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RESEARCH PAPER

A Mild Thermal Pre-treatment of the Organic Fraction of Municipal Wastes Allows High Ethanol Production by Direct Solid-state Fermentation

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Abstract A solid standard mixture (SSM) representing the annual composition of fresh fruits and vegetables residues generated at the Supply Center in Mexico City was used for bioethanol production. This type of residues allows bioethanol production with a single thermal pre-treatment instead of hard thermochemical or enzymatic treatments. The release of fermentable carbohydrates from the SSM by a mild thermal pretreatment was firstly optimized. After that, mixed and single cultures of *Saccharomyces cerevisiae*, *Scheffersomyces stipitis,* and *Schwanniomyces occidentalis* were evaluated for bioethanol production. The maximum ethanol production, 282.61 ± 13.09 L ethanol per ton of dry matter (DM), was reached using a severity factor (SF) of 2.35 and a mixed culture composed of *Saccharomyces cerevisiae*, *Scheffersomyces stipitis,* and *Schwanniomyces occidentalis.* The improved lab scale conditions were evaluated in a pilot scale (18 Kg) stirred bioreactor with an SF of 2.35 and the mixed culture, obtaining 245.72 ± 17.76 L ethanol per ton DM. The obtained results demonstrate for the first time the use of fresh fruits and vegetables residues for bioethanol production under solid-state culture conditions without any thermochemical or enzymatic pre-treatment.

Keywords: organic fraction of municipal solid wastes, thermal pre-treatment, solid-state culture, bioethanol production, helical ribbons rotating bioreactor

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1. Introduction

The increasing uncertainty of petroleum supplies, the rising demand, and greenhouse gas generation associated with fossil fuel usage have become a world priority for producing environmentally friendly renewable fuels. Production of liquid biofuels, such as bioethanol, is a sustainable option to tackle problems associated with fossil fuels [1]. Carbohydrates coming from a variety of substrates are used to produce bioethanol [2,3].

The organic fraction of municipal solid waste (OFMSW) is potential biomass for bioethanol production [4]. The total generation of OFMSW in Mexico is around 18 500 × 10^6 ton per year. Mexico City valley concentrates nearly 22% of these materials [http://www.gob.mx/sedesol, 2015].

Alternative treatment methods, such as composting, have been recently used for the treatment OFMSW. However, the increase in the production of organic wastes and the increasing demand of biofuels requires more approaches for the utilization of organic wastes [5-7].

Thermochemical and enzymatic approaches are used for the hydrolysis and release of carbohydrates from biomass for bioethanol production [8]. Different hydrolysis treatments have been investigated for the utilization of OFMSW [4,9,10].

Li [10] suggested that dilute acid hydrolysis (1%) followed by a steam treatment (121°C) and enzymatic hydrolysis (60 FPU/g substrate) is the most efficient hydrolysis method to obtain monomeric carbohydrates from municipal solid wastes.

However, the presence of inhibitors, such as weak acids and furans, generated during pre-treatment with dilute acids at high temperatures are inhibitors for the ethanologenic microorganisms [11-13].

Ballesteros [4] reported the use of OFMSW, containing 47 - 49% of carbohydrates, as a substrate to obtain a final concentration of 30 g/L of bioethanol under submerged fermentation. For that, the OFMSW was subjected to a thermal pre-treatment (TP) at 160°C for 30 min followed by amylases and cellulases addition for simultaneous fed-batch saccharification and fermentation. Under these conditions, more than 160 L of bioethanol per ton of dry matter could be produced from OFMSW.

Zhi-Min [14] proposed a TP (128 and 172ºC) with a low residence time (2 and 5 min) and a subsequent steam explosion to break down the structural fibers of residues in order to increase carbohydrates release for further fermentation. The TP also decreases microbial contaminants and improves fermentation yields, leading to an environmentally friendly technology.

Solid-state culture (SSC) has been used for the production of different products, such as pharmaceutical products, industrial chemicals, food, enzymes and fuel [15]. This bioprocess involves the growth and metabolism activity of microorganisms on moist solids without any free-flowing water. SSC represents a potential process for direct bioethanol fermentation, without separation of carbohydrates into a liquid phase. Bioethanol production from organic residues would be economically viable only if both, hexoses and pentoses, present in the hydrolysates are used [16].

Wild-type *S. cerevisiae* strains are incapable of assimilating pentoses [17], while some yeast such as *S. (Pichia) stipitis* have been used for xylose and glucose conversion to bioethanol [13,18]. However, bioethanol production from pentoses leads to lower bioethanol yield and productivity. In order to overcome this problem, the use of co-cultures of different yeast and bacteria can be evaluated.

Mixed culture fermentations might lead to an increased bioethanol yield, improving the control of the process and the product quality. Mixed cultures could also lead to the utilization of cheaper substrates and the potential for improving existing processes [19]. In this sense, yeasts of the genus *Schwanniomyces* have been selected because of their ability to produce amylases, particularly pullulanase [20].

In an SSC, the bioreactor provides the environment for the growth and activity of the microorganisms, which carry out the biological reactions. In recent years, the bioreactors used in SSC has been modified and modernized to maximize productivity in obtaining products of high industrial interest. However, research is needed to identify sustainable processes to maintain the productivity and quality of products with biotechnological application [21]. Pandey [22] described different types of bioreactors used in SSC including tray fermenter, drum fermenter (continuous rotating or discontinuous rotating drum bioreactor), column fermenter, packed bed fermenter, intermittently stirred bed fermenter and other different designs.

The potential use of column fermenter for the conversion of starch on biomass and bioethanol by *Schwanniomyces castellii* in an aerobic-anaerobic SSC, demonstrate the feasibility of using one single fermenter for aerobic growth to generate inoculum as well as simultaneous hydrolysis of the substrate to produce bioethanol [20].

On the other hand, helical ribbons rotating bioreactor has been reported for the spore production in SSC [23]. To the best of our knowledge, helical ribbons rotating bioreactor has not been reported for bioethanol production. This study attempts to demonstrate the optimization of bioethanol production at lab scale SSC, using static bioreactors and its production at pilot scale using stirred bioreactors.

Nevertheless, the use of lignocellulosic substrates for bioethanol production, requires hard thermochemical and/or enzymatic treatments, increasing the operating costs. Additionally, the organic fraction of municipal wastes is rich in fruits and vegetables residues with a high content of easily fermentable carbohydrates.

The larger Food Supply Center in Latin America (Central de Abasto), is located in the Iztapalapa neighborhood in Mexico City. It generates more than 1 291 ± 100 ton of municipal solid wastes per day; where the 70% (905.05 ± 70.06 ton of organic wastes per day) are fruits and vegetables residues (FVR), that are no longer commercialized. These side products can be used for several purposes; ethanol production [4], compost [6], animal feed [40] and others [7]. Utilization of these residues for bioethanol production allows to produce up to 281 m^3 of bioethanol per day, that can be used as a biofuel additive.

Some of the advantages of using this type of residues are i) they are directly generated in this Food Supply Center reducing costs of transportation of residues, ii) they are rich in easily metabolizable carbohydrates with very low content of lignocellulose, which facilitates the treatment prior to alcoholic fermentation, increasing the yield of bioethanol production. The latter significantly reduces the costs of bioethanol production and iii) the use of fresh organic residues reduces variations in the bioethanol production caused by the heterogeneity of residues and of microbial contaminations.

In this study, a feasible bioprocess for bioethanol production from fresh fruits and vegetables residues, with a mild thermal pre-treatment (TP), using a mixed culture of yeasts under solid-state conditions was developed. This approach avoids the use of hard thermochemical or enzymatic pre-treatments and considerably reduces water consumption. The TP was firstly optimized at lab scale and bioethanol production was evaluated at pilot scale (18 Kg) under stirred conditions.

The aim of this study was to evaluate the conditions (pressure-temperature and residence time) of the TP to increase the release of fermentable carbohydrates (FC) and reduce the acetic acid concentration. TP conditions were evaluated using a central composite factorial design (CCFD) coupled with surface response methodology. Bioethanol production was carried out using axenic and mixed yeast cultures. The best conditions were assayed for bioethanol production by the SSC at lab scale (0.1 Kg) and pilot scale (18 Kg) in a helical ribbon bioreactor.

2. Materials and Methods

2.1. Organic fraction of municipal solid waste and solid standard mixture

The fresh fruits and vegetables residues (FVR) were obtained from the residues generated from fruits juices and salads in food stores located in Iztapalapa neighborhood, Mexico City. The FVR composition was as follows (% w/w, wet basis) orange, 30; watermelon, 15; pineapple, 15; lemon, 10; carrot, 10; papaya, 10; banana, 5 and cucumber peels, 5. The FVR were manually cut into particles with an average size of 1.0 cm and stored for no more than 24 h at 4ºC. A solid standard mixture (SSM) with the following composition was used for different assays (% w/w, wet basis): FVR, 93; sawdust, 4; and shredded paper, 3, last components were used as volume agent. This SSM, with a carbon to nitrogen ratio (C/N) close to 34.6, was used for TP and ethanol production assays.

2.2. Yeasts selected and inocula production contidions

The yeasts used in this study were *Saccharomyces cerevisiae* ITD00196 [24] (*S. cerevisiae*), *Schwanniomyces occidentalis* ATCC26077 (*Schw. occidentalis*) and *Scheffersomyces stipitis* ATCC58785 (*S. stipitis*). For inocula production, *S. cerevisiae* and *S. stipitis* were cultivated in 250 mL baffled Erlenmeyer and *Schw. occidentalis* was cultivated in 250 mL Erlenmeyer standard flasks were filled with 50 mL of culture with the following composition (in g/L): glucose, 20; meat peptone, 3.5; yeast extract, 3; KH_2PO_4, 2; $MgSO_4.7H_2O$, 1 and $(NH_4)_2SO_4$, 0.5 [20]. The culture medium pH was adjusted at 5 with 1M HCl. Cultures were incubated at 30ºC and 180 rpm for 48 h in an orbital shaker with an inoculum of 1×10^6 cells/mL.

2.3. Thermal pre-treatment

The TP of the SSM was optimised using a central composite factorial design (CCFD). Pressure-temperature and residence time were used as independent variables. Actual and coded values used are shown in Table 3. The central point of experimentation for the pressure was 2 Kg/cm^2 with a variation unit of 0.7 Kg/cm^2. For the residence time, the central point was 10 min with a variation unit of 5 min. The analysis of variance (ANOVA) was achieved using FC release (glucose, fructose and sucrose concentrations) and acetic acid production as response variables. The interaction of the independent coded variables was determined using a second order polynomial model (1):

$$y = \beta_o + \Sigma\beta_i x_i + \Sigma\beta_{ij} x_i x_j + \Sigma\beta_{ii} x_i^2 \quad (1)$$

Where y is the predicted response, β_o is the offset term, β_i is the coefficient of the linear effect, β_{ij} is the coefficient of the of the quadratic effect when $i = j$ and the interaction effect when $i < j$, β_{ii} is the coefficient of the squared term, x_i is the ith independent variables.

On the other hand, the severity factor (SF), representing a function of the temperature and residence time (2) was determined according to the following expression [14]:

$$SF = log\left(t \cdot exp\left(\frac{T-100}{14.75}\right)\right) \quad (2)$$

Where t represents the residence time (min); T is the treatment temperature (ºC) and 100 is a reference temperature (ºC); the value of 14.75 is an empirical parameter that relates temperature and activation energy, assuming a first-order kinetics [25]. Standard thermal sterilisation condition (STS) with an SF of 1.79 was used as control (1.1 Kg/cm^2 for 15 min). TP assays were performed in a 180 L autoclave (SMI AVX 130E, France) modified to operate with steam provided by a steam boiler (Clayton EG-33, Mexico).

Samples of 100 g of the SSM in 740 mL unsealed bottles (15 cm height and 9.5 cm diameter) were autoclaved and closed with aluminium foil. The autoclave was firstly purged at atmospheric pressure; after that, the conditions described in the central composite design were applied (Table 2); at the end of the TP, the steam from the autoclave was released to the atmosphere in less than 2 min (steam explosion). The pre-treated, non pre-treated and control samples were stored at -20ºC until extraction of FC and acetic acid for analysis purposes. All experiments were conducted by duplicate. Dry samples (105ºC) without and with pre-treatment (2.7 Kg/cm^2 for 15 min, SF=2.35) were used for chemical characterization and determination of the structural composition of SSM.

2.4. SSC at laboratory scale for ethanol production

The SSC for ethanol production was performed in static tubular bioreactors (TBRs) of 5 cm diameter and 15 cm height, filled with 100 g of the pre-treated SSM. Two conditions of TP were evaluated (2.2 Kg/cm^2 and 12 min, SF=2.11; and 2.7 Kg/cm^2 and 15 min, SF=2.35). The first condition was selected for being the optimal condition for

carbohydrates release determined by the surface response methodology and the second condition was selected for being the experimental condition producing the lowest concentration of acetic acid.

The SSM was inoculated with axenic and mixed culture composed of *S. cerevisiae*, *Schw. occidentalis* and *S. stipites* at a ratio of 10% (v/w). Once inoculated and packed under aseptic conditions, the TBRs were incubated at 30ºC for 30 h without aeration. In order to obtain the kinetics of bioethanol production and FC consumption, several TBRs were inoculated with mixed culture under aseptic conditions and sampled at regular intervals of time during 30 h. Samples were stored at -20ºC until further analysis by HPLC. All experiments were conducted by a duplicate.

2.5. Solid-state culture in a pilot scale bioreactor

For the pilot scale bioreactor assays, the TP condition of the SSM was carried out using an SF=2.35 (2.7 Kg/cm^2 and 15 min); the highest ethanol production was obtained at laboratory scale with mixed culture using this SF. The SSM residues were autoclaved as indicated above using recipients of 4.5 Kg capacity. Pilot scale cultures were carried in the modified stainless-steel SSC bioreactor reported by Nava [23].

The 70 L capacity bioreactor (75.6 × 32.6 cm, length × diameter) is provided with a helical ribbons rotating system. Twenty-four hours before use, the bioreactor was sterilised with direct steam at 92ºC for 90 min at atmospheric pressure (at 2,200 m over sea level at Mexico City). After cooling, the bioreactor was loaded aseptically with 18 Kg of PT inoculated SSM. Mixed cultures of *S. cerevisiae*, *Schw. occidentalis* and *S. stipitis* were used as inocula at a ratio of 3.3% (v/w) each of the pre-treated SSM.

After inoculation and filling, the bioreactor was incubated at 30ºC for 30 h without aeration. The bed height of the wet material was nearly half of the inner bioreactor height. Cultures were intermittently agitated as follows: Continuous agitation for two minutes, 1 min clockwise and 1 min counter clockwise at 1 rpm, then cultures were incubated during 15 min without agitation, the agitation cycle was repeated until the end of cultivation.

The culture conditions were similar to that used in laboratory scale. The temperature of the water jacket was maintained constant at 30ºC. Control assays were carried out under the same conditions using lab scale TBRs. Gas samples from the headspace of bioreactors were taken using a membrane air pump and analysed with a GOWMAC 580 gas chromatograph, after that, the analysed gas was returned to the fermenter.

2.6. Sampling and analytical methods

For analysis of carbohydrates, ethanol and acetic acid, 10 g of sample were placed in a 250 mL Erlenmeyer flask and extracted with 40 mL of distilled water and stirred at 180 rpm at 30ºC for 15 min. The supernatant was recovered by centrifugation at 3500 rpm, at 4ºC for 20 min and stored at -4ºC for further analysis.

The pH was determined in the supernatant using a previously calibrated potentiometer (Conductronic, model pH 120). Structural carbohydrates [26] and total solids in biomass [27] were determined according to the standard method recommended by the National Renewable Energy Laboratory (NREL), USA. Elemental analysis for carbon (C), hydrogen (H) and nitrogen (N) was made with an elemental analyser (PerkinElmer 2400 Series II CHNS/O analyser, USA).

The moisture content was determined by the decrease in weight after drying overnight at 105ºC [28], and the ash content was determined by combustion at 750ºC for 3 h (Table 1).

Sucrose (Suc), glucose (Glu), fructose (Fru), ethanol and acetic acid concentrations were determined using a Shimadzu Prominence HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with an LC-20 pump, a DGU-

Table 1. Chemical characterization of solid standard mixture with thermal pre-treatment (SF=2.35)

	Content (%)
Moisture	79.57 ± 2.50
pH	6.46 ± 0.77
Ashes	4.93 ± 0.99
Total solids	20.43 ± 2.49
Elemental analysis	
C	34.48 ± 2.59
H	5.84 ± 0.15
N	1.00 ± 0.06
C/N ratio	34.59 ± 0.97

Table 2. Structural carbohydrates of solid standard mixture with (SF=2.35) and without thermal pre-treatment

	Without pre-treatment	With pre-treatment
Sucrose	ND	ND
Glucose	21.16 ± 1.64	27.83 ± 1.74
Fructose	12.58 ± 0.99	15.06 ± 0.54
Xylose	2.70 ± 0.80	3.21 ± 0.88
Arabinose	2.52 ± 0.08	3.56 ± 0.18
Galactose	ND	ND
Mannose	1.39 ± 0.28	2.24 ± 0.12
Total sugars	40.88 ± 2.97	52.53 ± 3.35

Values reported are means of carbohydrate (%) in dry matter with standard deviations.
Analysis realized according standard method recommended by the National Renewable Energy Laboratory (NREL).
ND: not detected.

Table 3. Central composite factorial design with star points, used to evaluate the effect of pressure-temperature and residence time on thermal pre-treatment upon fermentable carbohydrates an acetic acid of the solid standard mixture of residues used

	Actual values		Coded values			Response variables (g/kg IDM)				
Treatments	Pressure[a] (Kg/cm^2)	Time (min)	X_1	X_2	SF[b]	Sucrose	Glucose	Fructose	FC[c]	Acetic acid
Without thermal pre-treatment	-	-	-	-	-	20.19 ± 7.80	72.78 ± 12.24	98.77 ± 10.67	191.74 ± 18.02	2.77 ± 1.10
STS	1.1 (121)	15	-	-	1.79	11.91 ± 0.02	84.16 ± 3.06	94.68 ± 4.66	190.74 ± 5.58	1.02 ± 0.62
Central composite factorial design										
1	2.7 (140)	15	1	1	2.35	30.74 ± 3.93	164.81 ± 7.54	126.15 ± 20.70	321.71 ± 22.38	0.83 ± 0.09
2	2.7 (140)	5	1	-1	1.87	19.06 ± 2.91	95.44 ± 8.79	82.33 ± 10.28	196.82 ± 13.84	0.59 ± 0.11
3	1.3 (124)	15	-1	1	1.88	19.33 ± 0.04	96.53 ± 0.39	89.14 ± 7.44	204.99 ± 7.45	0.64 ± 0.10
4	1.3 (124)	5	-1	-1	1.41	32.64 ± 5.53	90.08 ± 0.85	112.57 ± 6.08	235.30 ± 8.26	1.41 ± 0.46
5	2 (133)	10	0	0	1.35	36.33 ± 1.95	142.55 ± 15.03	150.31 ± 9.05	329.20 ± 17.65	6.22 ± 1.70
6	2 (133)	10	0	0	1.35	33.89 ± 975	142.34 ± 7.72	124.94 ± 8.95	301.18 ± 15.32	6.75 ± 2.00
7	2 (133)	17.05	0	1.41	2.20	24.00 ± 3.96	108.87 ± 0.33	102.33 ± 3.30	235.21 ± 5.16	0.81 ± 0.22
8	2 (133)	2.93	0	-1.41	1.44	13.65 ± 0.32	72.77 ± 15.38	70.48 ± 12.07	156.91 ± 19.55	2.49 ± 0.55
9	3 (143)	10	1.41	0	2.27	20.50 ± 0.78	92.38 ± 2.04	91.02 ± 8.68	203.90 ± 8.95	0.88 ± 0.06
10	1 (120)	10	-1.41	0	1.59	25.64 ± 0.80	72.78 ± 3.03	86.73 ± 1.57	185.15 ± 3.51	0.69 ± 0.02

[a]Values in parentheses correspond to temperature (° C).
[b]Severity factor (SF) as define in equation 2.
[c]Fermentable carbohydrates (FC) (Suc + Glu + Fru)
STS: Standard thermal sterilization.

20AS degasser, a SIL-20A HT auto-sampler, a CTO-20A oven and a RID-20A Refractive Index detector.

Furfural (275 nm) and HMF (284 nm) concentrations were determined with a SPD-M20A Diode Array detector. Separation was performed using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). An aqueous solution of 5 mM H_2SO_4 was used as the mobile phase. The flow rate and operating temperature used were 0.6 mL/min and 60ºC, respectively. The compounds were quantified based on the retention times and calibration curves were obtained for each compound.

The results were expressed as g/Kg DM. Evolution of the bioprocess at pilot scale was directly monitored through CO_2 and O_2 concentration. These were monitored through periodic measurement of the gaseous atmosphere obtained from the bioreactor [29]. The measurement of CO_2 and O_2 was done using a GOWMAC 580 gas chromatograph equipped with thermal conductivity detector (Gow-Mac Instrumentation Co., USA) and a packed column Alltech CTR1, which was maintained at 80ºC. The temperature of automatic injector, detector temperature and detector current were maintained at 80ºC, 110ºC and 125 mA, respectively. The carrier gas flow (helium) was maintained at 30 mL/min [29]. The results were expressed as the volumetric percentage of CO_2 and O_2 in the headspace of the different bioreactors used; experimental gases concentration pattern was expressed using a simple moving average (SMA) of 5 points, as follows (3):

$$SMA = \frac{\sum_{i=1}^{n} D_i}{n} \tag{3}$$

Where, n is the demand of periods in the moving average and D_i is the demand in period i.

2.7. Mathematical considerations

For modelling fermentable carbohydrates (FC) consumption, a typical first-order decay equation [30] was used as shown in the following equation (4):

$$-\frac{dFC}{dt} = k_{FC} FC \tag{4}$$

The integration of equation (4) gives equation (5):

$$FC = FC_0 \exp(-k_{FC}\, t) \tag{5}$$

Where, CF is the fermentable carbohydrates concentration (g/Kg DM) as a function of time, CF_0 (g/Kg DM) refers to the initial concentration of fermentable carbohydrates and k_{CF} is the first order constant (h^{-1}) of proportionality.

The Gompertz equation (6) was used to model ethanol production as shown below [6]:

$$\frac{dEtOH}{dt} = k_{EtOH} \cdot EtOH \ \mathrm{Ln}\left(\frac{EtOH_{max}}{EtOH}\right) \tag{6}$$

The integration of equation 6 gives (7):

$$EtOH = EtOH_{max}\exp(-b\ \exp(-k_{ETOH}\ t) \quad (7)$$

Where, at t = 0, $EtOH = EtOH_{(0)} = EtOH_{max}\ exp(-b)$; and b refers to a parameter of integration without any biological interpretation.

2.8. Statistical analysis

All analyses were carried out at least in duplicate and the results are expressed as mean ± standard deviation. Tukey's HSD test was used for comparison of means and to estimate significant differences between samples ($p<0.05$). Statistical analysis was performed using the statistical software Statgraphics Centurion XV.I (Statpoint, Inc.).

3. Results and Discussion

Chemical characterization of the SSM was carried out first; afterward, TP of the SSM to release carbohydrates for ethanol production was performed. The thermally pre-treated SSM was used for ethanol by axenic and mixed cultures of yeasts in lab and pilot scale bioreactors.

3.1. Chemical characterization of the SSM with and without thermal pre-treatment

The general chemical characterization and the content of structural carbohydrates of the SSM without and with TP (2.7 Kg/cm^2 for 15 min, SF=2.35) are shown in Tables 1 and 2, respectively.

SSM exhibited a moisture content between 76 and 80% (Table 1), similar values were found by Tang [17] in kitchen wastes (80.3%) and by Uçkun Kiran [7] in mixed food wastes (61.3-87.1%). This might be due to the high proportion of fresh fruits residues used in these mixtures. On the other hand, the relatively high pH may be explained for the rapid management and handling of fresh residues, avoiding microbial acidification. The best conditions for ethanol production, using a kitchen waste mixture, were obtained with a C/N ratio between 28 and 35 [31]. In this work, the SSM exhibited a C/N ratio close to 34.6 being appropriate for ethanol production.

The structural composition of carbohydrates (Table 2) of the SSM depends on the origin of the organic material, soil and culture conditions, among other factors. The TP increased the content of most of the structural components, excepting galactose and sucrose, that were not detected. An increase of 28.5% in total carbohydrates due to the TP was noticed.

Total carbohydrates are in the range of 40.9-52.5%, similar than those reported by for other types of organic residues, 47-49% reported by Ballesteros [4] and other mixtures of food residues, 35-70% [7]. Taking into account the nature of these wastes and their low cost, OFMSW is a potential alternative substrate for ethanol production and TP is useful to increase the content of most of the structural component contents and reduce contaminants in the SSM used.

3.2. Optimization of the release of fermentable carbohydrates and reduction of acetic acid concentration in the SSM using a CCFD

The effect of pressure-temperature and residence time have been extensively reported [4,9,13,14,25,38] for the thermal pre-treatment of organic material. In this work, the main goal of the TP was to release the highest content of FC with the lowest release of antiphysiological factors, such as acetic acid, furfural, and HMF. For that purpose, multiple linear regression couples with response surface methodology, as well as, a non-linear equation (severity factor) was used (Table 3).

For all the conditions assayed, the SSM samples presented average moisture content and pH values of 80.92 ± 1.72% and 6.48 ± 0.17, respectively.

Fresh SSM and thermally pre-treated SSM, under standard thermal sterilization conditions (STS, 1.1 Kg/cm^2 and 15 min), were firstly analyzed to determine the effect of the TP in the fermentable carbohydrates composition and the acetic acid concentration. The fermentable carbohydrates, defined in this study as glucose, fructose, and sucrose, presented similar concentrations, 190.74 ± 16.79 and 191.25 ± 16.79 g/Kg IDM in fresh and thermally pre-treated SSM, respectively (Table 3).

A significant decrease in sucrose concentration (41%) was determined after STS in comparison with fresh SSM (20.19 ± 7.80 g/Kg IDM) (Table 3). Acetic acid is an inhibitor of alcoholic fermentation, and it is naturally present in fresh SSM (2.77 ± 1.10 g/Kg IDM). After STS no significant reduction ($p<0.05$) in the content of acetic acid was detected (1.02 ± 0.62 g/Kg IDM). Furfural and HMF were not detected after the STS).

The inhibitory effect of acetic acid in yeasts is well known [13,32,33]. A reduction of 80% in the growth of *S. cerevisiae* at a concentration of 7.5 g/L of acetic acid have been reported [32]. Acetic acid, at 3.5 g/L, has an inhibitory effect on the fermentation of *S. stipitis* [33]. In all of our assays, a non-inhibitory range of acetic acid concentrations between 0.02 and 0.33 g/L of acetic acid, was found. No other inhibitors, such as the furfural or hydroxymethylfurfural (HMF) was detected.

The 3-dimensional response surface and contour plots (Fig. 1) are the graphical representations of the regression model. Fig. 1A, shows the FC (glucose, fructose and sucrose) release to the medium. According to the regression model, the optimal conditions indicated by the surface

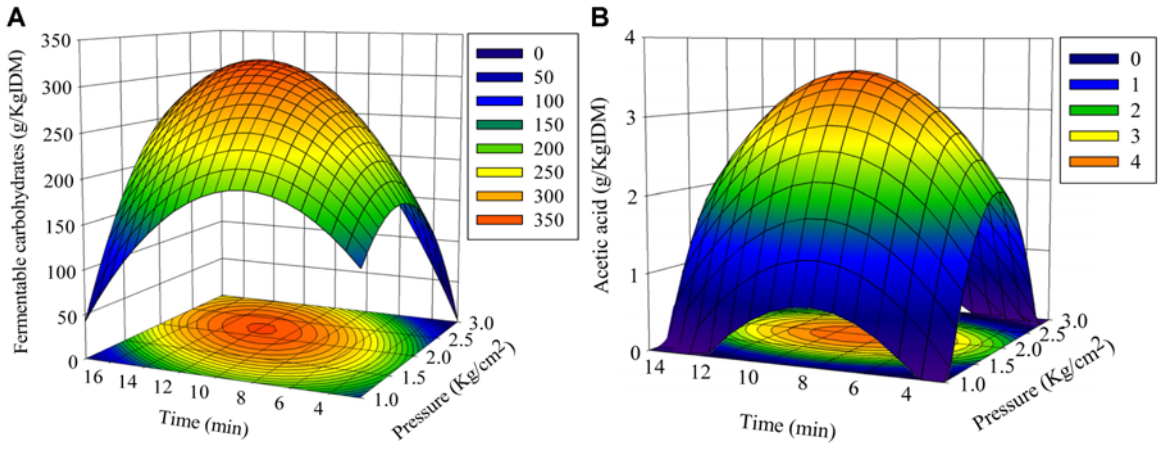

Fig. 1. Response surface and contour plots showing the effect of pressure and residence time in the fermentable carbohydrates (Suc + Glu + Fru) (A) and acetic acid (B) after thermal pre-treatment in the stated conditions.

Table 4. Regression coefficients and *p*-values obtained from the statistical analysis of the composite central factorial design with star points

	FC[b] (g/Kg IDM)		Acetic acid (g/Kg IDM)	
Term of the model[a]	Estimate	*p*-values[c]	Estimate	*p*-values[c]
Constant	-147.569		-10.232	
X_1	309.728	0.0253	11.1592	0.9278
X_2	21.7325	0.0004	0.6064	0.4549
X_1 X_1	-100.468	0.0000	-2.9851	0.0378
X_1 X_2	11.0848	0.0002	0.0719	0.7113
X_2 X_2	-1.9346	0.0000	-0.0411	0.1252

[a]X_1: Pressure (Kg/cm^2) and X_2: residence time (min).
[b]Fermentable carbohydrates (FC) (Suc + Glu + Fru)
[c]$p<0.05$ Indicate a significant effect on the response.

response methodology (SRM) for the release of FC were close to 2.2 Kg/cm^2 and 12 min yielding 321.80 g FC per Kg of initial dry matter (IDM). This FC content is 68.30% higher than the obtained under STS conditions (Table 3). However, under the same experimental conditions, the acetic acid concentration was 3.1 g/Kg DM (Fig. 1B).

The results of the coefficients of the second order polynomial model, the analysis of variance (ANOVA) and the test of goodness of fit of the model are reported in Table 4. The model presented a determination coefficient (R^2) of 0.81 for the FC concentration release. Considering the heterogeneity of the SSM samples, we found these results acceptable.

The effect of each factor on the different responses was determined by the *p*-values ($p<0.05$). The pressure-temperature (X_1), residence time (X_2) their interactions X_1X_2, and the quadratic terms X_1^2, and X_2^2, had a significant effect on the carbohydrates release (Table 4).

The optimal conditions for the maximum release of both FC (near to 2.2 Kg/cm^2 and 12 min, SF=2.11) and acetic acid (near to 2.0 Kg/cm^2 and 9 min, SF=1.93) are quite close.

Therefore, to increase the FC content, without the release of acetic acid, it is necessary to apply a higher SF in the TP that favors the release of FC and decrease the concentration of acetic acid. This condition is achieved by using an SF of 2.35 that allows a high FC concentration (321.71 ± 22.38 g/Kg IDM) and a low acetic acid concentration (0.83 ± 0.09 g/Kg IDM).

Concerning the release of FC, obtained only from thermal pre-treatment, these results agree to that reported by Ballesteros [4] and Zhi-Min [14]. The increase of pressure-temperature and time result in the release of carbohydrates, which is related to the increase of an SF. A combination of pressure-temperature, treatment duration and a rapid release of steam, reduce contaminants and possibly causes the rupture of the cell walls of the residues and the release of FC [14].

Zhi-Min [14] using an SF between 1.41 and 1.73, obtained the highest concentration of glucose when using a thermally pre-treated substrate composed of wheat bran and soybean meal without enzymatic hydrolysis. Using an SF of 1.52, these authors obtained an 80% increase in glucose.

For the subsequent ethanol production assays by SSC, a TP using an SF of 2.11 (2.2 Kg/cm^2 - 12 min) were used at lab scale; and an SF of 2.35 (2.7 Kg/cm^2 - 15 min) were used at lab and pilot scales. Axenic cultures of yeasts were used only at lab scale, and the mixed culture of yeasts were used at lab and pilot scales.

3.3. Ethanol production in the solid-state culture of the SSM at lab scale

Lab scale ethanol production from SSM, thermally pre-

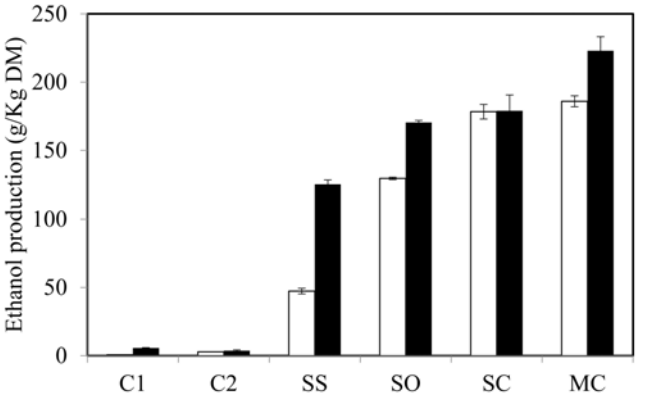

Fig. 2. Production of bioethanol using different inocula in the fermentation of the SSM with TP at SF=2.11 (2.2 Kg/cm^2 - 12 min; filled bars) and SF=2.35 (2.7 Kg/cm^2 - 15 min; open bars). Control 1 (C1) at the initial time, Control 2 (C2) at final of culture, *S. stipitis* (SS); *Schw. occidentalis* (SO); *S. cerevisiae* (SC) and mixed culture (MC).

treated at SF of 2.11 and 2.35, and inoculated with a different type of inocula was evaluated (Fig. 2). Axenic cultures and a mixed culture of *S. cerevisiae*, *Schw. occidentalis* and *S. stipitis* were used as inoculum. *S. cerevisiae* was selected because of its high ethanol productivity, *Schw. occidentalis* was selected because its amylolytic enzyme production and *S. stipitis* were selected to produce ethanol from the pentoses fraction [13,20,34].

The ethanol production from axenic and mixed cultures was higher with the SF of 2.35 than that obtained with an SF of 2.11. In the case of *S. stipitis* and *Schw. occidentalis,* ethanol production increases as the SF was increased. In the case of *S. cerevisiae,* no statistical significant differences was observed, when different SF was were applied for ethanol production.

The highest ethanol production was obtained using the mixed as inoculum, attaining 222.98 ± 10.33 g/Kg DM with an SF of 2.35. Under the conditions, an increase of 20% in ethanol production was noticed in comparison with the conditions of TP applying an SF of 2.11. This high ethanol concentration could be explained because the TP prior to fermentation enhances the release of FC, decrease the content of acetic acid and reduce the presence of microbial contaminants.

The use of a mixed culture avoids the use of genetically modified microorganisms and takes advantage of the fermentative characteristics of wild yeasts especially isolated for ethanol production [19]. In fact, the proposed mixed culture was formulated with the yeasts *S. cerevisiae*, *Schw. occidentalis* and *S. stipites*; in order to increase the ethanol production and the yield of ethanol from hexoses and pentoses; as well as, the production of amylolytic enzymes improve the hydrolysis of the SSM.

For the different strains used, acetic acid decreases as the SF was increased. Applying an SF of 2.11 during TP, a concentration of acetic acid between 14.19 ± 1.69 and 23.22 ± 1.20 g/Kg DM was observed. Applying an SF of 2.35 during TP, a concentration of acetic acid between 3.31 ± 1.22 and 6.69 ± 2.75 g/Kg DM was observed. In both cases, no inhibitory concentration of acetic acid was obtained (<0.57 ± 0.03 g/L). Under these conditions of SF, compounds from pre-treatment, such as acetic acid, were detected probably coming from the hydrolysis of acetyl groups [13]. The decrease in the concentration of acetic acid, at higher SF, may be due to the volatility of the inhibitor.

3.4. Kinetic characterization of ethanol production in the SSC of the SSM at lab and pilot scale

Once the TP conditions and the inoculum type were selected, kinetic studies of CO_2 and ethanol production, O_2 and FC consumption were carried out (Fig. 3). A similar profile for CO_2 and O_2 concentration (%), and ethanol production rate in both lab and pilot scale bioreactors was obtained (Figs. 3A and 3C). Real-time on-line analysis of the gas phase, without disturbing the system, provide a useful control process to determine the end of the process [29].

The maxima CO_2 concentrations in lab scale (22.6%) and pilot scale (27.9%) were attained at 12 and 8 h of culture, respectively (Figs. 3A and 3C). The CO_2 increase is mainly, due to the conversion of sugar into ethanol. Reduction of oxygen in the headspace is also a combined result of O_2 consumption for growth yeast during the first hours of aerobic conditions (less than 4 h) and the dilution effect because of CO_2 increase.

The consumption of FC was simulated using a first-order decay kinetic leading to coefficients of determination of 0.898 and 0.856, for laboratory and pilot scale bioreactors, respectively (Figs. 3B and 3D). The values of the first-order constant (k) were similar (0.06 ± 0.01 h^{-1} and 0.07 ± 0.02 h^{-1}) for both scale bioreactors, indicating a similar biochemical behavior. A successful scale-up was carried out, from lab to pilot plant level, with a scale factor close to 180.

The production of ethanol was successfully simulated using the Gompertz model, this sigmoidal equation is a very flexible model because it presents no-symmetry of ethanol production rate around the inflecton point. The goodness of fit was evaluated through the correlation coefficient between experimental and calculated data of ethanol concentration. It was 0.96 and 0.99 for lab and pilot scale bioreactors, respectively.

At lab scale, the highest ethanol concentration (205.96 ± 3.17 g/Kg DM) was reached at 24 h and is due to the rapid

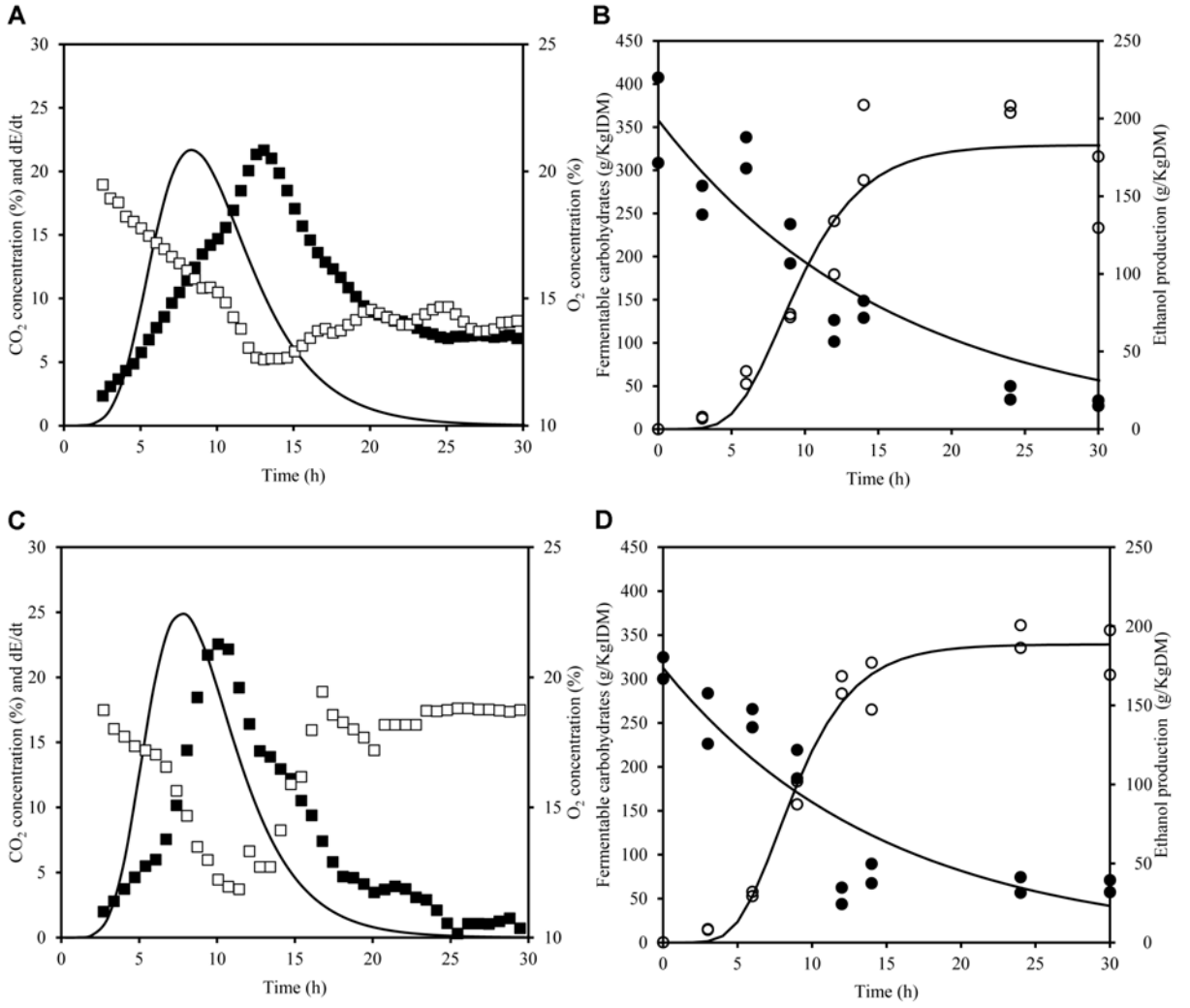

Fig. 3. CO_2 concentration (■) and O_2 concentration (□) in the bioreactor headspace, at the laboratory (A) and pilot scale (C); solid lines represent the ethanol production rate (dE/dt) simulated by differential Gompertz model. Fermentable carbohydrates consumption (●) and bioethanol production (○) during the fermentation of the SSM with TP at SF=2.35 using a mixed culture at the laboratory (B) and pilot scale (D), respectively; solid lines represent the first order model for fermentable carbohydrates consumption and integral Gompertz model for ethanol production.

consumption of FC plus the FC liberated by the amylases produced by *Schw. occidentalis* from some of the starchy material present in the initial SSM.

Simulation of the experimental data describes a similar general trend of the fermentation pattern at lab and pilot scale bioreactors. Considering the volatility of ethanol and heterogeneity of the system, the simulation of FC consumption and ethanol production is satisfactory according to the determination and correlation coefficients obtained. A proper kinetic description of the process is fundamental for bioreactor design [30].

A summary of ethanol production data from laboratory and pilot scale bioreactors is presented in Table 5. Assays at lab scale indicated that 185.92 – 222.98 g of ethanol per Kg of DM could be reached using an SF of 2.35 and a mixed culture of *S. cerevisiae*, *S. stipitis,* and *Schw. occidentalis*. These concentrations are close to 186.36 – 193.56 g/Kg DM reached at pilot scale. Cultures were inoculated with *S. cerevisiae* or mixed culture at lab scale. The highest ethanol production was obtained applying an SF of 2.35; no significant differences was observed when an SF of 2.11 was applied at lab scale. Higher SF improves ethanol production by using a mixed culture (Table 5).

The bioreactor with helical ribbons used at pilot scale is not completely sealed, so when using intermittent agitation ethanol losses due to evaporation could appear. Simulation of ethanol production is lower in the bioreactor, nevertheless, analysis of experimental data indicated that evaporation of ethanol in the bioreactor, could explain the higher concentration a lab scale than at pilot scale, 6.4% at 24 h

Table 5. Comparison of bioethanol production at lab and pilot scale, by SmC and SSC from organic residues pre-treated under different conditions

Scale	Substrate	Yeast	Conditions	SF	Culture	Ethanol production (g/KgDM)	References
Laboratory	OFMSW	*S. cerevisiae*	160°C-30 min + Cellulases/ amylases	3.24	SmC	126.20	[4]
	Kitchen waste	*S. cerevisiae*	121°C-30 min + α-amylase/ amyloglucosidase/cellulase/ β-glucosidase	2.10		161.00	[37]
	Lemon peel wastes	*S. cerevisiae* CECT 1329	160°C-5 min + Pectinase/ cellulase/β-glucosidase	2.47		254.85 ± 41.82	[38]
	Sugar cane bagasse	*Schw. castellii* CBS2863	121°C-30 min	2.10	SSC	89.30	[20]
	Rice husk	*S. cerevisiae*	121°C-20 min + α-amylase/ cellulase	1.92		135.00 ± 10.8	[39]
	Carob fruits/ Wheat bran	*Z. mobilis* PTCC1718	121°C-15 min	1.79		60.90	[41]
	SSM	*S. cerevisiae*	135°C-12 min	2.11	SSC	178.29 ± 5.36	This work
		Mixed culture				185.92 ± 4.04	
		S. cerevisiae	140°C-15 min	2.35		178.94 ± 11.67	
		Mixed culture				222.98 ± 10.33	
Pilot	Mongolian oak	*S. cerevisiae* DXSP	0.05% (w/w) H_2SO_4-380°C for 1 sec	-	SmC	141.00	[42]
	Sweet sorghum	*S. cerevisiae* TSH1	α-1,4-glucan-glucohydrolase	-	SSC	208.30	[43]
	SSM	Mixed culture[1]	140°C-15 min	2.35	SSC	193.56 ± 10.10	This work
		Mixed culture[2]				186.36 ± 3.54	

Abbreviations: Organic fraction municipal solid waste, OFMSW; solid standard mixture, SSM; severity factor, SF; submerged culture, SmC; solid-state culture, SSC, dry matter, DM; *S. cerevisiae* ITD00196, *S. stipitis* ATCC58785, and *Schw. occidentalis* ATCC26077, mixed culture.
[1]Run 1.
[2]Run 2.

(Figs. 3B and 3D), 15.2% at 30 h (Table 5). If the duration of the process is longer than 24 h losses of ethanol can occur by evaporation or biomass reassimilation. Differences among the lab and pilot scales are low; no statistically significant differences were observed.

Comparison of results is no easy because of differences of the scale used, the nature of organic residues and characteristic of bioprocess used for ethanol production (Table 5). The SSM used in this work, is a source of FC for direct ethanol fermentation, without separation of carbohydrates into a liquid phase. Several studies have been reported on the use of organic wastes as a substrate for ethanol production. However, in all cases, the solid fraction of organic wastes is suspended in a liquid medium for thermal or enzymatic pre-treatment; and in some other cases, carbohydrates are extracted in liquid culture for ethanol production. In all these cases, the number of process stages is increased [4,17,31,35-37].

Enzymatic hydrolysis is frequently used at lab [4,37-39] and pilot scale [43]. At lab scale, ethanol production obtained in this work, without using enzymatic hydrolysis, is higher than that reported at submerged culture (SmC) [4,37] and SSC [20,39,41]. At pilot scale, ethanol production obtained in this work, without using thermochemically pre-treated, is higher than reported at SmC [42]. Nevertheless, at lab and pilot scale, using enzymatically hydrolyzed lemon peel wastes [38] and sweet sorghum as substrate [43], that authors found an ethanol production 14.3% and 7.6% higher, than that reported in this paper, respectively.

These results demonstrate the potential of producing ethanol from thermally pre-treated SSM (282.61 ± 13.09 L ethanol/ton DM), without hard thermochemical and/or enzymatic treatments. SF is a practical criterion for comparison of TP of organic material.

Results indicate the OFMSW is a potential substrate for ethanol production been an alternative to sugar cane and starch from corn.

4. Conclusion

To the best of our knowledge, the above results demonstrate for the first time, that a mild thermal pre-treatment of the fruits and vegetables fraction from the OFMSW allows a high ethanol concentration when solid-state culture conditions are used in both static lab scale and stirred pilot

plant scale bioreactors. One of the advantages of using this type of residues is that they can be gathered and processed in the same place, avoiding transportation and storage fees as usually occurs with agroindustry by products such's as sugar cane, corn and wheat by products.

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