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METABOLIC ADAPTATION STRATEGY USING *Zymomonas mobilis* CP4 CELLS FOR THE PRODUCTION OF SECOND GENERATION ETHANOL

Danielle da Silveira dos Santos Martins, Elcio Ribeiro Borges, Viviane Castelo Branco Reis, Fernando Araripe Gonçalves Torres, Nei Pereira Jr.

Laboratories of Bioprocess Development, School of Chemistry, Federal University of Rio de Janeiro, 21949-900, Laboratories of Bioprocess Development, School of Chemistry, Federal University of Rio de Janeiro, 21949-900,
Laboratories of Bioprocess Development, School of Chemistry,Federal University of Rio de Janeiro, 21949-900,
Cellular Biology Department, University of Brasília, 70910-900, Cellular Biology Department, University of Brasília, 70910-900,

ABSTRACT

During the past few decades, considerable effort has been made to utilize agricultural and forest residues as biomass feedstock for the production of bioethanol as an alternative fuel. The bacterium *Zymomonas mobilis* was shown to be extremely attractive for the production of second-generation ethanol from glucose of the cellulose fraction due to its ability to uptake high amounts of this sugar, resulting in high ethanol productivity values. However, the wild-type strains are unable to metabolize xylose that arises from the hemicellulose fraction. Molecular biology techniques were incorporated to render the strain used in this study capable of fermenting xylose into ethanol and thus increase the efficiency of second-generation ethanol production. Thus, the aim of this study was to evaluate the performance of a recombinant strain of *Z. mobilis* in simultaneous saccharification and co-fermentation (SSCF) processes, in which the fermentation of both sugars (glucose and xylose) occurs in one step. Regarding the genetic transformation,the 1,565 kb *Z. mobilis* plasmid pZMO1 was chemically synthesized and cloned into a synthetic vector that contains the *E. coli* and *Z. mobilis* replication checkmark origin,the XI, XK, TAL, and TKL genes and tetracycline resistance. Metabolic adaptation was performed by transferring the recombinant strain to media containing increased xylose concentrations. Then, an experimental response surface methodology was used to evaluatethe addition of glucose and xylose with different concentrations, as well as the incorporation of hemicellulosic hydrolyzate in different proportions. The recombinant *Z. mobilis* CP4 strain reached 25 g/L ethanol, confirming that approximately 50% of this pentose was consumed in the SSCF process when using 30% solids, 20.5% hemicellulose hydrolysate, 10 mg/L tetracycline, an enzyme load of 25 FPU/g cellulignin, and 10% of the initial inoculum.

Indexing terms/Keywords

Ethanol 2G; Sugarcane bagasse; *Zymomonas mobilis*; Metabolic adaptation; SSCF.

Academic Discipline And Sub-Disciplines

Biotechnology, Biochemical, Microbiology, Bioprocess Engineering, Biochemical Engineering.

SUBJECT CLASSIFICATION

Biotechnology, Biochemistry, Applied Microbiology, Bioprocess.

TYPE (METHOD/APPROACH)

Statistics for Experimenters; Pretreatment of Lignocelulosic Materials; Basic Techniques in Molecular Biology; Metabolic Adaptation.

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INTRODUCTION

Sugarcane bagasse, the main solid residue generated in the production of ethanol from sugarcane juice, is considered an excellent lignocellulosic raw material despite being used for energy production in industrial distillery units. However, it is still produced in large amounts, and a large part of it is considered surplus. In this context, it is estimated that sugarcane production will reach approximately 660 million tons in the 2014/2015 harvest season, resulting in approximately 200 million tons of sugarcane bagasse [1].

For the efficient production of ethanol from sugarcane bagasse, i.e.,second-generation ethanol (ethanol 2G), the following steps are required to fraction the main lignocellulosic components: physical/physical-chemical pretreatments and enzymatic hydrolysis simultaneously or separately from fermentation. These steps are necessary to provide carbohydrates (hexoses and pentoses) that must be converted to ethanol by fermenting microorganisms. First, acid pre-treatment has been shown to be an interesting alternative [2] because in addition to disrupting the lignocellulosic complex, it causes hemicellulose hydrolysis, resulting in a xylose-rich hydrolysate. The liquid containing the hemicellulosic hydrolysate is separated from the solid fraction, which mainly consists of cellulose and lignin, i.e., cellulignin. A partial delignification stage of this solid fraction has been shown to be essential to increase the accessibility of the enzymes to the cellulose fibers [3], resulting in hydrolysates containing high glucose concentrations.

Zymomonas mobilis, which exists in natural sources, such as fruits, and as a “beneficial” contaminant in the alcohol fermentation industry, is capable of producing ethanol efficiently from glucose and fructose sugars via the Entner-Doudoroff pathway. This microorganism uses small fractions of sugar as a carbon source, and approximately 98% of the sugar is used for fermentation. Thus, *Z. mobilis* is widely used in the manufacture of beverages, milk enriched with fermentable sugars and traditional drinks,such as Pulque [4]. Due to its high potential as a fermentation agent, *Z. mobilis* has been the subject of numerous studies, many of which show promising results in terms of ethanol production when compared with the traditional *Saccharomyces cerevisiae*, which is largely used in industrial distilleries [5].

To enable the commercial production of lignocellulosic ethanol, an efficient conversion of the main carbohydrates arising from hemicellulose and cellulose hydrolysates into ethanol 2G is necessary. Although glucose fermentation is carried out efficiently by *Z. mobilis,* as well as by *Saccharomyces cerevisiae,* the same level of efficiency is not achieved with the conversion of xylose, the main component of the sugarcane bagasse hemicellulose fraction. According to Lynd & Zhang (2010), the use of microorganisms that are able to ferment glucose and xylose can increase ethanol production by approximately 20% with the adoption of the technological concept of simultaneous saccharification and co-fermentation (SSCF) compared to the production of only glucose from the cellulose fraction [6].

Because the ability to utilize only glucose, fructose or sucrose as an energy source limits the potential of *Z. mobilis* for the industrial production of ethanol [7], genetic engineering and metabolic adaptation were applied to enable this bacterium to ferment xylose, offering an excellent opportunity for process improvement.

Application of genetic engineering in *Z. mobilis* cells

General genetic tools have been developed for *Z. mobilis* since the 1980s, including native plasmids, broad host-range vectors or shuttle vectors, expression systems, and transfer, promoter, and reporter genes, as reviewed elsewhere [8]. The pioneering work of Liu *et al.* [9] and Feldman *et al.* [10], which introduced xylose isomerase (XI) and xylulokinase (XK) genes from *Xanthomonas campestris* and *Klebsiella pneumoniae* in *Z. mobilis*, obtained limited success. These enzymes are responsible for xylose assimilation; however, the enzymes transaldolase (TAL) and transketolase (TKT) promote the metabolism thereof, being essential for the production of ethanol [8,11].

The same genes encoding these enzymes from *E. coli* were also introduced into the bacterium *Zymobacter palmae*. After metabolic adaptation in xylose-containing medium, the microorganism reached 95% efficiency from the fermentation of glucose and xylose [12], higher than the efficiency reported in the study by Mohagheghi *et al.* [13], who achieved 76% efficiency with the use of 75% (v/v) of the pentose-derived hemicellulosic hydrolysate by *Z. mobilis.* Thus, Zhang *et al.* [14] demonstrated that the co-expression of XI, XK, TAL and TKT, derived from *Escherichia coli,* allowed *Z. mobiles* to co-ferment xylose and glucose, reaching 11 g/L ethanol and resulting in a yield of 0.44 g ethanol/g xylose consumed. This yield corresponds to a fermentation efficiency of 86%. However, with glucose and glucose/pentose media, the recombinant strain CP4 (pZB5) reached 94% and 95% efficiency after 16 and 30 h of cultivation, respectively.

Several articles reported the fermentation of xylose present in the hemicellulose fraction of agro-industrial waste. Mohagheghi *et al.* [15] used waste acid hydrolyzed corn processing, in which fermentation resulted in an ethanol concentration of 53 g/L. Zhang & Lynd [16] found that the *Z. mobilis* 8b microorganism (derived from 31821-pZB5) performed better than *S. cerevisiae* RWB222 when considering the waste paper industry hydrolyzate in the SSCF process. Both microorganisms performed quite similarly, producing ethanol concentrations of 29 and 23 g/L and obtaining yields of 0.47 and 0.40 g/product/g sugar consumed, respectively. Davis *et al.* [17] have also used hemicellulosic hydrolyzate from wheat kernels, reaching an ethanol concentration of 11 g/L and a residual xylose of 12 g/L using the ZM4 (pZB5) strain without supplementation. In subsequent studies, 5 g/L yeast extract and 40 g/L glucose was added to the medium, obtaining 28 g/L ethanol and 2.6 g/L residual xylose after 18 h.

Recently, some authors have reported different strategies: Zhang *et al.* [18] cited the creation of heat-tolerance strains that use low nutritional requirements, which would be beneficial for the ethanol industry; in addition, recombinant *Z. mobilis* mutants can produce ethanol efficiently at high temperatures using low nutritional requirements, yielding an ethanol concentration of 25 g/L from 60 g/L glucose added to an RM medium. Caimi *et al.* [19] reported that ribulose

accumulation reduces ethanol production in recombinant *Z. mobilis* colonies grown on xylose. Thus, the authors genetically engineered a microorganism that produces the ribose-5-phosphate isomerase at high concentrations, reducing ribulose accumulation, and the production of ethanol increased from 5% to 15% (w/ v) using 100 g/L xylose.

The incorporation of molecular biology techniques is necessary to make progress in second-generation ethanol production and to provide the strains used in this study that are also able to ferment xylose into ethanol. In this context, the aim of this work is SSCF process development (simultaneous saccharification and co-fermentation), in which the fermentation of both sugars occurs in one step.

In this context, the theoretical yield of the ethanol production from xylose is 0.51 g ethanol/g xylose (1.67 mol/mol), generating 1 ATP mol/mol pentose. Aristidou & Penttilä [20] found that the new metabolic pathway promotes the conversion of 5 moles of ethanol from 3 moles of xylose according to the following stoichiometric equation:

$$\mathbf{3\ xylose + 3ADP + 3\ Pi \rightarrow 5\ ethanol + 5CO_2 + 3ATP + 3H_2O.}$$

MATERIALS AND METHODS

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

Pretreatment of Cellulignin: The following conditions were used for sugarcane bagasse acid pretreatment: H_2SO_4 1% (v/v), solid/liquid ratio 1:2, temperature 121°C, and duration of 30 min [2]. After pretreatment, the aqueous phase was removed by pressing filtration, and the remaining solid (acid cellulignin) was subjected to an alkaline delignification with NaOH at a concentration of 4% (w/v) and with a solid/liquid ratio of 1:20. Then, the acid cellulignin was exposed to a temperature of 121°C for 30 min [21]. 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). The enzymatic activities were determined by filter paper activity, as recommended by Ghose, [22] and are expressed as filter paper units (FPU) per milliliter of mixture.

Microorganism and Inoculum: The native *Z. mobilis* CP4 used in this work was kindly provided by the Department of Antibiotics of the Federal University of Pernambuco (Brazil). The strain was grown in a liquid medium (20 g L^{-1} of glucose and 5 g L^{-1} of yeast extract), as recommended by Swing and De Ley, at 30°C for 24 h and maintained at 4°C.

SSCF Experiments*:* Batch fermentation experiments were performed in 500mL 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 carriedout in a 1.5L bioreactor (BIOFLO III, New Brunswick Scientific, USA) with temperature, pH, and agitation controlled. The reactor was 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 SSCF performed in a bioreactor were evaluated under the optimum conditions established in the shake flask experiments. The experiments were performed under the principles of the statistical methodology of response surfaces, that is, a statistical model widely used to study the aggregate effect of several variables and to seek optimum conditions for a multivariable system [23]. The data were statistically analyzed using “Design Expert” software (7.1.6., Stat-Ease).

Analytical Methods: Samples were analyzed for glucose, cellobiose, and ethanol concentrations by high-performance liquid chromatography (HPLC) using a chromatographic system (WATERS) consisting of an HPX-87p (Bio-Rad) column, WATERS 510 pump, 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 by the stabilization of ethanol production, as verified by the reading of two consecutive and equal values of alcohol content.

Applications of Genetic Engineering

Gene designer: The synthetic genes encoding xylose isomerase, xylulokinase, transaldolase and transketolase as well as two operons, each under the control of a strong constitutive promoter pGAP of *Z. mobilis*, were constructed by chemical synthesis at Advanced Life Sciences Company Technologies, Inc., from genome data for *E. coli* and *Z. mobilis*. The *Z. mobilis* Plasmid pZMO1 (1,565 kb) [24], which demonstrated stability in the bacteria, was also chemically synthesized and cloned into a synthetic bifunctional vector for *Z. mobilis* and *E. coli* (Figure 1). This vector presents the *E. coli* origin of replication and tetracycline resistance. In this context, the well-defined segments of *E. coli* facilitate the maintenance of plasmid because the gene of *Z. mobilis* includes sequences responsible for replication.

A control that has a plasmid containing only the genes for thetetracycline resistance and the replication origin from *Z. mobilis* and *E. coli* was also developed, named *ORI*; the plasmid containing all genes described, in addition to the genes of xylose metabolism (XI, XK, TAL and TKT), was named *ORI Zymo.* Figure 1 shows the organization of the dual-system operon (~ 3.2 kb on average) for the assimilation and metabolism genes of xylose, under the control of the pGAP promoter, inserted into the plasmid pZMO1.

Gene amplification: One microliter(50 ng) of pZM01 was used for the amplification of genes by genetic transformation into *E. coli* DH5α. The plasmid was added to each aliquot of competent cells and incubated on ice for 45 min. Subsequently, heat shock was performed at 42°C for 90 s, followed by incubation at 37°C for 45 min and stirring at 200 rpm using 1 mL of LB medium Then, the cells were plated on LB medium in the presence of the antibiotic (tetracycline, 10 mg/L) at 37°C for 24 h. This method, adapted from Sambrook & Russell (2001), has often been used without sophisticated equipment [25].

DNA extraction: Initially, the transformed *E. coli* cultures were grown in 200 mL of LB medium containing 10 mg/L tetracycline at 37°C for 24 h. Large-scale DNA extraction, developed according to the methodology of Azevedo *et al.*[25], was performed after the *E. coli* transformation procedure (discussed above).

Competent cells of *Z. mobilis*: Regarding the insertion of genes by processing,the cells must be made competent to enable the introduction of exogenous DNA in the selected host. Therefore, the competent cell protocol of *Z. mobilis* developed in this work was based on Liang Lee's methodology [26]. After reaching 0.36 of Abs_{600}, the inoculum (100 mL) was centrifuged at 4,000 rpm for 5 min at 4°C,the supernatant was discarded and the pellet was resuspended in 10 mL of sterile distilled water solution (added with 10% glycerol supplemented with 0.85% NaCl). The centrifugation step was repeated under the same conditions, followed by the addition of 2 mL of solution containing distilled water (10% glycerol).

Exogenous Plasmid: The plasmid was extracted after lyophilization concentration, and the volume was then adjusted to 30 µL. After measuring absorbance (260-280 nm),its concentration was calculated to be 10 mg/µL for ORI Zymo and 23 mg/µL for ORI.

Electroporation: The *Z. mobilis* cells were transformed by electroporation using BioRad GenePulser Xcell ™ equipment. The colonies were transferred to an electroporation cuvette (0.2 cm), maintained at a low temperature and then processed as follows: 1.5kV/cm^3, 75kV/cm, 25µF and 200Ω.

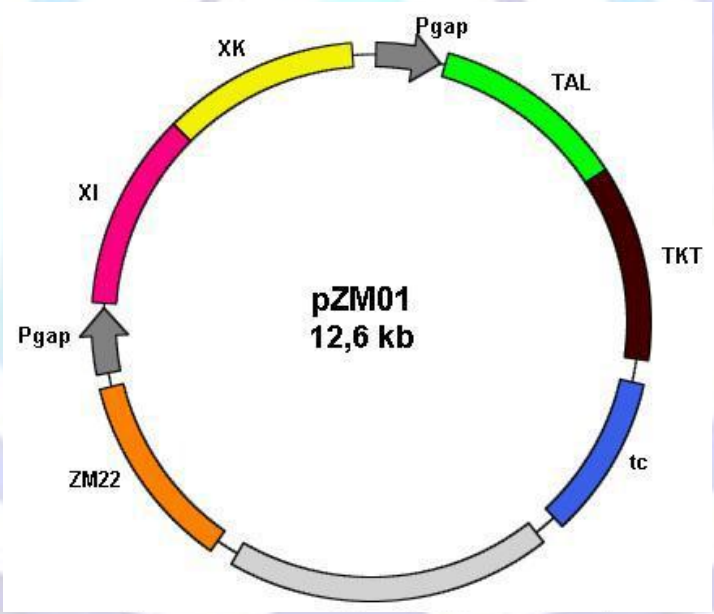

Fig 1: Map of the pZM01 plasmid, which has the double operon system for the genes of xylose metabolism.pGAP, glyceraldehyde-3-phosphate promoter; XI, xylose isomerase; XK, xylulokinase; TAL, transaldolase; TKT, transketolase; Tc, tetracycline resistance gene (4.2 kb); ZM22, origin of replication *Z. mobilis* (5.9 kb).

Post-Processing

Immediately after the bacterial cultures of *Z. mobilis* [ORI, ORI Zymo and the control without plasmid] were transformed by electroporation, they were incubated in RMG medium for 16 h as described by Picataggio *et al.* [27]. Subsequently, the three cultures were plated with RMG agar medium (20 g/L) at 30°C for 7 days. Thus, transformed clones resistant to the antibiotic were grown in RM medium, and xylose and glucose were added at different concentrations to assess and enhance bacterial growth. Subsequently, isolated colonies that produced more biomass and rapid growth in these substrates were tested in terms of ethanol production.

Growth medium: The synthetic RM medium (rich medium) was used in the fermentation trials with a recombinant strain, as described by Mohagheghi *et al.* [28], Zhang [29] and Jeon *et al.* [30]. Glucose, xylose, and a combination of the two sugars were used as the RMG, RMX and RMGX mediums,

respectively, for a carbon source at 20 g/L concentrations for each sugar.

RESULTS AND DISCUSSION

After genetic modification of *Z. mobilis*, its cells were grown on glucose (20 g/L) and RM medium and 10 mg/L tetracycline were added, as shown in Figure 2.The naturally occurring strain did not grow, unlike the genetically modified strains ORI and ORI Zymo, which displayed several colonies, confirming that the genes exhibited tetracycline resistance. Because the antibiotic resistance gene is associated with the gene for xylose metabolism, the ORI Zymo strain apparently also changed simultaneously, as noted in the subsequent experiments.

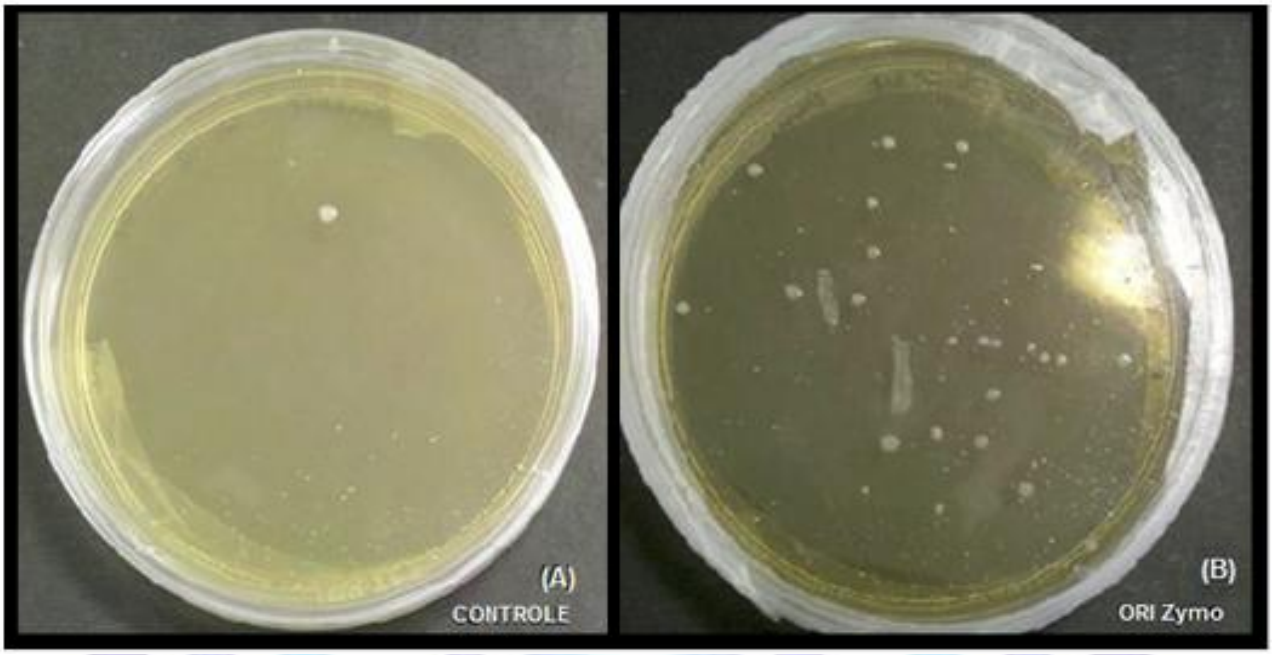

Fig 2: Growth of different strains of *Z. mobilis* after 168 h compared to the RMG medium containing glucose (20 g/L), KH_2PO_4 (2 g/L), yeast extract (10 g/L), agar (20 g/L) and tetracycline (10 mg/L) at a temperature of 30°C. (A) naturally occurring *Z. mobilis*; (B) ORI Zymo: bacterial plasmid that contains the antibiotic resistance gene and the gene for xylose metabolism.

Metabolic adaptation in synthetic medium

Several authors observed that the *Zymomonas* cells had difficulty converting xylose into ethanol after genetic transformation.The low ethanol yield from glucose and xylose mixtures can be ascribed to the preferential glucose uptake, generating high ethanol and acetic acid concentrations, which in turn hinder further xylose metabolism and reduce the rate of utilization of this pentose (Leksawasdi *et al.,* 2001). Furthermore, the exogenous acetate stress or non-native xylose utilization shiftsthe redox balance, which leads to toxic intermediate xylitol formation and glycolysis inhibition [31]. Additionally, the competition for the only sugar transporter causes a reduction in the xylose metabolism compared to glucose [32]. Thus, some authors, e.g., Zhang *et al.* [33] and Viitanem *et al.* [34], have successfully developed the methodology of metabolic adaptation to circumvent this problem. Yanase *et al.* [35] reported the need for successive adaptation in recombinant species of *Zymobacter palmae* to increase ethanol yields.

Agrawall *et al.* [36] also reported that metabolic adaptation was essential for ethanol production by modified strains of*Z. mobilis* ZM4 (pZB5). Additionally, this strain has been identified as one of the best strains for xylose metabolism and has a high tolerance to ethanol, acetic acid and furfural. Zhang [29] found that the CP4 strain, which produced 24 g/L ethanol from 15 g/L glucose plus 35 g/L xylose, was not as effective as the 8b strain [6], which yielded 40 g/L ethanol from the hydrolysate of paper waste. With the strain bearing the pZB5 plasmid, an ethanol production rate of 90 g/L was achieved by applying the methodology of metabolic adaptation for 80 days with 30 adaptation cycles. The first colonies were grown in RMGX medium, and subsequently, the authors gradually increasedthe xylose concentration. Later, the selected strain had higher enzyme activity values for xylose isomerase and provided a higher conversion rate to ethanol and lower production of xylitol from this pentose. Nonetheless, the XI activity was still low compared to the other three enzymes, XK, TAL, and TKL. Additionally, the adapted and original colonies were sequenced, but no genetic difference could be identified. The authors reported a possible mutation involving transcription XI for both cultures, i.e., before and after metabolic adaptation.

Thus, the first adaptation cycles performed in the present study contained high glucose and low xylose concentrations. As the cycles advanced, the glucose concentration decreased, and the xylose concentration increased (Table 1). As noted

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above, immediately after genetic transformation, the microorganism showed slow growth, circa 170 h, compared with the results developed by the native strain, which fermented glucose within 48 h. The bacterial growth time after the 20th cycle was reduced to approximately 72 h, and the xylose uptake and the cell growth were increased.

The cell growth after the 26th cycle reached 0.166 g/L in approximately 70 h, with xylose and glucose concentrations of 15 and 5 g/L, respectively. Cultures for the first adaptation cycles reached values of 0.05 g/L in 96 h with medium containing 15 g/L glucose and 5 g/L xylose, which is more favorable for bacterial growth (more glucose). Therefore, although improvements regarding fermentation with this genetically modified *Z. mobilis* are still needed, the fermentation time and the ability to ferment xylose were both improved.

Table 1. Metabolic adaptation process during 50 cycles with varying glucose and xylose concentrations in RM medium.

Cycles	Time(Days)	Initial Glucose (g/L)	Initial Xylose (g/L)	Biomass*(g/L)	Ethanol*(g/L)
1-10	70	15	5	0.05	2.3
11-19	30	10	10	0.08	4.1
20-25	20	7.5	13.5	0.100	3.9
26-30	15	5	15	0.166	4.6
31-40	15	2.5	17.5	0.159	4.1
41-50	10	1.5	18.5	0.158	4.2

***** Values correspond the results at the end of the last cycles.

Figure 3 summarizes the metabolic adaptation process insynthetic medium, suggesting that the recombinant *Z. mobilis* colonies increased ethanol production in the earlier cycles (1-30)but maintained such levels in subsequent cycles (31-50). This strategy allowed the recombinant to produce ethanol from xylose more efficiently becausehigher participation in ethanol production is derived from this pentose in the latter cycles.

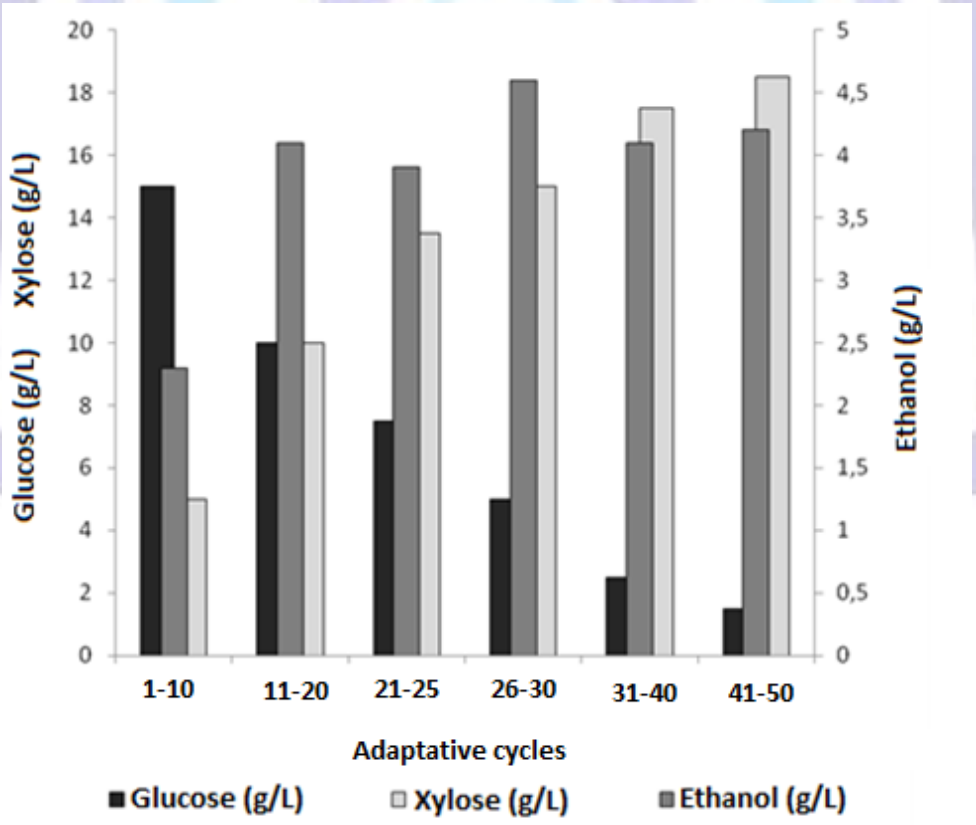

Fig 3: **Metabolic adaptation of a recombinant strain of *Z. mobilis* during 50 cycles to increase ethanol production from xylose.**

Evaluation of ethanol production from SSCF through sequential experimental design

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Two Sequential 2^2 Response Design Surfaces were developed to evaluatethe solid concentration of pretreated sugarcane bagasse and the proportion of hemicellulose hydrolysates in the SSCF process. The highest ethanol production was 25.0 g/L, which was achieved with a pretreated sugarcane bagasse concentration of 2.21:10 g:mL and proportion of hemicellulose hydrolysates of 20% v/vin the first Experimental Design. The second highest ethanol final concentration (10 g/L) was obtained in experiment 6 (Table 2), with a volumetric proportion of 45% of hemicellulose hydrolysate. Additionally, for the other experiments using HH=45%, the ethanol production was low, indicating cell inhibition by toxic compounds generated during pretreatment (HMF, furfural and acetic acid), as widely reported by Padilla *et al.*

Subsequently, another Sequential 2^2 Response Design Surface was developed to assess the same independent variables, although with higher levels of the S:L variable and lower levels of the HH variable. Table 3 shows that the highest ethanol concentration (27.3 g/L) was obtained under the following conditions: 3:10 pre-treated sugarcane bagasse and 20% v/v hemicellulose hydrolysate. When the solid content was low, the ethanol concentration was severely reduced for any acid hydrolysate percentage (experiments 2, 4 and 10).

Table 2. First Sequential 2^2 Response Design Surface for the evaluation of the percentage of hemicellulose hydrolysate (%) and the solid:liquid ratio (g:mL) of the pretreated sugarcane bagasse using the SSCF process for ethanol production by the recombinant strain *Z. mobilis CP4*, adapted after the 25th cycle in synthetic medium.

Exp.	Independent Variables		Conditions		Response
	HH (%)	S:L (g:mL)	Initial Glucose(g/L)	Initial Xylose (g/L)	Ethanol (g/L)
1	45.00	2.2:10	67.3	24.5	7.5
2	**20.00**	**2.0: 10**	**57.4**	**9.6**	**25.0**
3	80.36	1.5: 10	38.9	61.1	0.0
4	70.00	2.0: 10	61.4	53.6	0.0
5	9.64	1.5: 10	36.5	5.3	7.4
6	45.00	1.5: 10	33.8	27.9	11.0
7	45.00	1.5: 10	39.2	30.1	2.2
8	45.00	1.5: 10	36.1	32.0	2.2
9	70.00	1.0: 10	25.0	51.7	1.5
10	45.00	1.5: 10	34.6	33.3	4.3
11	20.00	1.0: 10	23.3	10.1	7.6
12	45.00	0.8: 10	14.9	40.5	3.3
13	45.00	1.5: 10	37.5	35.0	4.0

H. H= hemicellulosic hydrolyzed; S:L= solid:liquid ratio.

Table 3. Second 2^2 Response Surface Design for the evaluation of the percentage of hemicellulose hydrolysate (%) and the solid: liquid ratio (g:mL) of the pretreated sugarcane bagasse using the SSCF process for ethanol production by recombinant *Z. mobilis* CP4 after the 25th cycle in a synthetic medium.

Ex.	Independent Variables		Conditions		Response
	HH (%)	S:L (g:mL)	Initial Glucose(g/L)	Initial Xylose (g/L)	Ethanol (g/L)
1	20.50	2.0:10	59.3	12.5	18.2
2	6.50	1.3:10	34.0	5.1	1.6
3	**20.50**	**3.0:10**	**48.9**	**11.0**	**27.3**
4	34.50	1.3:10	36.1	18.7	5.1
5	20.50	2.0:10	57.8	14.2	15.2
6	0.70	2.0:10	62.6	0.6	24.0
7	40.30	2.0:10	65.2	23.4	5.70
8	20.50	2.0:10	63.7	8.0	15.6
9	6.50	2.7:10	51.3	3.9	23.3
10	20.50	1.0:10	23.5	7.7	4.5

11	34.50	2.7:10	73.0	21.3	5.2
12	20.50	2.0:10	62.1	8.2	17.0
13	20.50	2.0:10	60.4	12.6	8.8

HH= hemicellulosic hydrolyzed; S:L= solid:liquid ratio.

According to the variance analysis of SSFC Experimental Design 1 and SSCF Experimental Design 2, the linear model (2FI: two-factor interactions) and quadratic model adequately fit the experimental results, with correlation coefficients of 0.7 and 0.8, insignificant pure error and insignificant lack of fit for the 90% confidence interval, as shown in Tables 4 and 5, respectively. Furthermore, the two variance analyses show that the models are significant. For the first design, parameter A (hemicellulosic hydrolyzate) mainly influenced the final ethanol concentration, followed by the AB interaction and parameter B (solid:liquid ratio). According to the model referring to SSCF Experiment 2, parameter B (solid:liquid ratio) was the most influential parameter, followed by A (hemicellulosic hydrolyzate). The combinations that showed the strongest influence on the model were between AB, followed by A^2 and B^2.

Table 4. Variance analysis of the ethanol concentration reached in the first Experimental Planning SSCF1 process from sugarcane bagasse by recombinant *Z. mobilis*.

	SS	DF	MS	F-Value	*p-value*
Model	366.91	3	122.30	6.76	0.0111
A	217.04	1	217.04	12.00	0.0071
B	60.22	1	60.22	3.33	0.1013
AB	89.66	1	89.66	4.96	0.0530
Residue	162.73	9	18.08		
Lack of Fit	109.96	5	21.99	1.67	0.3204
Pure Error	52.77	4	13.19		
Total Cor.	529.64	12			

A= hemicellulosic hydrolyzed, B= solid:liquid ratio; SS= Sum of squares; DF= Degree of freedom; MS= Mean square.

Table 5. Variance analysis of the ethanol concentration reached in the second Experimental Planning SSCF2 process from sugarcane bagasse by recombinant *Z. mobilis*.

	SS	DF	MS	F-Value	*p-value*
Model	700.29	5	140.06	5.71	0.0205
A	201.31	1	201.31	8.20	0.0242
B	360.93	1	360.93	14.71	0.0064
A^2	114.43	1	114.43	4.66	0.0677
B^2	18.21	1	18.21	0.74	0.4175
AB	8.19	1	8.19	0.33	0.5817
Residue	171.80	7	24.54		
Lack of Fit	120.36	3	40.12	3.12	0.1502
Pure Error	51.43	4	12.86		
Total Cor.	872.08	12			

A= hemicellulosic hydrolyzed, B= solid:liquid ratio; SS= Sum of squares; DF= Degree of freedom; MS= Mean square.

The model of SSCF1 and SSCF2 processes are represented by equations (2) and (3), respectively:

[Ethanol]: +5.84 - 5,21A + 2,74B - 4,73AB (2)

[Ethanol]: +14.96 - 5,06A 6,75B- 1,70A2 + - 1.17 B2 - 5.42 AB (3)

Figure 4 shows the contour surface, evaluating the hemicellulose hydrolysate percentage (%), as well as the solid:liquid (g:mL) ratio of pretreated sugarcane bagasse through the SSCF1 process, in relation to ethanol production by the recombinant *Z. mobilis* CP4 strain. The surface indicates a reduction in parameter A (hemicellulose hydrolysate) and an increasein the concentration of parameter B (solid/liquid ratio). However, another design was necessary to optimize the experiments.

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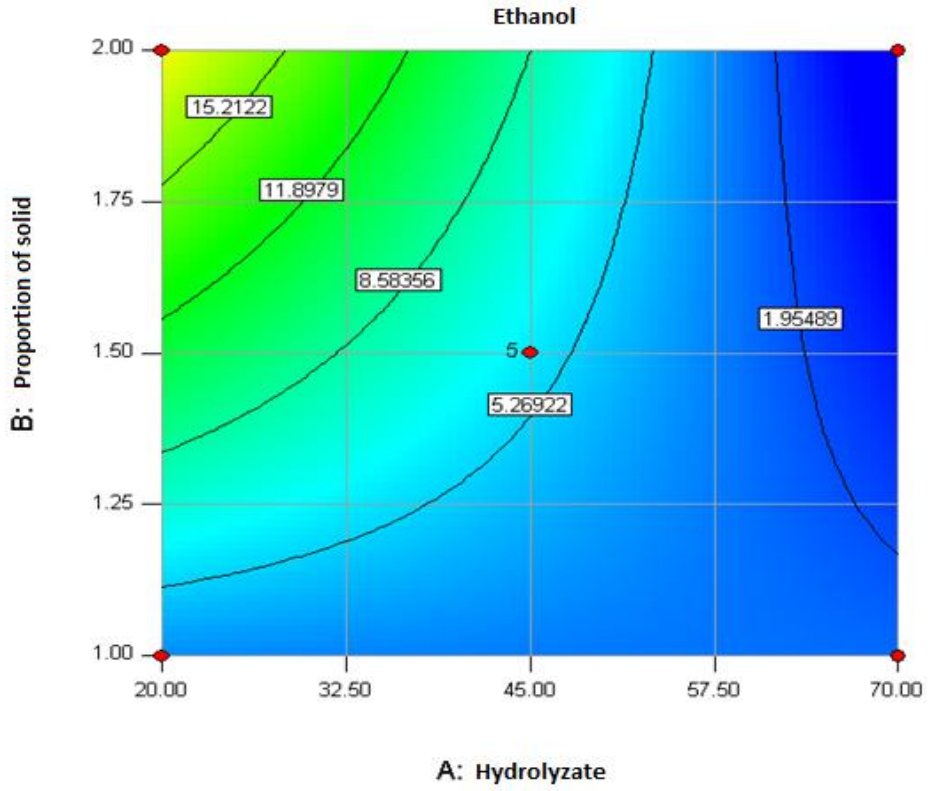

Fig 4: Contour surface evaluating the combined effects of the parameters on the production of ethanol from the first Experimental Planning regarding the SSCF 1 process by the recombinant *Z. mobilis* strain.

Figure 5 shows the contour surface for the second Experimental Design, assessing the hemicellulose hydrolysate (%) v/v and the solid:liquid ratio (g:mL); these were the same parameters used in the previous series of experiments, although with higher solid concentrations and lower volumetric proportions of acid hydrolysate.

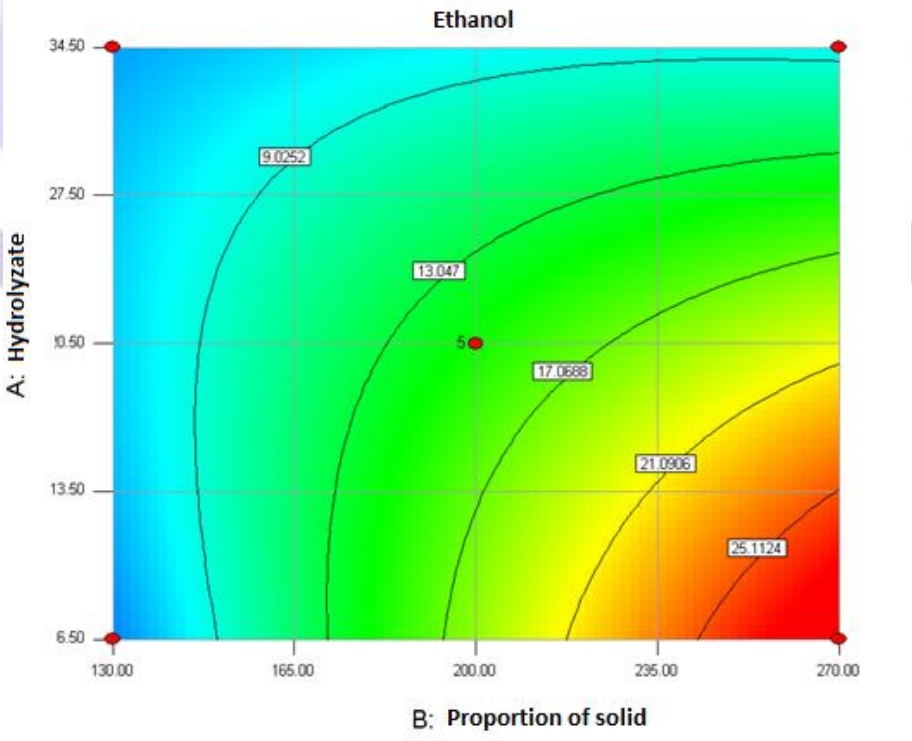

Fig 5: Contour surface evaluating the combined effects of the parameters on the production of ethanol in the second planning related by the SSCF 2 Process by the recombinant strain of *Z. mobilis*.

According to the contour plot, the optimum region (in red) was reached through these experiments, and the highest ethanol production,approximately 25 g/L, was obtained, close to the maximum experimental value of 27 g/L, using the SSCF process, employing 30% of solids, 20.5% of hydrolyzed hemicellulosic, 10 mg/L of tetracycline, enzyme load of 25 FPU/g cellulignin, and 10% of the initial inoculum.

It was observerd that the recombinant bacterium *Z. mobilis* CP4 produces promising results as far as the metabolic adaptation cycles are performed. Some researchers also found it difficult in the pentose fermentation, generating residual concentrations xylose, as described by Davis *et al*. [17], reaching 11 g/ L of bioethanol and 12 g/ L of residual xylose concentration during 11 hours of process. Therefore, as indicated by several authors and proven in this study, the metabolic adaptation processes, as well as optimizations using the recombinant strain of *Z. mobilis* must be continuous with respect to maximizing the production of ethanol and microbial biomass.

CONCLUSIONS

Genetic and metabolic engineering still present the best and possibly only alternatives to making *Z. mobilis* capable of fermenting lignocellulosic hydrolysates on an industrial scale. However, the difficulties in metabolizing xylose by recombinant *Z. mobilis* strains might be related to the inefficient xylose transport, as this approach was not envisaged in the present work. The vector insert in the wild strain contained only the genes responsible for xylose metabolism and did not include the genes coding for xylose assimilation. The evidence that the recombinant strain consumed xylose was due to its transportation through hexose permeases [37]. Additionally, the relatively low ethanol concentration might be ascribed to the inhibition of microbial growth by toxic compounds generated in the hydrolysates, which arise from acid pretreatment. Brazilian studies using molecular biology techniques in this organism are very recent. The LADEBIO research center,a pioneer in this subject, employs such bacteria, transforming and developing the optimization of enzymatic hydrolysis and simultaneous co-fermentation (SSCF) from an abundant agroindustrial waste - sugarcane bagasse.

This study demonstrated a positive aspect of adopting the metabolic adaptation strategy. The recombinant strain exhibited increased ability to uptake and ferment xylose upon transfer into media containing an increased xylose concentration.

Efforts were made to optimize the simultaneous saccharification and co-fermentation of sugarcane bagasse cellulignin with different proportions of hemicellulosic acid hydrolysate. This investigation, carried out with two sequential experimental designs, resulted in ethanol concentrations varying from zero to 27.3 g/L. The optimum conditions for achieving the highest ethanol was a solid:liquid ratio of approximately 3:10 and a volumetric hemicellulose hydrolysate proportion of 20.5%. These findings were statistically validated using the linear model (2FI: two-factor interactions) and the quadratic model adequately in the designs, which presented correlation coefficients of 0.7 and 0.8, respectively; insignificant pure error and insignificant lack of fit for the 90% confidence interval.

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Author' biography with Photo

Danielle da Silveira dos Santos Martins is PhD in Sciences from the Pos Graduation Program on Tecnology in Biochemical and Chemical Process from School of Chemistry/ Federal University of Rio de Janeiro, Brazil. The postdoctoral researcher acts specifically in biotechnology with emphasis on Industrial Microbiology and Fermentation; participates of theoretical- experimental projects development, scientific research and development in bioprocesses, student´s guides, reports development, as lectures and conferences. Are subjects of research, the process development aimed at biofuels production, enzymes, biosurfactants, organic acids, waste treatment and industrial effluents, as molecular biology techniques development.