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Ethanol Production from Sugarcane Bagasse by *Zymomonas mobilis* Using Simultaneous Saccharification and Fermentation (SSF) Process

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Abstract Considerable efforts have been made to utilize agricultural and forest residues as biomass feedstock for the production of second-generation bioethanol as an alternative fuel. Fermentation utilizing strains of *Zymomonas mobilis* and the use of simultaneous saccharification and fermentation (SSF) process has been proposed. Statistical experimental design was used to optimize the conditions of SSF, evaluating solid content, enzymatic load, and cell concentration. The optimum conditions were found to be solid content (30%), enzymatic load (25 filter paper units/g), and cell concentration (4 g/L), resulting in a maximum ethanol concentration of 60 g/L and a volumetric productivity of 1.5 g $L^{-1}h^{-1}$.

Keywords Lignocellulosics · Sugarcane · SSF · Bioethanol · *Z. mobilis*

Introduction

Bioethanol is currently produced from conventional raw materials such as sugarcane, beet, or starch. Substantial replacement of fossil fuels by bioethanol will require its production from lignocellulosic raw materials such as agricultural and forestry residues because they are inexpensive, plentiful, and renewable. On top of that they do not compete with the use of land for feedstock production. In the Brazilian context, sugarcane bagasse is considered a lignocellulosic feedstock par excellence.

Despite being used as solid fuel for energy generation in the production units there exist enormous surpluses. However, all these surpluses have applicability on other industries. A balance made in the 2007/2008 harvest season revealed that 143.340 million tons of sugarcane bagasse was generated in Brazil, from which 93.3% was used for heat and 6.7% for electricity production. The burning of sugarcane bagasse impacts significantly the

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energy balance, and this is one of the characteristics that turns the sugar and ethanol industrial complex so competitive [1].

The utilization of lignocellulosic biomass has been closely associated with a new technological concept, so called Biorefinery, which emerges as key to the significant expansion of the desired production of ethanol. Fermentative processes stand out, where microbial metabolism is used for the transformation of simple raw materials in products with high aggregate value [2]. Experts believe that the biorefineries are likely to be a key industry of the twenty-first century, even responsible for a new industrial revolution, because of the importance of the technologies they employ and their effects on the actual industrial model [3].

Lignocellulosic biomass is a potential source of cheap sugars for producing fuels and chemicals, and a pretreatment stage is essential to make the cellulose accessible to the hydrolysis by cellulases [4]. This processes use chemicals or enzymes to break the molecules of cellulose into its sugar monomers that are fermented by ethanol-producing microorganisms.

Research in ethanol has been targeted for the development of second-generation technology, including the strategy of simultaneous saccharification and fermentation (SSF) process, which combines in a single unit the cellulose enzymatic hydrolysis and the ethanol fermentation [5].

Before the SSF process, two steps are required for cellulose enzymatic hydrolysis: pretreatment for disorganizing the lignocellulosic complex and delignification. Both steps aim at increasing internal surface area, reduction of cellulose crystallinity, and separation of structural linkages between lignin and carbohydrates, resulting in an increase of the accessibility of the enzymes to the cellulose microfibrils [6]. The cellulose enzymatic hydrolysis is carried out by cellulases, which are generally a mixture of several enzymes mainly composed of endoglucanase, exoglucanase, and β-glucosidase.

In this study a strain of *Zymomonas mobilis* was used for ethanol concentration. It is a gram-negative bacterium, which uses sucrose, glucose, and fructose as carbon and energy source, producing an equimolar quantity of ethanol and CO_2 [7]. This bacterium has been the subject of numerous investigations because of its high potential for ethanol fermentation, which is comparable or even higher than that obtained by the traditional yeast *Saccharomyces cerevisiae* [8]. *Z. mobilis* ferments 1 mol of glucose to almost 1.6 mol of ethanol, 1.8 mol of CO_2, and small amount of other byproducts such as lactate, acetaldehyde, acetic acid, and others. Therefore, like most of the chemoorganotrophic organisms, it needs sources of nitrogen, phosphorus, sulfur, and micronutrients for the functioning of metabolism.

The bacterium *Z. mobilis* ferments sugars by Entner–Doudoroff pathway. Glucose is broken into 2 mol of ethanol and produces 1 mol of ATP, in contrast to the classical Embden–Meyerhoff–Parnas, which generates two molecules of ATP for each mole of glucose, producing ethanol. When compared with *S. cerevisiae*, *Z. mobilis* shows higher sugar uptake and ethanol production rates and lower cell growth, since approximately 2% of the carbon sources are converted into biomass [9].

Objective

The aim of this work was to analyze the optimum operational conditions for the production of ethanol from sugarcane bagasse by the bacterium *Z. mobilis*, using SSF process.

Materials and Methods

Substrate Sugarcane bagasse (*Saccharum* spp.) was kindly provided by Costa Pinto Distillery (SP, Brazil). This lignocellulosic residue undergoes acid pretreatment, followed by solid separation, alkali delignification, resulting in a material used for enzymatic hydrolysis and fermentation as detailed as follows.

Pretreatment of Cellulignin Acid hydrolysis was performed for disorganizing the lignocellulosic matrix and fractioning the hemicellulosic component. The following condition for sugarcane bagasse acid pretreatment was used: H_2SO_4 1% (*v*/*v*), solid/liquid ratio 1:2, temperature 121 °C, and time of 30 min [10]. After pretreatment, the aqueous phase was removed by pressing filtration, and the remaining solid (cellulignin) was subjected to an alkaline delignification, with NaOH in a concentration of 4% (*v*/*v*) and with a solid/liquid ratio of 1:20. Then, the cellulign was exposed a temperature of 121 °C for 30 min [11]. Thereafter, the alkaline-pretreated cellulignin was washed with distilled water several times until the aqueous phase remained clear. This solid matter with increased cellulose accessibility was subjected to enzymatic hydrolysis by a commercial preparation (Multifect, Genencor, USA). First, an enzymatic pretreatment was carried out with varying enzyme loads, at 50 °C for 12 h. The enzymatic activities were determined by the filter paper activity as recommended by Ghose [12] and expressed as filter paper units (FPU) per milliliter of mixture.

Microorganism and Inoculum Preparation Z. mobilis CP4 used in this work was kindly provided by the Department of Antibiotics of the Federal University of Pernambuco. The strain was grown in a liquid medium (20 g L^{-1} of glucose and 5 g L^{-1} of yeast extract) recommended by Swing and De Ley, at 30 °C for 24 h, and maintained at 4 °C. Monthly transferences were performed for maintenance of cell viability. The inoculum was grown in a medium composed of glucose 20 g L^{-1}, yeast extract 2.5 g L^{-1}, ammonium sulfate 1 g L^{-1}, potassium phosphate 1 g L^{-1}, and agnesium sulfate 0.5 g L^{-1}. The cultures were shaken in a rotary shaker at 150 rpm, at 30 °C for 20 h. After growth, the cells were centrifuged (8,000 rpm, 20 min), and their concentration was determined by measuring the optical density of a diluted sample at 600 nm (SPECTRUMLAB 22 PC), using a standard curve of absorbance against dry cell mass. The centrifuged cells were inoculated in the enzymatically pretreated solids, with different solid/liquid ratios (Table 1).

Fermentation Assays The fermentation medium (enzymatically pretreated solids) was supplemented with the same nutrients used for the inoculum preparation medium, without glucose, providing the technical implementation of the SSF process.

Table 1 Independent variables in the experimental design.

Parameters		Real levels				
		$-\alpha$	−	0	+	$+\alpha$
A	Solid/liquid Ratio	0.32:10	1:10	2:10	3:10	3.68:10
B	Enzyme load (FPU/g)	4.89	10	17.5	25	30.11
C	Cell concentration (g/L)	0.02	1	2.5	4	5.02

SSF Experiments Batch fermentations experiments were performed in 500-mL Erlenmeyer flasks with a working volume of 100 mL to define the optimum process conditions (solid/liquid ratio; enzyme load and cell concentration). Additionally, fermentations were carried out in a 1.5-L bioreactor (BIOFLO III, New Brunswick Scientific, USA) with control of temperature, pH, and agitation. The reactor operated with a working volume of 500 mL, and the temperature and pH were set at 30 °C and 5.0, respectively. The pH was monitored using a sterilizable pH electrode and controlled by adding 1 M KOH. The kinetics of SSF in bioreactor was evaluated in the optimum conditions established in the shake flask experiments.

Analytical Methods Cell quantification was determined as described previously. Samples were analyzed for glucose, cellobiose, and ethanol concentrations by high-efficiency liquid chromatography (HPLC) using the chromatographic system (WATERS) consisting of a HPX-87p (Bio-Rad) column, WATERS 510 pump, a refractive index detector WATERS 410, and HP 3390A integrator. The standard solution consists of cellobiose, glucose, and ethanol concentrations of 5, 10, and 15 g/L, respectively. The end of fermentation was determined through the stabilization of the production of ethanol, as verified by the reading of two consecutive and equal values of alcohol content.

Optimization of Ethanol Concentration The experiments were performed under the principles of statistical methodology of response surfaces, that is, a statistical model widely used to study an aggregate effect of several variables and to seek optimum conditions for a multivariable system [13]. Full 2^3 factorial central composite experimental design with six axial points and six replications at the center points leading to a total number of 20 experiments was employed. Final ethanol concentration, volumetric productivity, and SSF initial glucose concentration were the response (dependent) variables. The parameters analyzed shown in Table 1 were the solid/liquid ratio (*A*; delignified cellulignin/nutrient medium), enzyme load (*B*), and cell concentration (*C*). The statistical analysis of the data was performed using "Design Expert" software (7.1.6., Stat-Ease).

Results and Discussion

The results of the experimental design are depicted in Table 2. Statistical significance of the respective model equations was checked using *F* test analysis of variance.

The best fitted model for the SSF initial glucose concentration after enzymatic prehydrolysis was the reduced quadratic model, since it provided the highest R^2 value of 0.8884 (Table 3). The lack of fit is presented as significant ($p<0.05$), but the model remained significant (p value less than 0.0001). Parameter *C* (cell concentration) and its interactions were removed from the model, since it represents the produced glucose during the enzymatic prehydrolysis, i.e., before cell inoculation. For this reason, probably, the lack of fit has shown significance. The parameter *B* (enzyme load) was the most influential in the release of glucose. The resulting glucose after enzymatic prehydrolysis model is represented by Eq. 1.

$$[\text{Glucose}] = -69.28050 + 67.60248*S : L + 4.70742*\text{FPU/g} + 1.21333*S : L*FPU/g - 19.75705*S : L^2 - 0.14319*\text{FPU/g}^2 \quad (1)$$

Table 2 3^3 Central composite design investigating effects of ratio solid/liquid, enzyme load, and cellular concentration on ethanol concentration, SSF initial glucose concentration, and volumetric productivity.

Run	Variables in coded levels			Measured response		
	A	*B*	*C*	Glucose (g L^{-1})	Ethanol (g L^{-1})	Q_P (g $L^{-1}h^{-1}$)
1	$-\alpha$	0	0	12.3	6.5	0.23
2	0	0	$+\alpha$	70.7	35.2	1.49
3	0	0	0	69.9	33.8	1.42
4	+	−	+	33.8	13.7	0.5
5	0	0	0	69.9	33.8	1.45
6	+	−	−	35.5	14.9	0.5
7	−	−	−	17.2	5.9	0.2
8	+	+	+	80.3	60.7	1.52
9	0	0	0	70.5	30.6	1.38
10	0	0	0	69.5	33.4	1.42
11	−	−	+	20.1	13.4	0.41
12	−	+	−	26.3	14.7	0.85
13	−	+	+	33.8	17.9	0.88
14	0	$+\alpha$	0	76.6	36.8	0.94
15	+	+	−	80.7	48.7	0.98
16	0	0	$-\alpha$	50.5	6.9	0.06
17	$+\alpha$	0	0	10.6	6.2	0.42
18	0	0	0	70.6	29.1	1.41
19	0	$-\alpha$	0	12.8	5.4	0.21
20	0	0	0	70.6	34.9	1.39

The best model for the ethanol concentration was the reduced cubic model, since it resulted in the highest value for R^2 (0.9926), indicating 99.2% of the response variability. The model remained significant, with Fisher's value of 78.31 and lack of fit not significant ($p > 0.05$), as shown in Table 4. The probability p value less than 0.0001

Table 3 Analysis of variance for glucose [partial sum of squares].

Source	Sum of squares	DF	Mean square	*F* value	$p>F$
Model	11,694.875	5	2,338.97	22.29	<0.0001
A	1,313.64	1	1,313.64	12.52	0.0033
B	3,460.54	1	3,460.54	32.98	<0.0001
A^2	5,681.33	1	5,681.33	54.15	<0.0001
B^2	944.23	1	944.23	9.00	0.0096
AB	662.48	1	662.48	6.31	0.0248
Residual	1,468.91	14	104.92		
Lack of fit	1,467.84	9	163.09	759.75	<0.0001
Pure error	1.07	5	0.21		
Cor total	13,163.78	19			

[Glucose]: [*R*-squared=0.8884, Adj *R*-squared=0.8486]

also indicated that the model was highly significant and that the experimental data obtained are in a good agreement with the model [14]. All the three parameters evaluated (solid/liquid ratio, enzyme load, and cell concentration) were significant and were kept in the model, although of the three existing interactions only the solid/liquid ratio and the enzyme load (*AB*) were kept in the model and were very representative (high *F* value). Solid/liquid ratio (parameter *A*) had the most significant factorial design effect, followed by the enzyme load (parameter *B*), and finally the cell concentration (parameter *C*), which had lesser influence in ethanol concentration. The resulting ethanol concentration model is presented in Eq. 2

$$\begin{aligned}[\text{Ethanol}] = {} & +32.60 + 16.70{*}S:L + 13.09{*}\text{FPU/g} + 808.35{*}[\text{Cell}] - 9.28{*}S:L^2 \\ & - 4.07{*}\text{FPU/g}^2 + 4.48{*}[\text{Cell}]^2 + 8.44{*}S:L{*}\text{FPU/g}{*} - 5.93{*}S:L^3 \\ & - 1.33{*}\text{FPU/g}^3 - 287.21{*}[\text{Cell}]^3 - 516.46{*}S:L^2{*}[\text{Cell}] \\ & + 2.19{*}S:L{*}\text{FPU/g}{*}[\text{Cell}]\end{aligned} \tag{2}$$

The best fitted model for the ethanol volumetric productivity was also the reduced cubic model, since it resulted in the best R^2 value (0.8848). The lack of fit was shown as significant ($p<0.05$), however not invalidating the model, since the predicted values are in accordance with the experimental values. The hierarchical model was maintained with their parameters and interactions. The parameter *B*, enzymatic load, was the most influential, followed by the cell concentration, and the solid/liquid was less influential in the ethanol

Table 4 Analysis of variance for ethanol concentration [partial sum of squares].

Source	Sum of squares	DF	Mean square	*F* value	*p*>F
Model	4,693.84	12	391.15	78.31	<0.0001
A	792.07	1	792.07	158.58	<0.0001
B	486.79	1	486.79	97.46	<0.0001
C	244.36	1	244.36	48.92	0.0002
A^2	1,033.59	1	1,033.59	206.94	<0.0001
B^2	198.37	1	198.37	39.72	0.0004
C^2	76.08	1	76.08	15.23	0.0059
AB	569.53	1	569.53	114.03	<0.0001
A^3	390.22	1	390.22	78.13	<0.0001
B^3	19.52	1	19.52	3.91	0.0886
C^3	239.23	1	239.23	47.90	0.0002
A^2C	244.67	1	244.67	48.99	0.0002
ABC	38.28	1	38.28	7.66	0.0278
Residual	34.96	7	4.99		
Lack of fit	9.90	2	4.95	0.99	0.4350
Pure error	25.06	5	5.01		
Cor total	4,728.80	19			

[Ethanol]: [*R*-squared=0.9926, Adj *R*-squared=0.9799]

volumetric productivity (Table 5). The resulting ethanol volumetric productivity model is presented in Eq. 3

$$\begin{aligned}\text{Ethanol volumetric produtivity} = &-3.17128 + 1.61333*S:L + 0.20374*\text{FPU/g} \\ &+0.67448*[\text{Cell}] - 0.012667*S:L*\text{FPU/g} \\ &-0.10500*S:L*[\text{Cell}] \\ &-9.00000\text{E}{-}003*\text{FPU/g}*[\text{Cell}] - 0.33342*S:L^2 \\ &-4.35605\text{E}{-}003*\text{FPU/g}^2 - 0.078147*[\text{Cell}]^2 \\ &+7.00000\text{E}{-}003*S:L*\text{FPU/g}*[\text{Cell}]\end{aligned} \quad (3)$$

Figures 1, 2, and 3 show the 3D response surface plots, which represent the regression equations. Plots showing interaction between solid/liquid ratio, enzyme load, and cell concentration for final ethanol concentration, volumetric productivity, and SSF initial glucose concentration are depicted.

Figure 1 shows the response contour and surface plots for the optimization of SSF initial glucose concentration. The effects of solid/liquid ratio (*A*) and enzyme load (*B*) indicate that the SSF initial glucose concentration is higher in maximum levels of enzyme load and solid/liquid ratio, obviously since higher solid content associated to a higher enzyme load result in more released sugars.

Figure 2 shows that the maximum ethanol concentration occurs when the parameter solid/liquid ratio (*A*) and enzyme load (*B*) are at their highest levels. It adds up significantly from the central point of the two parameters analyzed for their higher levels. The increase in the solid/liquid ratio associated to the increase of enzyme load improved the hydrolysis

Table 5 Analysis of variance for volumetric productivity [partial sum of squares].

Source	Sum of squares	DF	Mean square	*F* value	*p*>F
Model	4.63	10	0.46	6.92	0.0038
A	0.14	1	0.14	2.11	0.1800
B	1.14	1	1.14	16.97	0.0026
C	0.79	1	0.79	11.85	0.0074
A^2	1.60	1	1.60	23.96	0.0009
B^2	0.87	1	0.87	12.94	0.0058
C^2	0.43	1	0.43	6.47	0.0315
AB	0.011	1	0.011	0.16	0.7011
AC	5.513E−003	1	5.513E−003	0.082	0.7806
BC	0.025	1	0.025	0.38	0.5538
ABC	0.050	1	0.050	0.74	0.4116
Residual	0.60	9	0.067		
Lack of fit	0.60	4	0.15	242.90	<0.0001
Pure error	3.083E−003	5	6.167E−004		
Cor total	5.23	19			

Volumetric productivity: [*R*-squared=0.8848, Adj *R*-squared=0.7569]

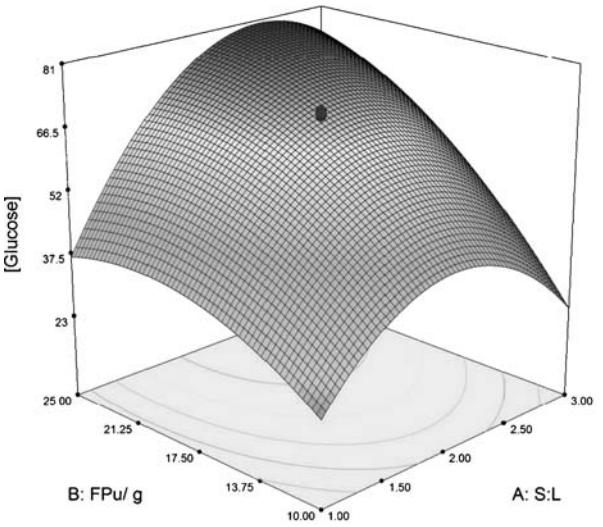

Fig. 1 Responses surface plots showing the effect of solid/liquid ratio (parameter *A*) and enzyme load (parameter *B*) and their combined effects on SSF initial glucose concentration

efficiency and therefore generated a higher SSF initial glucose concentration available in the medium. The efficiency of the fermentation process depends on the initial SSF glucose concentration produced during the enzymatic prehydrolysis [15]. Although there is a tendency for a curvature in the ethanol 3D plot, the maximum value is clearly reached, as it can be seen in the plots of volumetric productivity

The interaction between enzyme load and cell concentration (*BC*), as well as solid/liquid ratio and cell concentration (*AC*), had no significant influence in this analysis, with values of $p>F$ less than 0.05, and therefore removed from the model.

Figure 3a–c represents the response contour and surface plots for the optimization of volumetric productivity. The effect of solid/liquid ratio (*A*) and enzyme load (*B*), keeping the cell concentration in the central point, clearly indicates that optimum point for volumetric productivity is around the maximum level of enzyme load and around the

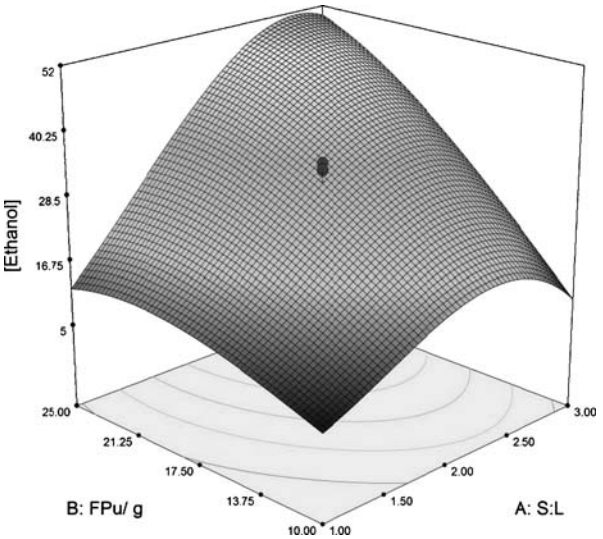

Fig. 2 Responses surface plots showing the effect of solid/liquid ratio (parameter *A*) and enzyme load (parameter *B*) and their combined effects on ethanol concentration

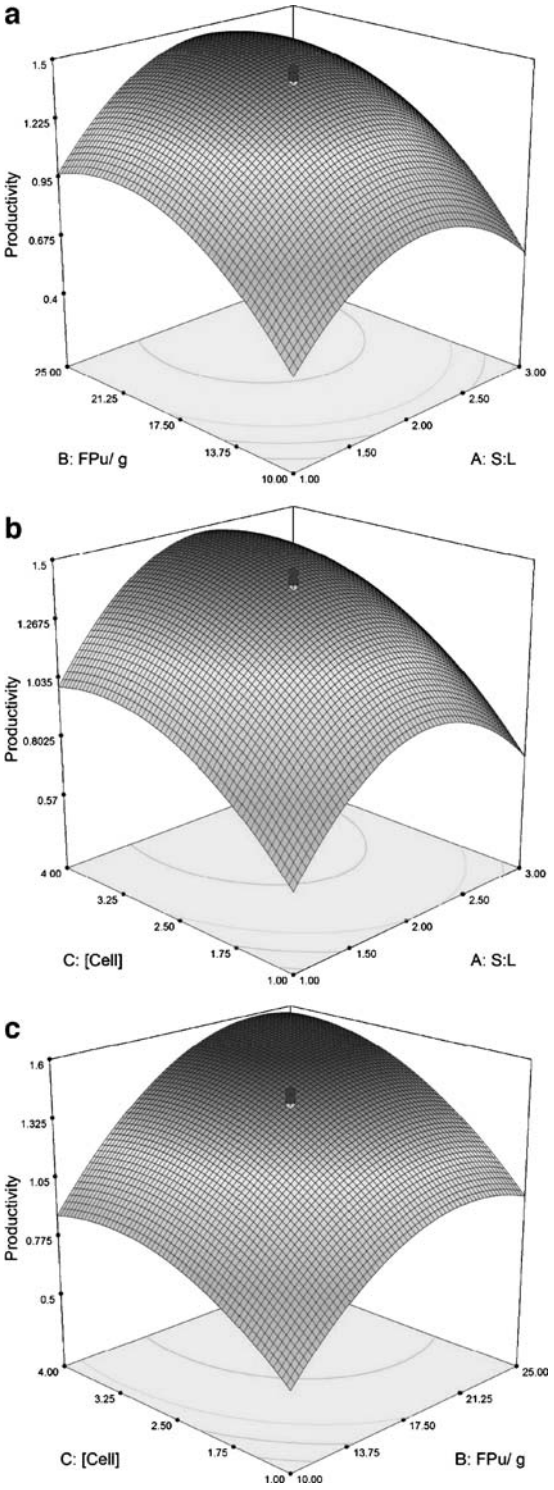

Fig. 3 Responses surface plots showing the effect of solid/liquid ratio (parameter *A*), enzyme load (parameter *B*), and cell concentration (parameter *C*) and their combined effects on volumetric productivity (**a**–**c**)

central point of the solid/liquid ratio (Fig. 3a). Therefore, when the SSF process occurs in conditions of high levels of solids, the fermentation time and the ethanol concentration are affected. The interaction between solid/liquid ratio (*A*) and cell concentration (*C*), keeping the enzyme load in its central point, indicates that the highest volumetric productivity occurs in the maximum levels of enzyme load and cell concentration (Fig. 3b). When the enzyme load is low, the release of glucose in the process is very slow, so that the ethanol concentration and its volumetric productivity decrease. When there is high cell concentration in medium, the fermentation proceeds more rapidly, increasing ethanol volumetric productivity. The effect of enzyme load and cell concentration (*BC*), when solid/liquid ratio is in its central point, indicates that the highest volumetric productivity occurs in the maximum levels of cell concentration and enzyme load (Fig. 3c). When there is an increase in solid content and the enzyme load does not increase proportionately, the enzymatic hydrolysis takes place slowly, and thus the release of glucose in the process is low, reducing the ethanol concentration and its volumetric productivity because the fermentation rate is limited by the low substrate concentration.

The validation of the experimental design was carried out either in shake flask (Fig. 4) or in bioreactor (Fig. 5). The highest values of SSF initial glucose concentration (76 g/L), final ethanol concentration (60 g/L), and volumetric productivity (1.5 g $L^{-1}h^{-1}$), obtained in shake flasks, were in agreement with the conditions predicted by the model. The glucose was converted to ethanol within 36 h without pH control, at temperature 30 °C and orbital agitation at 150 rpm. The experimental validation in bioreactor yielded a SSF initial glucose concentration of 80 g/L, final ethanol concentration of 55 g/L, and volumetric productivity

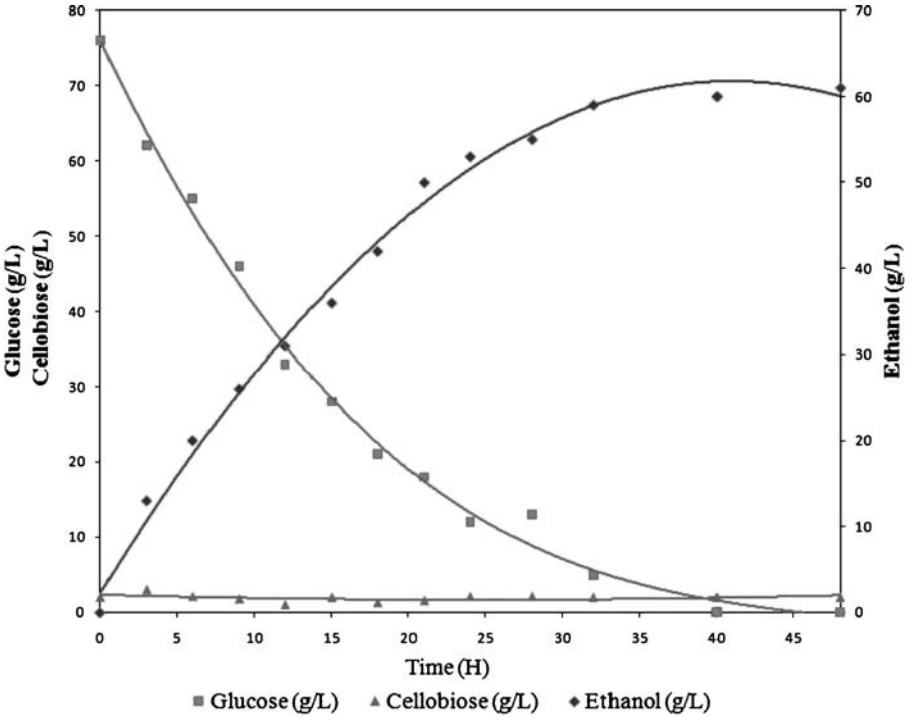

Fig. 4 Validation of the experimental design optimum conditions (*S*/*L*=1 g/3 mL; EL=25 FPU/g; X_o=4 g/L) for the SSF process in shake flask with *Z. mobilis* CP4

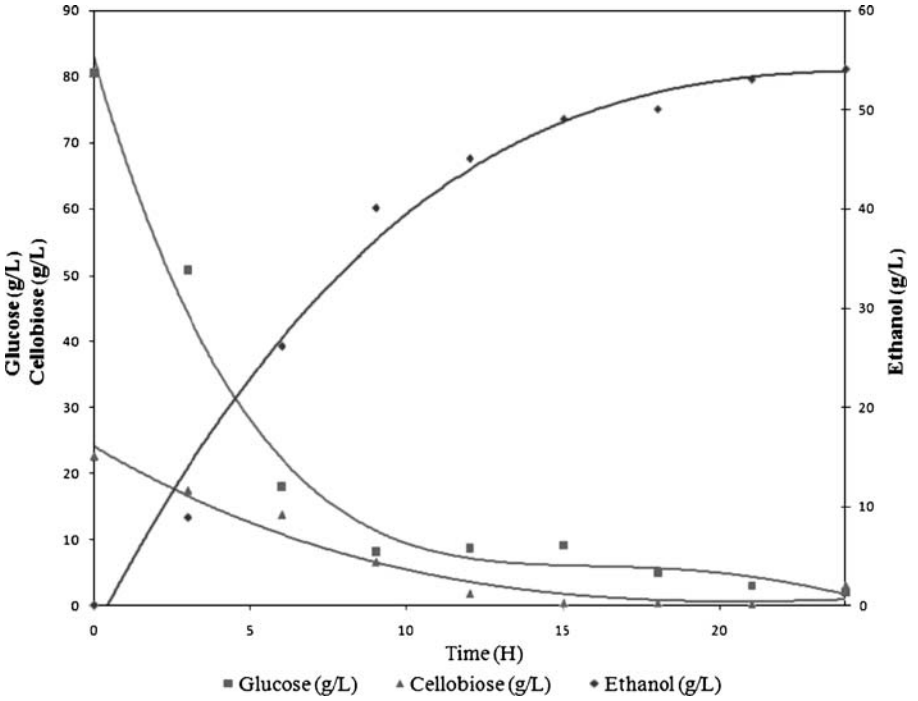

Fig. 5 Validation of the experimental design optimum conditions (S/L=1:3; EL=25 FPU/g; X_o=4 g/L) for the SSF process in bioreactor with *Z. mobilis* CP4

of 2.3 g $L^{-1}h^{-1}$, with a fermentation time of approximately 24 h, at temperature of 30°C, orbital agitation at 150 rpm, and pH 5.

Finally, Table 6 shows the most prominent results reported in literature using strains of *Z. mobilis* in the ethanol fermentation of lignocellulosic feedstocks. Through literature review using the *Z. mobilis* in second generation materials, it is observed that the most studies uses starch materials [16], the association with another microorganisms [17], or genetically engineered alteration [18].

As analyzed in this work, *Z. mobilis* has the potential to revolutionize the fuel ethanol industry commercially; laboratory and pilot-scale operations indicate that it can generate nearly theoretical maximum yields from several feedstocks, including sugarcane [19], cassava and sago [20], and enzymatic hydrolyzate of wood-derived cellulose [21, 22].

A limitation to the potential of the bacterium as a universal biocatalyst in the fuel ethanol industry is its capacity to utilize only glucose, fructose, or sucrose. It lacks a complete pentose metabolism pathway necessary for xylose fermentation, unless it is genetically manipulated [18, 23]. However, if the two-stream model is adopted, for the utilization only of the cellulose faction naturally occurring strains of *Z. mobilis* seem to be a promising alternative.

Conclusions

The experiments showed that it was possible to optimize the ethanol production, volumetric productivity, and glucose concentration through the experimental designs of response surfaces. SSF initial glucose concentration and ethanol concentration were affected by the

Table 6 Main results reported in literature for the SSF process with strains of *Zymomonas mobilis*.

Feedstock	Conditions	Ethanol concentration	Reference
Sigmacell	Isothermal co-cultures of *K. oxytoca* P2 with *Z. mobilis* ZM4	36 g/L	[17]
	Substrate 100 g/L of sigmacell		
	Enzyme load 15 FPU/g		
	Cell concentration 0.320 g/L		
	Temperature of 35°C		
	Time 120 h		
	Agitation 100 rpm		
	pH 5		
Steam-pretreated willow	Substrate 61 g/L of glucose	28.8 g/L	[24]
	Enzyme load 18 FPU/g		
	Cell concentration 3 g/L		
	Temperature 37°C		
	Time 72 h		
	Agitation 150 rpm		
	pH 5		
Kitchen garbage	Substrate 70 g/L of reducing sugar	52 g/L	[25]
	Enzyme load 100 U/g wet mass		
	Cell concentration 10% (*v*/*v*)		
	Temperature 30°C		
	Time 40 h		
	Agitation –		
	pH 6		
Sugarcane bagasse	Substrate 80 g/L of glucose	60 g/L	this work
	Enzyme load 25 FPU/g		
	Cell concentration 4 g/L		
	Temperature 30°C		
	Time 36 h		
	Agitation 150 rpm		
	pH 5		

increase of solids content (parameter *A*) as well as increasing the enzyme loading (parameter *B*) resulting in higher concentrations of ethanol. The optimum conditions were found to be solid content (3:10), enzymatic load (25 FPU/g), and cell concentration (4 g/L), resulting in the maximum ethanol concentration of 60 g/L, with 76 g/L of SSF initial glucose concentration, reaching the highest volumetric productivity of 1.52, at temperature 30°C, orbital agitation at 150 rpm in shaken flasks and 55 g/L, with 80 g/L of SSF initial glucose concentration, reaching the volumetric productivity of 2.29 g $L^{-1}h^{-1}$, at temperature 30°C, orbital agitation at 150 rpm, pH 5 in bioreactor.

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