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ORIGINAL PAPER

Succinic acid production from sugarcane bagasse hemicellulose hydrolysate by *Actinobacillus succinogenes*

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Abstract Succinic acid, a four-carbon diacid, has been the focus of many research projects aimed at developing more economically viable methods of fermenting sugar-containing natural materials. Succinic acid fermentation processes also consume CO_2, thereby potentially contributing to reductions in CO_2 emissions. Succinic acid could also become a commodity used as an intermediate in the chemical synthesis and manufacture of synthetic resins and biodegradable polymers. Much attention has been given recently to the use of microorganisms to produce succinic acid as an alternative to chemical synthesis. We have attempted to maximize succinic acid production by *Actinobacillus succinogenes* using an experimental design methodology for optimizing the concentrations of the medium components. The first experiment consisted of a 2^{4-1} fractional factorial design, and the second entailed a Central Composite Rotational Design so as to achieve optimal conditions. The optimal concentrations of nutrients predicted by the model were: $NaHCO_3$, 10.0 g l^{-1}; $MgSO_4$, 3.0 g l^{-1}; yeast extract, 2.0 g l^{-1}; KH_2PO_4. 5.0 g l^{-1}; these were experimentally validated. Under the best conversion conditions, as determined by statistical analysis, the production of succinic acid was carried out in an instrumented bioreactor using sugarcane bagasse hemicellulose hydrolysate, yielding a concentration of 22.5 g l^{-1}.

Keywords Sugarcane bagasse · Fermentation · Organic acid · Carbonic gas

Introduction

Succinic acid is currently produced from crude oil by catalytic hydrogenation of maleic anhydride to succinic anhydride and subsequent hydration or by the direct catalytic hydrogenation of maleic acid [29]. Population growth and the associated demand for energy and goods coupled with more restrictive environmental regulations and growing concern about carbon emissions from the burning of fossil fuels have intensified the search for renewable energy feedstocks to substitute or complement fossil fuel sources [21, 23]. Sugarcane bagasse, a lignocellulosic material composed of 50% cellulose, 25% hemicellulose and 25% lignin, is one of the most important agricultural residues produced in Brazil, amounting to approximately 250 kg per ton sugarcane [28]. A diluted acid pretreatment of sugarcane bagasse generates a liquid phase (hemicellulose hydrolysate) composed mainly of xylose, which can, in turn, be used as a substrate for biotechnological and chemical processes [7, 11]. The use of a renewable agricultural feedstock for the production of succinic acid reduces the need to draw upon limited oil reserves, thus diversifying a biorefinery's potential product portfolio [17]. The utilization of such feedstocks within the concept of biorefinery has been successfully investigated for the fermentative production of a range of products, including lactic acid, ethanol, polyhydroxybutyrate (PHB) and succinic acid [6].

Succinic acid, also known as amber acid or butanedioic acid, is a dicarboxylic acid with the molecular formula

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$C_4H_6O_4$. It can be used as a precursor for the production of many chemicals for use in the agricultural, food processing and pharmaceutical industries [for example, as surfactants, detergents, adipic acid, 1,4 butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts, gamma-butyrolactone, various green solvents, biodegradable polymers, such as polybutyrate succinate (PBS) and ingredients to stimulate animal and plant growth] [22, 29]. Several research teams are working on the development of industrial-level fermentation processes for succinic acid production using strains of *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens* [14, 24, 30] and *Corynebacterium glutamicum* and recombinant *Escherichia coli* strains [3, 19]. *A. succinogenes* is able to produce a relatively large amount of succinic acid from a broad range of carbon sources, such as arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sorbitol, sucrose, xylose or salicin under anaerobic conditions [8]. This microbial species is a facultatively anaerobic, Gram-negative bacterium that naturally produces high concentrations of succinate as a fermentation end product in addition to formate, acetate and ethanol [8, 26]. CO_2 functions as an electron acceptor and alters the flux of phosphoenolpyruvate (PEP), which induces the metabolism of the substrate to pyruvate and lactate/ethanol at low CO_2 levels and succinate at high CO_2 levels [26].

The composition of culture medium significantly affects the yield of any fermentation product. Physiological and nutritional factors, such as the initial sugar concentration, complex nitrogen sources, inoculum size and cell acclimatization, carbonate ion concentrations, magnesium, pH and the temperature of the growth medium, are reported to be the most critical factors affecting both cell growth and succinic acid production [1, 14].

Objective

The ultimate aim of the study reported here was to develop a cheap and suitable medium for succinic acid production from the hemicellulose hydrolysate of sugarcane bagasse in a batchwise culture of *A. succinogenes*. To this end, we studied the influence of the medium components ($NaHCO_3$, Yeast Extract, K_2HPO_4, $MgSO_4$) and their interaction on succinic acid production using an experimental design strategy.

Materials and methods

Microorganism and inoculum preparation

The strain of *A. succinogenes* CIP 106512 was obtained from the Pasteur Institute. The culture stock was maintained on Trypticase Soy Agar (TSA) slants at 4°C. The inoculum was grown in a liquid medium composed of xylose, 20 g l^{-1}; yeast extract, 2.0 g l^{-1}; $MgSO_4$, 1.0 g l^{-1}; $NaHCO_3$, 12.0 g l^{-1}; K_2HPO_4, 1.5 g l^{-1}. The cultures were shaken at 150 rpm and 37°C in an orbital shaking incubator for 24 h, which was the time required for the microorganisms to enter the exponential growth phase. After growth, the cells needed for each fermentation assay were separated from the media by centrifugation at 8,000 rpm and 10°C for 20 min.

Fermentation assays in flasks

The fermentation was initially carried out in 500-ml flasks containing 200 ml of a synthetic medium containing the same nutrients as those in the medium for the inoculum preparation. The medium was inoculated with 10% (v/v) of inoculum and the fermentation under anaerobic conditions was carried out as described elsewhere [12] at 150 rpm and 37°C in a shaker for 24 h. The system was set to create anaerobic conditions by introducing the desired CO_2 [12, 24]. The injection of CO_2 into the flasks was achieved using a gas cylinder, with the flow rate monitored with a gas manometer. Tygon tubes were used to introduce the gas into the interior of the flasks via connections in which membranes were present to purify the gas as it entered. Disposable syringes attached to the end of the tubes penetrated the cork on the surface of the flasks, thus promoting the flow of gas into the region between the fermentation environment and the Erlenmeyer surface (headspace). The pH was adjusted to 7.0 through the addition of sodium hydroxide (NaOH). A separately autoclaved solution of carbohydrate was added aseptically to the medium after autoclaving (Fig. 1).

The results were analyzed by experimental design to define the optimal conditions for succinic acid production, and the predicted values were validated experimentally in batch fermentation.

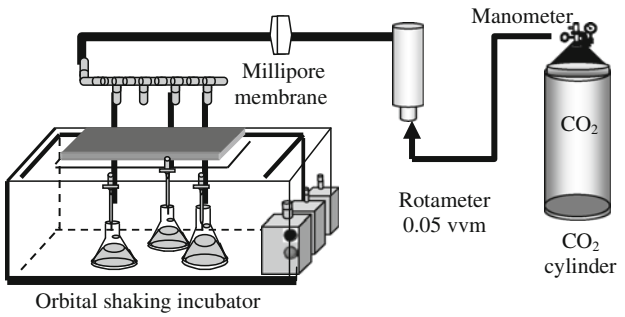

Fig. 1 Schematic representation of CO_2 distribution in shake flasks for medium optimization in succinic acid fermentation

Fermentation assays in bioreactor using synthetic medium

All batch fermentations were carried out at 37°C in a 2-l bioreactor (BIOFLO III; New Brunswick Scientific, Edison, NJ) with a working volume of 1.5 l. The initial xylose concentration in the medium was 20 g l^{-1}, and the temperature and agitation speed were controlled at 37°C and 150 rpm, respectively. The bioreactor was continuously supplied with CO_2 from the gas cylinder at a flow rate of 0.05 vvm during the whole fermentation period. A second bioreactor was operated without a supply of external CO_2 to compare how essential the feed of this gas was in the succinic acid production. The pH was monitored using a sterilizable pH electrode and maintained at 7.0 through the addition of 1 M NaOH. The kinetics of the fermentation process were evaluated under the optimal conditions established in the shake flask experiments. In the second part of this study, the fermentation was performed using the hydrolysate obtained from sugarcane bagasse with a high xylose concentration (52 g l^{-1}).

Sugarcane diluted acid pretreatment/hemicellulose hydrolysis

The sugarcane bagasse (*Saccharum* spp.) was provided by Costa Pinto Distillery (SP, Brazil). An acid pretreatment was developed to disrupt the lignocellulosic matrix of the sugarcane bagasse and hydrolyze the hemicellulosic component [H_2SO_4, 1% (v/v); solid:liquid ratio, 1:2; 121°C; 40-min pretreatment] [4]**.** The hydrolysate obtained was separated from the solid phase by pressure filtration and neutralized until pH 6.0 with $Ca(OH)_2$; it was then filtered again to remove the generated precipitate.

Fermentation assays in bioreactor using sugarcane hemicellulose hydrolysate

The cells were initially activated in the synthetic medium described above and then cultured in two acclimatization stages on a hydrolysate-containing medium; first, with 25% (v/v) medium and second, with 50% (v/v) medium. The cultures were grown in a rotatory shaker at 37°C and 150 rpm. After acclimatization, the cells required for each fermentation assay were separated from the media by centrifugation at 8000 rpm and 10°C for 20 min and inoculated into the bioreactor with all of the same nutrients used in the inoculum preparation medium, with the exception of xylose, which was substituted by the hemicellulose hydrolysate (xylose concentration of 52 g l^{-1}) derived from sugarcane bagasse pretreatment. The kinetics of the fermentation process operating in the bioreactor were evaluated under the optimal conditions established in the shake flask experiments using the statistical software package Design-Expert ver. 6.0 (Stat-Easem Minneapolis, MN).

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^{4-1} fractional factorial design with four variables ($NaHCO_3$, yeast extract, K_2HPO_4, $MgSO_4$) was carried out on two levels, namely, minimum and maximum, coded as "−1" e "+1", respectively, with two replicates of the center point (Table 1). A second experimental central composite rotational design (CCRD) (Table 2) was conducted to study the influence of the medium components on succinic acid production.

The response surface methodology (RSM) and the Pareto chart were also used to investigate the aggregate effect of multiple variables and to seek the optimal conditions for this multivariable system [5].

Analytical methods

Cell concentration was determined by measuring the optical density of a diluted sample at 600 nm (Spectrum Lab 22 PC; S) using a standard curve of absorbance against dry cell mass [16, 30]. Xylose concentration in the samples was determined by high-performance liquid chromatography (HPLC) using the Waters chromatographic system (Waters, Milford, MA) consisting of a HPX-87p column (Bio-Rad, Hercules, CA), a Waters 510 pump, a refractive index detector (Waters model 410) and HP 3390A integrator. The succinic acid concentration produced also was estimated on high performance liquid chromatography (HPLC). Samples (20 µl) were injected into a column (C18, 250 length × 4.6 mm internal diameter, 9 µm; StrodsII Peek) at a flow rate of 0.9 ml min^{-1}. Degassed and filtered sulfuric acid (0.01 N) was used as the mobile phase [8]. Succinic acid yield was defined as the amount of

Table 1 Parameters and levels used in the experimental 2^{4-1} fractional factorial design

Parameters		Minimum value	Maximum value
Code	Component	−1	+1
A	K_2HPO_4 (g l^{-1})	0.0	10.0
B	$MgSO_4$ (g l^{-1})	0.0	2.0
C	Yeast extract (g l^{-1})	0.0	5.0
D	$NaHCO_3$ (g l^{-1})	0.0	10.0

Table 2 Parameters and levels used in the experimental central composite rotational design (CCRD) for optimization of succinic acid production

Parameters		Real levels				
		Axial	Minimum value	Center point	Maximum value	Axial
Code	Component	$-\alpha$	−	0	+	$+\alpha$
A	K_2HPO_4 (g l^{-1})	2.5	5.0	7.0	10.0	12.5
B	$MgSO_4$ (g l^{-1})	1.0	2.0	3.0	4.0	5.0
C	Yeast extract (g l^{-1})	0.0	1.0	3.0	5.0	7.0
D	$NaHCO_3$ (g l^{-1})	1.5	5.0	10.0	15.0	20.0

succinic acid produced from 1 g of xylose (expressed as a percentage).

Results and discussion

Fractional factorial design and analysis

The results of the experimental 2^{4-1} fractional factorial design with four variables are presented in Table 3. A set of ten trials was generated (8 independent and 3 repetitions of the central point). Statistical significance of the respective model equations was checked using ANOVA. The final succinic acid concentration ranged from 4.74 to 9.02 g l^{-1}. The highest succinic acid concentrations (experiments 3, 5, 8, 9 and 10) were coincident with the presence of $NaHCO_3$ and $MgSO_4$, and the maximum succinic acid concentration of 9.02 g l^{-1} was achieved in 24 h of incubation at 37°C and an agitation speed of 150 rpm, with the following medium composition: xylose, 20 g l^{-1}; K_2HPO_4, 10.0 g l^{-1}; $MgSO_4$, 2.0 g l^{-1}; yeast extract, 5.0 g l^{-1}; $NaHCO_3$, 10.0 g l^{-1}.

According to the variance analysis, the best model for succinic acid concentration was the Reduced Quadratic Model, and the values of R^2 (0.952) and pure error (0.064) show that the model is in agreement with the experimental data. The model remained significant, with a Fisher's value of 29.85 and a statistically nonsignificant lack of fit (0.120) ($P > 0.1$), as shown in Table 4.

These results enabled plotting the histogram of the influence of the process variable (Fig. 2a), which provided important data on the statistical relevance of the factors and their interactions. Pareto diagram analysis (Fig. 2b) revealed that the variables D ($NaHCO_3$) and B ($MgSO_4$) had significant positive linear effects on succinic acid production. $NaHCO_3$ (parameter D) had the most significant factorial design effect, followed in decreasing order of effect by $MgSO_4$ (parameter B), yeast extract (parameter C) and K_2HPO_4 (parameter C); as independent variables, these had a lesser influence on succinic acid concentration.

Figure 2 reveals that $NaHCO_3$ had a significant effect on achieving a high succinic acid concentration, indicating that in the next trial the concentration of this parameter should be increased. In contrast, K_2HPO_4 showed no significant effect (0.1159), suggesting that K_2HPO_4 could be eliminated from the medium. However, the interaction between K_2HPO_4 and yeast extract (AC) had a significant influence in this analysis, with values of $P > F$ of less than 0.1 (0.0952). $MgSO_4$ and K_2HPO_4, with their components magnesium and phosphorus, play an important role in cell physiology and morphology [8].

Table 3 Matrix of experimental 2^{4-1} fractional factorial design and corresponding results on succinic acid concentration

Number of trials[a]	Parameters[b]				Measured response
	A K_2HPO_4 (g l^{-1})	B $MgSO_4$ (g l^{-1})	C Yeast extract (g l^{-1})	D $NaHCO_3$ (g l^{-1})	Succinic acid (g l^{-1})
1	0.0	0.0	0.0	0.0	5.53
2	10.0	0.0	0.0	10.0	4.74
3	0.0	2.0	0.0	10.0	8.36
4	10.0	2.0	0.0	0.0	5.96
5	0.0	0.0	5.0	10.0	7.66
6	10.0	0.0	5.0	0.0	4.96
7	0.0	2.0	5.0	0.0	6.08
8	10.0	2.0	5.0	10.0	9.02
9 (CP)	5.0	1.0	2.5	5.0	7.16
10 (CP)	5.0	1.0	2.5	5.0	7.58
11 (CP)	5.0	1.0	2.5	5.0	7.23

[a] Trials: eight independent and three repetitions of the center point (CP)

[b] The code for variables A, B, C, D are given in Table 1

Table 4 Variance analysis (ANOVA) of succinic acid concentration [partial sum of squares] in the experimental 2^{4-1} fractional factorial design

Source[a]	Sum of squares	*df*	Mean square	*F* value	*P* > F
Model	18.0797875	7	1.80797875	29.85843805	<0.0001
A	1.9110125	1	1.9110125	19.49093364	0.1159
B	3.0876125	1	3.0876125	47.64834105	0.0916
C	2.9701125	1	1.9701125	41.40297068	0.0982
D	7.7815125	1	7.7815125	120.0850694	0.0579
AB	0.8385125	1	0.8385125	12.94000772	0.1726
AC	2.4090125	1	2.4090125	31.17611883	0.0922
AD	0.0820125	1	0.0820125	1.265625	0.4626
Curvature	1.3140625	1	1.3140625	30.27874228	0.0952
Lack of fit	45.641850	9	4.564185	27.43712	0.1204
Pure error	1.5634	5	0.0648		
Correlation total	19.04526	19			
[Succinic acid]: [R^2 = 0.9526, Adjusted R^2 = 0.9399]					

ANOVA, Analysis of variance

[a] The code for variables A, B, C, D are given in Table 1

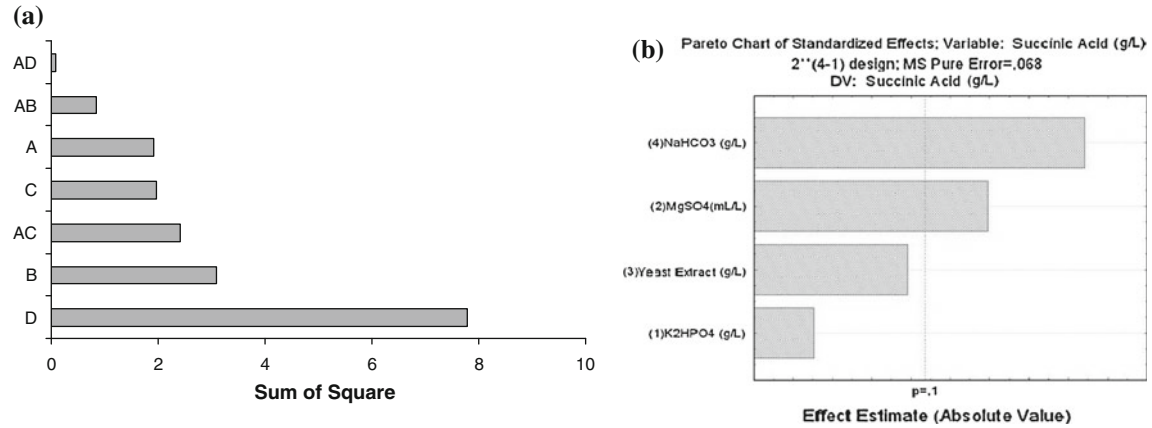

Fig. 2 **a** Histogram of the influence of variables and their interactions, **b** Pareto chart for minimal confidence range of 90%, according to the sum of squares in 2^{4-1} fractional factorial design. *A* K_2HPO_4, *B* $MgSO_4$, *C* Yeast extract, *D* $NaHCO_3$

According to the results of the first experimental design, the greatest efficiency was obtained in experiment no. 8. ANOVA analysis showed that the maximum succinic acid concentration occurred when the highest levels of parameters (B) and ©) were combined with a lower concentration of K_2HPO_4, indicating that there is a maximum. However, the values of the central points are close to the maximum obtained in the investigated range, indicating the need to add axial points to a subsequent trial in order to optimize succinic acid production (in factorial design only linear models can be evaluated). Thus, the next step was to evaluate succinic acid production using the CCRD technique, since this approach enables the response surface to be predicted with curvature plots and the maximum values of the response variables to be visualized. The maximum succinic acid concentration (9.02 g l^{-1}) can be enhanced, as the center points are still lower than the factorial points, showing an increasing tendency in the direction of the maximum point. All four parameters evaluated were significant (high *F* value) and kept in the model. The analysis of effects revealed that the levels of $NaHCO_3$ and $MgSO_4$ could be increased. However, any increase in K_2HPO_4 concentration may reduce succinic acid production since impurities, such as other organic acids (acetic and formic acids) and salts (HPO_4^{2-}), can cause a decrease in the extraction yield of succinic acid from an aqueous phase due to their ionic strength. Thus, the concentration of K_2HPO_4 should be minimized in the fermentation process to optimize the downstream processing of succinic acid separation [9].

Central composite rotational design and analysis

The results of the CCRD are presented in Table 5. A set of 30 trials was generated (27 independent and 3 repetitions of the central point). According to the results of the second

Table 5 Matrix of experimental central composite rotational design (CCRD) investigating the effects of the concentrations of K_2HPO_4, $MgSO_4$, yeast extract and $NaHCO_3$ on succinic acid production

Number of trials[a]	Parameters[b]				Measured response
	A K_2HPO_4 (g l^{-1})	B $MgSO_4$ (g l^{-1})	C Yeast extract (g l^{-1})	D $NaHCO_3$ (g l^{-1})	Succinic acid (g l^{-1})
1	5.0	4.0	3.0	5.0	11.55
2	5.0	2.0	3.0	10.0	12.85
3	10.0	3.0	5.0	15.0	11.12
4	10.0	3.0	1.0	15.0	11.42
5	7.5	2.0	0.0	10.0	6.04
6	7.5	4.0	3.0	1.5	10.45
7	5.0	4.0	1.0	5.0	10.26
8	7.5	2.0	3.0	10.0	9.42
9	10.0	2.0	1.0	15.0	9.10
10	10.0	1.0	5.0	15.0	8.45
11	10.0	4.0	5.0	5.0	12.15
12	10.0	3.0	1.0	5.0	12.10
13	12.5	4.0	3.0	10.0	12.27
14	10.0	4.0	3.0	20.0	12.45
15	5.0	2.0	5.0	15.0	7.85
16	7.5	2.0	3.0	10.0	7.12
17	7.5	3.0	3.0	10.0	12.20
18	5.0	4.0	1.0	15.0	12.41
19	5.0	3.0	5.0	5.0	11.02
20	7.5	3.0	5.0	5.0	10.78
21	5.0	4.0	1.0	5.0	7.56
22	10.0	3.0	1.0	5.0	10.23
23	2.5	2.0	3.0	10.0	8.86
24	5.0	2.0	1.0	15.0	6.54
25	5.0	3.0	5.0	15.0	7.45
26	7.5	3.0	7.0	10.0	11.24
27	7.5	2.0	3.0	10.0	9.25
28 (CP)	7.5	3.0	3.0	10.0	10.15
29 (CP)	7.5	3.0	3.0	10.0	11.23
30 (CP)	7.5	3.0	3.0	10.0	11.12

[a] Trials: eight independent and three repetitions of the CP

[b] The code for variables A, B, C, D are given in Table 1

experimental design, an increase in succinic acid concentration was achieved, increasing from a maximum of 9.05 g l^{-1} (first experimental design) to 12.8 g l^{-1} (second experimental design—CCRD). The final succinic acid concentration varied between 6.04 and 12.8 g l^{-1}. A maximum succinic acid concentration was obtained in experiment no. 2, with the following conditions: xylose concentration, 20 g l^{-1}; K_2HPO_4, 5.0 g l^{-1}; $MgSO_4$, 2.0 g l^{-1}; yeast extract, 3.0 g l^{-1}; $NaHCO_3$; 10 g l^{-1}.

The data presented in Table 6 corroborate the results of the experimental 2^{4-1} fractional factorial design in showing that the best model for succinic acid concentration was the Reduced Quadratic Model, based on the highest value for R^2 (0.995), indicating 99.5% of the response variability. This model remained significant, with a Fisher value of 34.81, thus denoting a curvilinear plane, as can be observed in the ANOVA (Table 4). Moreover, the residue was low and statistically nonsignificant, which did not invalidate the model for predictive purposes because the equation had a high R^2 value. The lack of fit was not significant ($P > 0.05$) which, combined with the F values of the parameters and the determination coefficient (R^2), showed that the model adequately adjusts to the experimental points, representing the confidence of the results [2].

As observed in the ANOVA of Table 4, there was an interaction between K_2HPO_4 and yeast extract, and the combined effect of both was maintained in the model. The resulting succinic acid concentration model is presented in Eq. 1.

Table 6 ANOVA for succinic acid concentration [partial sum of squares] in the experimental CCRD

Source[a]	Sum of squares	*df*	Mean square	*F* value	*P* > F
Model	149.903075	14	14.9903075	34.81492204	<0.0001
A	0.881666667	1	0.881666667	0.624275956	0.4418
B	22.08001667	1	22.08001667	45.63405313	0.0013
C	8.401666667	1	8.401666667	35.948913221	0.0276
D	88.16666667	1	88.16666667	62.42759559	<0.0001
AB	7.1824	1	7.1824	5.085595039	0.0395
AC	0.126025	1	0.126025	10.089233698	0.7693
AD	1.177225	1	1.177225	20.833550014	0.3757
BC	4.3264	1	4.3264	33.06336578	0.1000
BD	4.2849	1	4.2849	8.033981146	0.1020
CD	0.912025	1	0.912025	0.645771583	0.4342
Residual	21.18454167	15	1.412302778		
Lack of fit	39.18699167	9	3.918699167	35.80263114	0.0486
Pure error	5.99755	5	0.39951		
[Succinic acid]: [R^2 = 0.995, Adjusted R^2 = 0.9699]					

[a] The code for variables A, B, C, D are given in Table 1

$$\begin{aligned}[\text{Succini. ac}] = &+ 12.02 + 3.13 * \text{A} + 0.60 * \text{B} + 0.32 * \text{C} \\ &+ 0.75 * \text{D} + 0.88 * \text{A} * \text{B} + 0.91 * \text{A} \\ &+ 1.31 * \text{A} * \text{D} - 0.39 * \text{B} * \text{C} \\ &+ 0.73 * \text{B} * \text{D} + 0.38 * \text{C} * \text{D} \\ &+ 0.55 * \text{A}^2 - 0.58 * \text{B}^2 - 0.50 * \text{C}^2 \\ &+ 0.29 * \text{D}^2. \end{aligned} \quad (1)$$

The histogram of variable influence and interactions in the CCRD experimental design are presented in Fig. 3. Parameter D ($NaHCO_3$) was the most influential, followed by parameter B ($MgSO_4$) and parameter C (Yeast extract) and their interactions. The interaction between AB, followed by BC, AD and CD presented more significant effects than those of parameter A (K_2HPO_4).

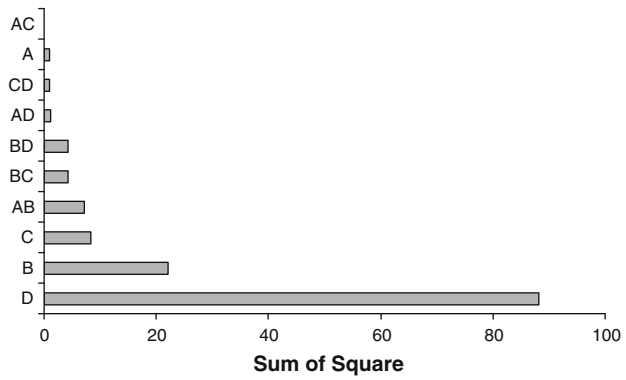

Fig. 3 Histogram of the influence of the process variables and their interactions, according to the sum of squares in the experimental central composite rotational design (CCRD). *A* K_2HPO_4, *B* $MgSO_4$, *C* yeast extract, *D* $NaHCO_3$

Figure 4 shows the three-dimensional (3-D) response contour and surface plots, which represent the regression equations. Analysis of Fig. 4a and b shows that increasing concentrations of $MgSO_4$ and yeast extract can result in increases in the final succinic acid concentration. Figure 4c shows that the increase in K_2HPO_4 concentration contributes to a reduced succinic acid production. In contrast, Fig. 4b shows that the interaction between K_2HPO_4 and yeast extract (AC) has a significant effect. The interaction between these four variables is also shown with high Fisher values (Table 6), indicating the large degree of synergism between the nutrients being fermented by *A. succinogenes*, producing larger amounts of succinic acid. Figure 4d shows that the highest interaction was between $MgSO_4$ and $NaHCO_3$, which confirms the statistical analysis. In all experiments, the highest levels of these nutrients promote an increase in succinic acid concentration.

Succinic acid production was increased by *A. succinogenes* when an optimal combination of nutrients was used. Analysis of the response measured (concentration of succinic acid) emphasizes experimental condition number 2 since it is the most efficient. The effect of $NaHCO_3$, keeping $MgSO_4$ to a minimum level of (2.0 g l^{-1}) and yeast extract at the center point (3.0 g l^{-1}), indicates that the optimal point for succinic acid production is around the maximum level of $NaHCO_3$ (10.0 g l^{-1}). Therefore, when the fermentation process occurs in the presence of high levels of K_2HPO_4, succinic acid concentration is affected. All of the nutrients were significant and essential to the metabolism of the bacteria. The concentrations of amino nitrogen (yeast extract) in the fermentation broth usually reflect the concentrations of proteins and amino acids [21].

Liu et al. [16] demonstrated that there was no significant inhibition of cell growth or succinic acid production by magnesium ions using *A. succinogenes* strain CGMCC1593. Nitrogen sources generally play a significant role because these nutrients are directly linked with cell proliferation and metabolite biosynthesis. It has been shown that yeast extract, which contains protein, lipid, vitamins and others, supplies important growth factors for succinic acid production, but its cost may be a limitation for its application in industrial processes [25]. Recent studies on similar types of fermentations have demonstrated that the nitrogen deficiency stimulated the biosynthesis of other metabolites, such as acetic and formic acids [20]. Variance analysis showed the adequacy of the model, which was validated experimentally in the bioreactor.

Thus, the medium obtained after optimization by the strategy of sequential experimental design had the following composition: xylose, 20 g l^{-1}; yeast extract, 5.0 g l^{-1},

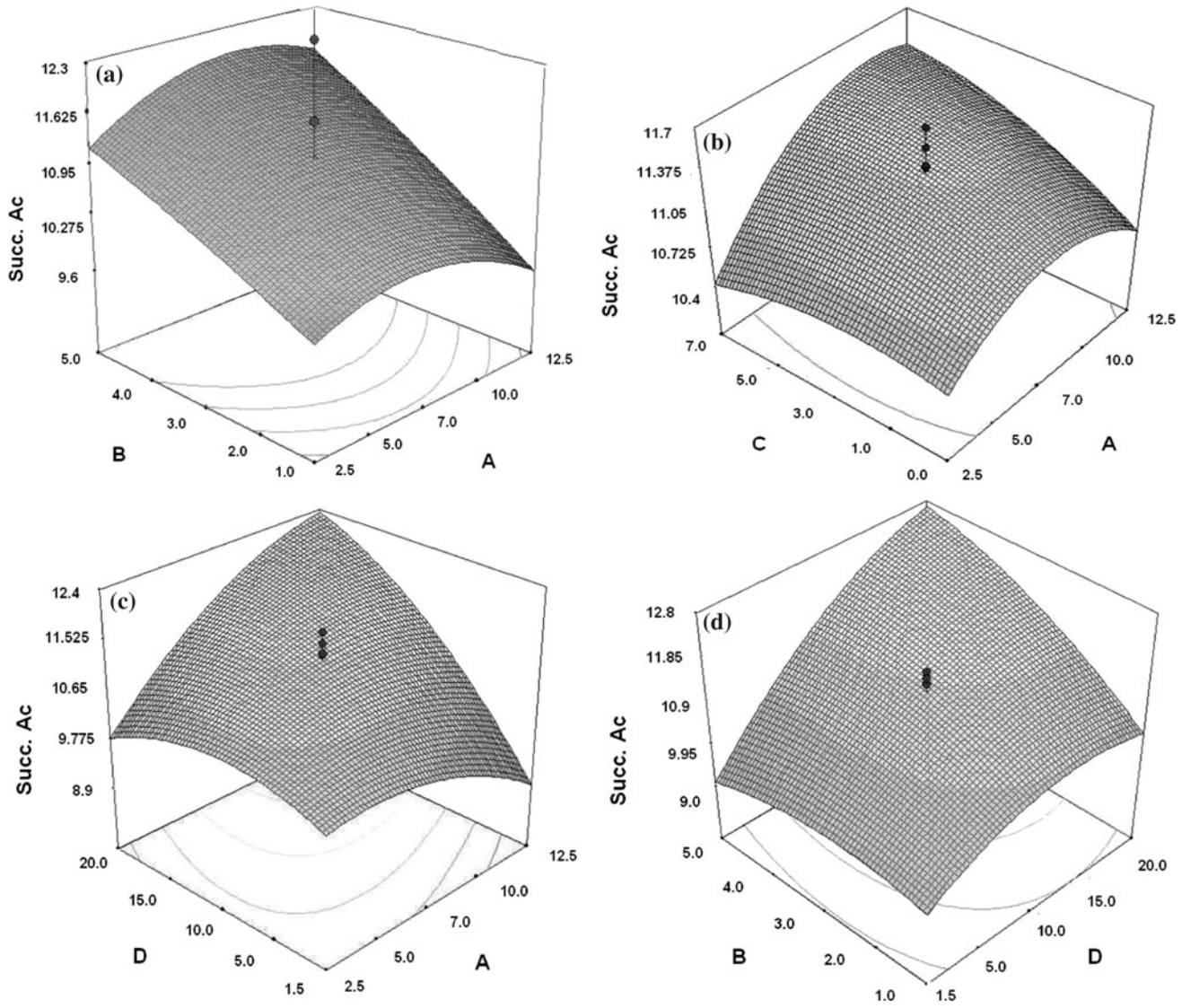

Fig. 4 Response surface plots showing the effect of parameters and their combined effects on succinic acid concentration. Interaction between the parameters AB (**a**), AC (**b**), AD (**c**), BD (**d**). *A* K_2HPO_4, *B* $MgSO_4$, *C* Yeast extract, *D* $NaHCO_3$

$MgSO_4$, 3.0 g l^{-1}; $NaHCO_3$, 10 g l^{-1}. The validation of the experimental design was carried out in the bioreactor (Figs. 5, 6).

Succinic acid fermentation

The best conditions for the fermentation process indicated by CCRD (Table 5) were used to prepare the fermentation medium for succinic acid production in the bioreactor experiment. Under response surface methodology (RSM)-optimized conditions, a concentration of 10.04 g l^{-1} succinic acid, a volumetric productivity (Q_P) of 0.478 g l^{-1} h^{-1} and an efficiency (E_f) of 58.4% were obtained in 24 h (Fig. 5a). An increase in succinic acid production was subsequently obtained in a second bioreactor operated with a supply of external CO_2, resulting in a final succinic acid concentration of 14.22 g l^{-1}, a volumetric productivity of 0.677 g l^{-1} h^{-1} and an efficiency (E_f) of 82.8% (Fig. 5b). A similar trend was reported by Lee et al. [13], who observed that 13.5 g l^{-1} of succinic acid was produced when 100% CO_2 was supplied to the bacterium *M. succiniciproducens*. These outcomes were likely achieved because of the supply of carbon dioxide. Similarly, van der Werf et al. [26] suggested that the production of succinate requires CO_2 fixation and also that CO_2 concentration regulates the level of the key enzymes of the PEP carboxykinase pathway in *A. succinogenes*. These researchers further confirmed that high levels of CO_2 stimulated PEP carboxykinase levels, whereas the levels of alcohol dehydrogenase and lactate dehydrogenases were significantly decreased.

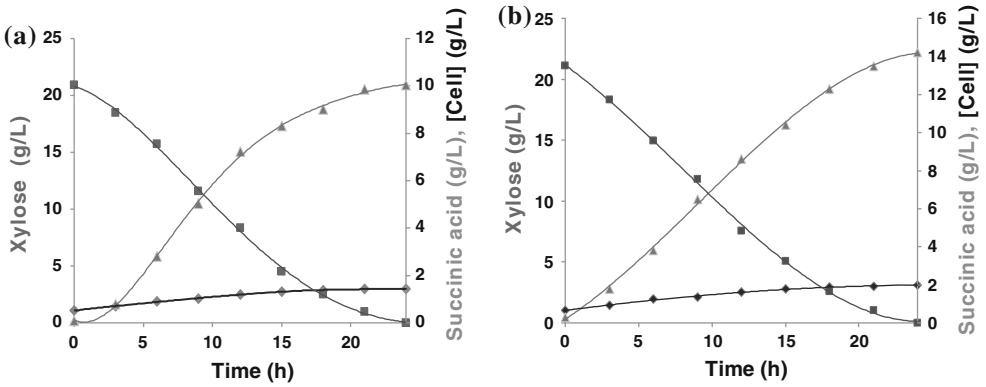

Fig. 5 Concentration of cells (*filled diamond*), xylose (*filled square*) and succinic acid (*filled triangle*) during the fermentation process carried out under controlled conditions (37°C, pH 7.0, 150 rpm) using synthetic medium with 22.0 g/l of xylose. Medium with low (**a**) and high (**b**) CO_2 supply

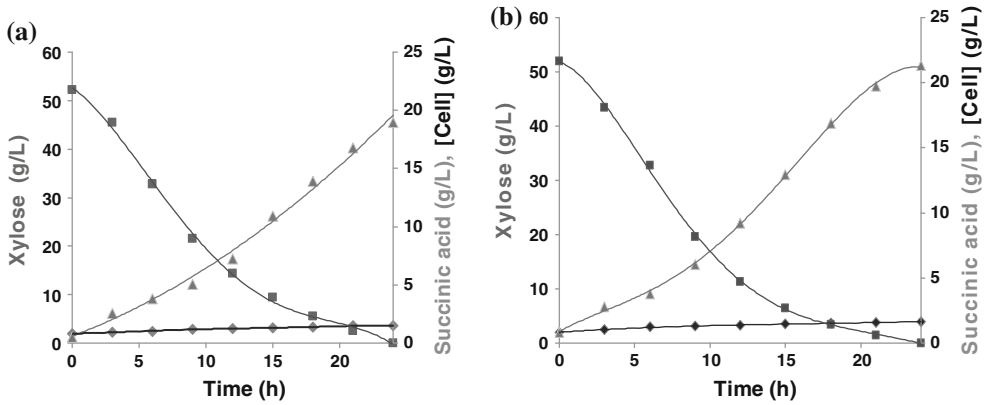

Fig. 6 Concentration of cells (*filled diamond*), xylose (*filled square*) and succinic acid (*filled triangle*) during the fermentation of sugarcane bagasse hemicellulose hydrolysate containing 52.0 g l^{-1} xylose, carried out under controlled conditions (37°C, pH 7.0, 150 rpm). Medium with low (**a**) and high (**b**) CO_2 supply

Succinic acid fermentation of sugarcane bagasse hemicellulose hydrolysate

The final verification of the statistical model for succinic acid production was also carried in a 2-l bioreactor. Figure 6 shows the behavior of the hydrolysate during the fermentation process under the conditions predicted in the statistical analysis. The hydrolysate was inoculated with a cell concentration of 1.02 g l^{-1}; the fermentation was run at 37°C for 24 h, pH 7.0, with an agitation of 150 rpm, and the initial xylose concentration was 52 g l^{-1}.

The succinic acid concentration reached its maximum value (19.0 g l^{-1}) after 24 h of fermentation, corresponding to a yield of product per substrate consumed of 0.465 g g^{-1}, a volumetric productivity of 0.903 g l^{-1} h^{-1} and a fermentation efficiency of 45.6% (Fig. 6a).

The effect of 10 g l^{-1} of $NaHCO_3$ was similar to that of $MgCO_3$ used by Vemuri et al. [27], who obtained a final succinic acid concentration of 10.53 g l^{-1}; nonetheless, concentrations of $NaHCO_3$ higher than 10.0 g l^{-1} did not significantly increase the succinic acid concentration. The results of this analysis also suggest strategies for improving CO_2 utilization. For example, at intermediate CO_2 concentrations, overexpression of the carbonic anhydrase enzyme, which catalyzes the hydration of CO_2 to HCO_3^-, may be effective. If pure CO_2 were to be used, further improvement in succinate production should result from increased enzyme expression, as has been previously observed with pyruvate carboxylase [27].

Similarly, Fig. 6b shows the fermentation of hemicellulose hydrolysate in the presence of carbon dioxide, which was continuously introduced into the medium at 0.05 vvm throughout the process. The final succinic acid concentration, volumetric productivity and efficiency were 22.5 g l^{-1}, 1.014 g l^{-1} h^{-1} and 55.4%, respectively, with a fermentation time of approximately 24 h, at a temperature of 37°C, agitation of 150 rpm and pH 7.0 (Table 7).

Table 8 shows the results previously reported in the literature using various bacterial strains in succinic acid fermentation processes as well as the main results obtained in our study. A review of the relevant literature reveals that many published studies used other materials, such as wood hydrolysate and corn steep liquor [1, 14], and other microorganisms, such as naturally occurring *M. succiniciproducens* [9, 10, 13], *A. succiniciproducens* [12, 18] and genetically engineered *E. coli* [15, 19]. Other authors

Table 7 Results of the fermentation process for succinic acid production carried out in a bioreactor under controlled conditions (37°C, pH 7.0, 150 rpm)

Fermentation process	Conditions		Maximum values							
	S_o (g l^{-1})	Time (h)	Low CO_2 tension				High CO_2 tension			
			AS (g l^{-1})	Q_P (g l^{-1} h^{-1})	$Y_{P/S}$ (g/g)	E_f (%)	AS (g/l)	$Y_{P/S}$ (g g^{-1})	E_f (%)	Q_p (g l^{-1} h^{-1})
Synthetic medium	22.0	24	10.04	0.47	0.45	58.4	14.2	0.64	82.8	0.67
Hydrolysate	52.0	24	18.5	0.90	0.35	45.6	22.5	0.43	55.4	1.01

S_0 Initial xylose concentration (g l^{-1}), *AS* succinic acid (g l^{-1}), Q_P productivity (g l^{-1} h^{-1}), $Y_{P/S}$ product yield in relation to the substrate, E_f efficiency

Table 8 Main results reported in the literature for succinic acid production

S_o (g l^{-1})	Q_p (g l^{-1} h^{-1})	$Y_{p/s}$ (g/g)	Carbon source	Organism	Cultivation	Reference
35.6	1.01	0.82	Wheat	*Actinobacillus succinogenes*	Fed-batch	Du et al. [6]
43.0	0.72	0.53	Glucose	*Escherichia coli*	Batch	Lin et al. [15]
58.3	1.08	0.62	Glucose	*Escherichia coli*	Fed-batch	Lin et al. [15]
83.0	10.40	0.89	Glucose	*Anaerobiospirillum succiniciproducens*	Fed-batch/electrodialysis	Meynial-Salles et al. [18]
55.2	1.15	–	Cane molasses	*Actinobacillus succinogenes*	Batch	Liu et al. [16]
52.0	1.80	0.76	Glucose	*Mannheimia succiniciproducens*	Batch	Lee et al. [13]
22.0	0.67	0.64	Xylose	*Actinobacillus succinogenes*	Batch	This work
52.0	1.01	0.43	sugarcane bagasse	*Actinobacillus succinogenes*	Batch	This work

reported that *A. succinogenes* could use a wide range of carbohydrates as carbon sources and ferment straw hydrolysate or cane molasses, making this species a promising agent for succinic acid production [16].

Conclusions

The sequential experimental design strategy used to study the addition of nutrients in the fermentation medium was an important tool for maximizing final succinic acid concentration through RSM.

The results of our study on succinic acid production from sugarcane bagasse hemicellulose hydrolysate by means of *A. succinogenes* fermentation indicate that it is a promising procedure provided that an optimal combination of nutrients is used. Increased levels of $NaHCO_3$ and $MgSO_4$ were primarily responsible for the increased succinic acid concentration, followed by yeast extract and KH_2PO_4, which were less significant as independent variables, but revealed themselves to be important when they were involved in the interactions.

The conversion yield of succinic acid from sugarcane bagasse was relatively high in the batch cultivation of *A. succinogenes*. Under the best conversion conditions, dictated by the results of our statistical analysis and validated experimentally, a succinic acid concentration of 22.5 g l^{-1} was achieved in hemicellulose hydrolysate fermentation.

Succinic acid will likely be one of the future platform chemical derivatives from renewable resources. Microbial production of succinic acid, in particular, may prove a novel "green technology," since CO_2 fixation is integrated to carboxylation and decarboxylation in both catabolism and anabolism. Based on recent research results, cost reduction and scale-up are the main objectives of new process development in this area.

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