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Biotechnological Production of Succinic Acid by *Actinobacillus Succinogenes* Using Different Substrate.

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Succinic acid, also known as butanoic acid, is widely applicated in the food industry, pharmaceuticals, agriculture and as a precursor of many chemical compounds including: adipic acid, 1,4-butanediol tetrahydrofuran *N*-methyl pyrrodidinone, 2-pyrrolidinone, succinate salts and gamma butyrolactone. The biotechnological production of succinic acid is an interesting alternative, presenting an economic advantage when compared with the chemical process. In addition, the agroindustrial residue glycerol can be used to obtain succinic acid by a fermentative process. Glycerol is a by-product of the production of biodiesel and with the increase in production of biodiesel in Brazil, a large volume of glycerol has accumulated in the industries' manufacturing biofuels. The biological production of succinic acid from glycerol is an attractive process, since it produces a high added-value compound from this by-product while decreasing environmental pollution. Some bacteria have the potential to produce succinic acid from glycerol, amongst which the strains of *Actinobacillus succinogenes.* Up to now, no economically interesting technology for the production of acid succinic has been developed in Brazil. The biotechnological conversion of the industrial by-product glycerol into acid succinic using cells of *Actinobacillus succinogenes* would allow for the production of a high added-value product, contributing to a reduction in the excessive volume of glycerol on the market, and would indirectly make the biodiesel production a process more complete. This work studied the succinic acid production by *Actinobacillus succinogenes* using glucose, sugar cane molasses, xylose, glycerol P.A. and glycerol from the biodiesel industry as substrate. The fermentative process was conducted at temperature 37°C, agitation 150rpm in different time periods (24, 48, 72, 96 hours) using free cells. The best result was observed in glycerol from biodiesel as substrate 1.62 g L^{-1} in 48 hours of fermentation.

1. Introduction

Succinic acid, also known as amber acid or butanedioic acid, is a dicarboxylic acid having the molecular formula of C4H6O4. After its first purification of succinic acid from amber by Georgius Agricola in 1546 (Song and Lee, 2006). Succinic acid can be used in food, pharmaceutics and agriculture industry, as a precursor of many industrially important chemicals including adipic acid, 1,4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts and gamma-butyrolactone (Khan et al. 2009; Beauprez et al., 2010). Furthermore, the increasing demand for succinic acid is expected as its use is extended to the synthesis of biodegradable polymers such as polybutyrate succinate (PBS) and polyamides (Nylon®x,4) and various green solvents (Cheng et al. 2012)

At present, the commercial succinic acid is mostly produced by the chemical process form maleic anhydride derived from petroleum; which limits the use of succinic acid for a wide range of applications due to the high conversion cost. However, recent works showed that succinic acid production by fermentation form renewable resources and a greenhouse gas, CO_2 could be more cost-effective than the petroleum-based process (Jiang et al., 2010). Many researchers in the literature had shown biotechnological production of succinic acid using by-products as a carbon source such as: sugar cane molasses, glycerol and others.

The fermentative production of succinic acid has been investigated with a wide variety of bacteria, but the most intensively researched are *Anaerobiospirillum succiniciproducens* (Lee et al. 2003; Jabalquinto et al.

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2004; *Actinobacillus succinogenes* (Li et al., 2011, Xi et al. 2012); *Mannhei succiniciproducers* MBEL 55E (Lee et al. 2002) and recombinant *Escherichia coli* recombinant (Kahn et al. 2009). However, the anaerobic bacterium, *Anaerobiospirillum succiniciproducens,* is considered among the best succinic acid producers (Khan et al. 2009).

The usage of waste-glycerol as a carbon source for biotechnological applications is not new, but is becoming an emerging field nowadays. A growing number of studies are focused on marketable uses for waste-glycerol (Varbanov et al. 2012). Generically, fermentations based on glycerol might have relative slow growth rates, and/or lower yields than the classical ones based on other carbon sources. But the production of added value metabolites would compensate the low volumetric productivities, compared to biofuels or bulk commodities. A positive economical yield might be achieved when considering a final industrial scale application (Abad et. al. 2012).

Efforts to develop a biological process for the production of succinic have been focused by many researchers. Lee et al. (2001) studied succinic acid by bacteria *A. succiniciproducens* using a mix of glycerol and glucose as substrate and obtained a productivity of 1.35 g L^{-1} h^{-1} and 0.99 gg^{-1}. Urbance et al. 2004 evaluated the succinic acid production by *Actinobacillus succinogenes* using glucose and observed a productivity of 0.88 g L^{-1} h^{-1}. Liu et al. (2008) obtained 1.15 g L^{-1} h^{-1} of productivity by the bacteria *Actinobacillus succinogenes* using glucose as a carbon source.

The present work had an objective to study the effect of different sources of carbon in the succinic acid production, and optimized the succinic acid production using glycerol from biodiesel industry by the bacteria *Actinobacillus succinogenes*.

2. Materials and Methods

2.1 Chemical and gas

All the chemicals used were of reagent grade and were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Fluka Chemical (Buchs, Switzerland). CO_2 Gas was obtained from Air liquid Brasil LTDA.

2.2 Microorganism and inoculums preparation

Actinobacillus succinogenes DSM 22257 was used in all experiments. The liquid medium for inoculums preparation contained the following (in grams per liter): 15 triptona; 5 soybean peptone, 5 sodium chloride. The cultures were shaken at 150 rpm and 37°C in an orbital shaking incubator for 24h, which was the time required for the microorganism to enter the exponential growth phase.

2.3 Fermentation process

First, the fermentative process was done using different carbon sources (glucose, sugar cane molasses, glycerol grade P.A., glycerol from biodiesel industry), under aerobic and anaerobic conditions. The fermentative medium contained the following (in grams per litter): salt solutions (including: 4.4 K_2HPO_4, 1.3 KH_2PO_4, 2 $NaSO_4$, 2 NH_42CO_3), 5 yeast extract, 5 peptone and 20 carbon source.

The batch fermentation process was carried out in flasks with 60 mL capability containing 30 mL of fermentation medium and 3 mL of inoculun medium, temperature at 37°C and agitation 150rpm. Anaerobic conditions were obtained for injection of CO_2 into the read space of flasks.

2.3.1 Optimization of conditions for succinic acid production

The data collected on succinic acid production were subjected to analysis of variance (ANOVA), in order to evaluate statistical significance. The mathematical relationship between the independent and response variables was calculated. The strategy of sequential experimental design was adopted for optimization of succinic acid production. The first experimental 2^{7-3} Plackett & Burman (PB) with 12 experiments and 7 variables (Glycerol, Temperature, pH, yeast extract, $(NH_2)SO_4$, urea and corn steep licor) was carried out on two levels, namely, minimum and maximum, coded as “-1” and “+1,” respectively, with triplicates of the center point (Table 1).

Table 1: Parameters and levels used in the experimental 2^{7-3} Plackett & Burman (PB) with 12 experiments and 7 variables

Parameters	-1	0	1
Glycerol (g. L^{-1})	2	7	10
Temperature (°C)	30	33.5	37
pH	6	7	8
Yeast Extract (g.L^{-1})	0	5	10
$(NH_2)SO_4$ (g. L^{-1})	0	1.5	3
Urea (g. L^{-1})	0	2	4
Corn steep licor (v^{-1})	20	30	40

2.4 Analytical methods

The glycerol and fermentation products were analyzed by high-performance liquid chromatography.

2.5 Statistical analysis

The Statistical® 10.0 software from Stat soft Inc. (Tulsa, Oklahoma, USA) was employed for experimental design, data analysis, and model building.

3. Results and Discussion

The succinct acid production by bacteria *A. succinogenes* was studied using a different carbon source, supplemented or not CO_2 gas. The highest succinic acid production (1.36 g.L^{-1}) was in anaerobic condition using glycerol from a biodiesel industry as a carbon source in 48 hours of fermentation. The second best conditions to succinic acid production were medium containing glucose and sugar cane molasses as substrate. Anaerobic conditions demonstrated a positive effect on succinic acid production when compared to aerobic process fermentation (Figure 1 and 2). Lee et al. (1999) demonstrated in our study an increase of 84% in the succinic acid production by *A. succinogenes*, when CO_2 was added in the fermentation medium to obtain anaerobic conditions. Datta (1999) report in your work that to ssucinic production for bacteria, the culture medium needs containing carbon source, sodium ions, dissolved carbon dioxide and other nutrients.

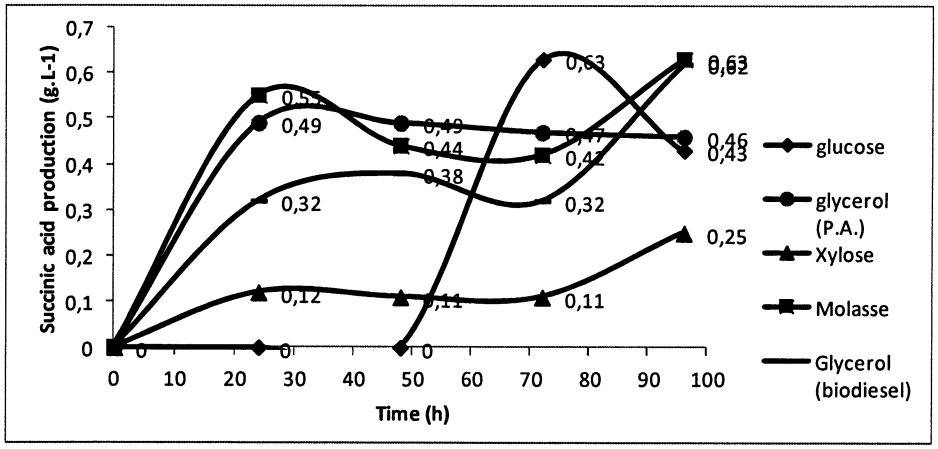

Figure 1: Succinic acid production by A. succinogenes in medium with different substrate and aerobic conditions.

Lee et at. (2008) have first published a paper on the anaerobic production of succinate from glycerol using the bacterium *Anaerobiospirillum succiniciproducens* in a 2.5 L bio-reactor. The authors produced 4.9 g.L^{-1} in batch fermentation by adding to the medium apart from glycerol (6.5 g.L^{-1}) yeast extract and polypeptone. This bio-process illustrated high yield (1.30 g/glycerol consumed) and hence low formation of by-products (succínico acid: acetic acid = 25.8:1 g/g) together with a productivity of 0.155 g- g.$L^{-1}h^{-1}$ /L/h

and a final product titer of 4.9 g.L^{-1}. The authors have also performed fed-batch experiments by adding yeast extract and glycerol as they have found that complex nitrogen sources contain important nutrients for the growth of this bacterium and help on the consumption/production of glycerol/succinate. The fed-batch experiment resulted in increased yield (1.60 g-acid succinic/g-glycerol), final succinate titer (19 g.L^{-1}) and succínico acid to acetic acid ratio (31.7:1 g succínico acid/g-acetic acid). Lee et. al. (2010) reported high succinic acid production by *A. succiniciproducens* using glycerol as substrate under anaerobic conditions.

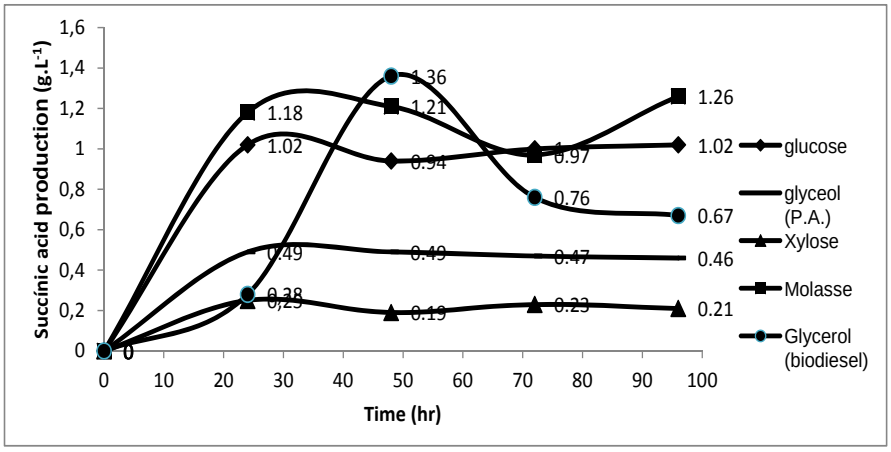

Figure 2: Succinic acid production by A. succinogenes in medium with different substrate and anaerobic condition.

The data presented in Tables 2 and 3 corroborate the results of the experimental 7^{2-3} (PB) with 3 center points. This experiment evaluated the effect of glycerol concentration, temperature, pH, urea, ammonium sulfate and corn steep licor concentration on succinic acid production (Figures 3 and 4). The best succinic acid production was obtained in the 7 and 1 experiments. The highest succinic acid concentration 1.51 g L^{-1} was obtained in the medium containing 2 g L^{-1} glycerol, 19 g L yeast extract, 4 g L^{-1} urea, pH 8 and temperature at 37°C. According to effect analysis at 10%, significant temperature, pH, yeast extract and corn steep licor demonstrated a positive effect on succinic acid production. However, glycerol concentration showed a negative on succinic acid production in the concentrations evaluated.

Table 2: Matrix of experimental PB 12 trials and corresponding results on succinic acid concentration.

Numbers of trials	Parameters							Measured response Succinic acid (g L^{-1})
	Glycerol (g L^{-1})	Temperature (°C)	pH	Yeast Extract (g L^{-1})	$(NH_2)SO_4$ (g L^{-1})	Urea (g L^{-1})	Corn steep licor (g L^{-1})	
1	10 (1)	30 (-1)	8 (1)	0 (-1)	0 (-1)	0 (-1)	20 (-1)	1.30
2	10 (1)	37 (1)	6 (-1)	10 (1)	0 (-1)	0 (-1)	20 (-1)	0.52
3	2 (-1)	37 (1)	8 (1)	0 (-1)	3 (1)	0 (-1)	20 (-1)	1.01
4	10 (1)	30 (-1)	8 (1)	10 (1)	0 (-1)	4 (1)	20 (-1)	0.67
5	10 (1)	37 (1)	6 (-1)	10 (1)	3 (1)	0 (-1)	40 (1)	1.02
6	10 (1)	37 (1)	8 (1)	0 (-1)	3 (1)	4 (1)	20 (-1)	0.97
7	2 (-1)	37 (1)	8 (1)	10 (1)	0 (-1)	4 (1)	40 (1)	1.51
8	2 (-1)	30 (-1)	8 (1)	10 (1)	3 (1)	0 (-1)	40 (1)	1.11
9	2 (-1)	30 (-1)	6 (-1)	10 (1)	3 (1)	4 (1)	20 (-1)	0.66
10	10 (1)	30 (-1)	6 (-1)	0 (-1)	3 (1)	4 (1)	40 (1)	0.92
11	2 (-1)	37 (1)	6 (-1)	0 (-1)	0 (-1)	4 (1)	40 (1)	1.27
12	2 (-1)	30 (-1)	6 (-1)	0 (-1)	0 (-1)	0 (-1)	20 (-1)	0.88
13(CP)	7 (0)	33.5 (0)	7 (0)	5 (0)	1.5 (0)	2 (0)	30 (0)	1.05
14(CP)	7 (0)	33.5 (0)	7 (0)	5 (0)	1.5 (0)	2(0)	30 (0)	1.06
15(CP)	7 (0)	33.5 (0)	7 (0)	5 (0)	1.5 (0)	2 (0)	30 (0)	1.10

Peng et. al. (2008) studied the effect of different inorganic and organic nitrogen sources (in same percent equivalent) were compare with yeast extract (15 g.L^{-1}) in anaerobic bottles, including NH_4Cl (5.3 g.L^{-1}), peptone (12 g.L^{-1}), corn steep liquor CLS (2,4% v/v) and beef extract (15 g.L^{-1}). The authors observed poor cell growth with the entire inorganic nitrogen source tested, and yeast extract was found to be the best nitrogen source for succínico acid production by *A. succininogenes*. The same was observed in this work. According to Vlysidis et al. (2009) the most importants parameters to acid succínico production are CO_2 supply, pH value, redox parameters and their interactions.

Vlysidis et al. (2012) evaluate the production of succinic acid in different concentrations of glycerol. The best productivity of succinic acid was 0,26 g.$L.^{-1}h.^{-1}$ with 36,4 g.L^{-1}glycerol concentration of. Xi et al. (2012) studied the effect of biotin on acid succinic production by *A. Succinogenes.* The authors observed an increased of the succínic acid production with addition of biotin in the fermentative medium.

Table 3: Results of the analysis of effects on succinic acid production of by Actinobacillus succinogenes.

Factor	Effects	Standard Error	t (7)	p-value
Means/Interactions	1	0.03	36.33	<0.0005
(1) Glycerol (g L^{-1})	- 0.17	0.06	-2.79	0.026
(2) Temperature (°C)	0.13	0.06	2.04	0.08
(3) pH	0.22	0.06	3.5	0.009
(4) Yeast Extract (g L^{-1})	0.14	0.06	-2.29	0.055
(5) $(NH_2)SO_4$ (g L^{-1})	- 0.08	0.06	-1.25	0.249
(6) Urea (g L^{-1})	0.03	0.06	0.42	0.685
(7) Corn steep licor (g L^{-1})	0.4	0.06	6.47	0.0003

Variables statistically significant at p <0.1. (R^2= 0.91277, Adjusted R^2= 0.82544)

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