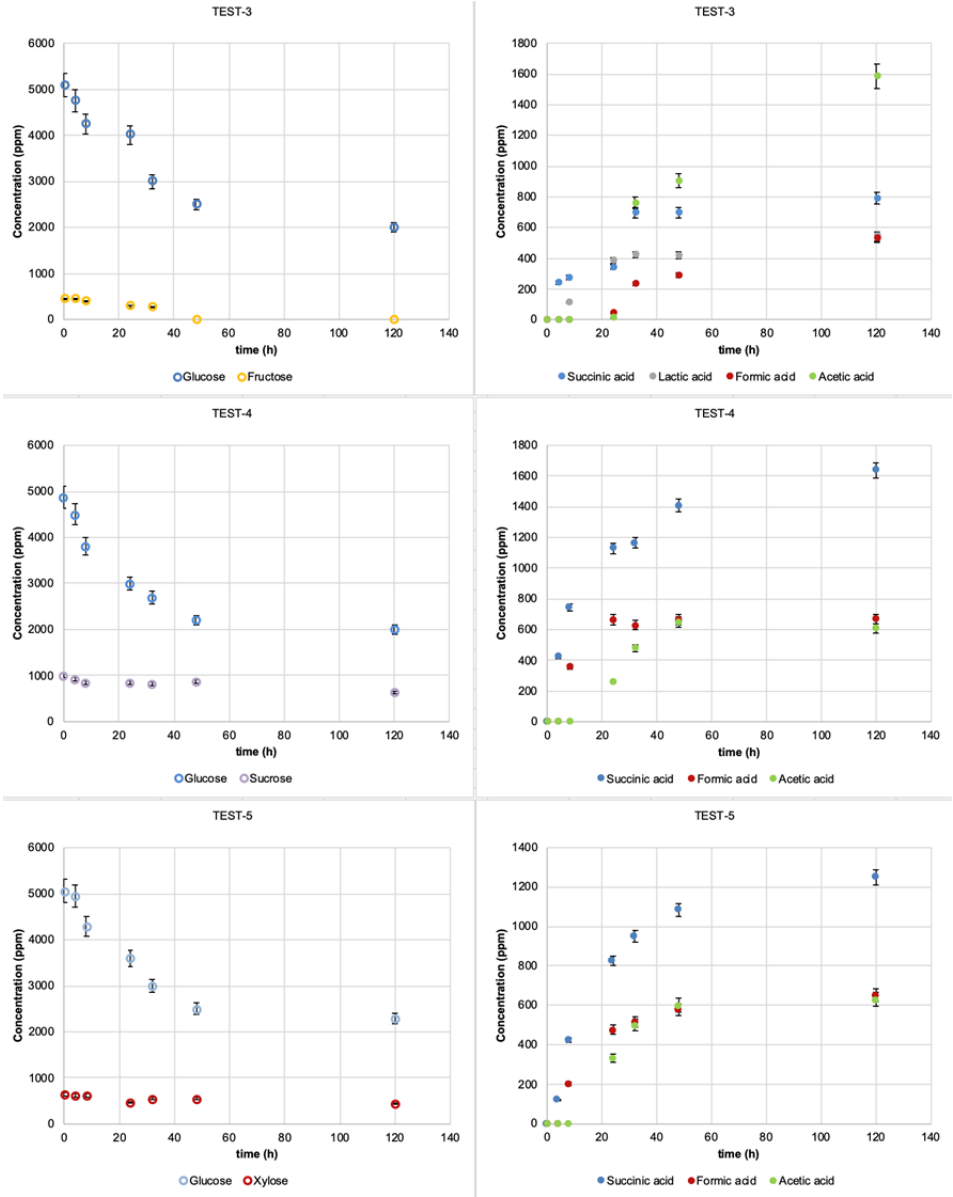

Figure 2: Glucose, SA, Formic acid, Acetic acid, Lactic acidversus time. TEST-3: Glucose: 5.1g/l – Fructose:0.45g/l. TEST-4: Glucose: 4.9g/l – Sucrose:1.0g/l. TEST-5:Glucose: 5.1g/l – Xylose:0.65g/l

Figure 2 shows that in all the experimental tests, glucose concentration achieves value of about 2000ppm at the end of the experiment. At the same timeit is possible to evaluate that the SA concentration does not reach the maximum concentration of 2g/l obtained in the test by using only glucose at initial concentration of 2g/l. In terms of sugars concentration, all the fructose is consumed during the anaerobic dark fermentation while sucrose and xylose are consumed for 30-35% of their initial concentration.
In the table 4 are reported the values related to the performance of the process for experimental tests performed in presence of sugars mixture in comparison with analogue tests with only glucose:

Table 4: SA concentration maximum value, yield and productivity for test 3-4-5 with sugars mixtures in comparison with test 1

			SA Productivity at: (g/l/h)		
	max SA conc. (g/l)	SA Yield (g/g)	8h	24h	48h
TEST-1	2.0	0.41	0.06	0.06	0.02
TEST-3	0.79	0.14	0.03	0.01	0.01
TEST-4	1.64	0.28	0.09	0.05	0.03
TEST-5	1.25	0.22	0.05	0.03	0.02

Table 4 shows that SA yield for experiments with sugars mixture is lower than the only glucose feed. In terms of productivity it is possible to observe that in the case of glucose in mixture with sucrose, after 8h the productivity is 90mg/(l*h) while by using only glucose is 60mg/(l*h). After 48h the productivity of SA for glucose:sucrose mixture is higher than the test with only glucose and this is in accordance with literature data (Cao et, al., 2018). The other two experimental tests conducted with glucose:fructose and glucose:xylose after 8h reflect a productivity of 30 and 50mg/(l*h) respectively that are lower than the same values obtained with only glucose but, in these cases the final productivity in these two conditions (120h) is similar to that in which only glucose is used (20mg/(l*h)).

4. Conclusions

SA production with dark anaerobic concentration can be considered a good strategy for renewable production of SA. More and more importance will assume the use of complex feedstock like lignocellulosic biomass of organic fraction of wastes. In this direction, the role of inhibitor represents the Achille'shell for the development of the integrated process.
In this work, the conversion of sugars mixture, mainly containing glucose, into SA by *Actinobacillus succinogens*, was investigated. The effect of changes in the content of glucose as well as that of other sugars as fructose, xylose, sucrose was evaluated by working under anaerobic conditions at 37°C and in a pH range 7-8. Sugar analyses indicated that the fructose, xylose and sucrose metabolism rate was slower than that of glucose. Meanwhile, it seemed that *A. succinogenes* did not take up most of the sucrose, xylose and sucrose through a direct transport system. After 48h of digestion the mixture of glucose:sucrose showeda higher productivity respect the other two mixtures (glucose:fructose and glucose:xylose). During the firsts 8h the mixture glucose:sucrose showed a productivity of 90mg/(l*h) higher than 30 and 50mg/(h*l) obtained with glucose:fructose and glucose:xylose.

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