Production of vinyl derivatives from alkaline hydrolysates of corn cobs by recombinant *E. coli* containing the phenolic acid decarboxylase from *Lactobacillus plantarum* CECT 748^{T}

José Manuel Salgado[1,*], Raquel Rodríguez[1], José Antonio Curiel[2], Blanca de las Rivas[2], Rosario Muñoz[2], José Manuel Domínguez[1]

[1]*Laboratory of Agro-food Biotechnology, CITI-Tecnólopole, Parque Tecnológico de Galicia, San Cibrao das Viñas, Ourense, SPAIN. Phone: (+34) 988 387416. Fax: (+34) 988 470 330*

[1]*Department of Chemical Engineering, Sciences Faculty, University of Vigo (Campus Ourense), As Lagoas s/n, 32004 Ourense, SPAIN,*

[2]*Laboratorio de Biotecnología Bacteriana, Instituto de Ciencia y Tecnología de Alimentos y Nutrición, ICTAN-CSIC, Juan de la Cierva 3, 28006 Madrid, SPAIN*

Phone: 34-988-387416, Fax: 34-988-387001, E-mail: jmanuel@uvigo.es

* To whom correspondence should be addressed

Abstract

The enzyme PAD from *Lactobacillus plantarum* CECT 748^T decarboxylates some cinnamic acids namely *p*-coumaric acid (*p*-CA), caffeic acid (CA), and ferulic acid (FA) into their corresponding 4-vinyl derivatives (4-VD): 4-vinyl phenol (4-VP), 4-vinyl catechol (4-VC), and 4-vinyl guaiacol (4-VG), respectively, which are valuable food additives mainly employed as flavoring agents. The gene encoding this enzyme was cloned and overexpressed in *Escherichia coli*. Two consecutive experimental designs using these recombinant *E. coli* cells provided valuable information about the experimental conditions to be used in experiments carried out in 250 ml Erlenmeyer flasks containing synthetic media. In this media, recombinant *E. coli* cells overproducing *L. plantarum* PAD showed a preference to degrade mainly *p*-CA and CA, meanwhile FA was hardly decarboxylated. These cells were also tested on corn cob hydrolysates as they are renewable feedstock for the generation of *p*-CA and FA. Sterilized liquors obtained after alkaline hydrolysis of corn cob or alkaline hydrolysis of the solid residue coming from acid hydrolysis of corn cob were employed as growth media in fermentations performed in shaker or bioreactor. The fermentative process allowed converting 2222.8 mg/L *p*-CA into 993.9 mg/L 4-VP ($Q_{4\text{-}VP}$ = 33.128 mg/L·h, $Y_{4\text{-}VP}$ = 0.81 mg/mg). The process described here allowed the production with a high-yield of a valuable food additive from a by-product of the food industry.

Keywords: Phenolic acid decarboxylase (PAD), Decarboxylation, 4-vinyl derivatives, 4-vinyl phenol, *Lactobacillus plantarum*, *Escherichia coli*, cinnamic acid

1. Introduction

Corn cobs are an important by-product of corn industry as its annual generation is estimated to be approximately of 700 MM tons/year (Rivas, Torres, Domínguez, Converti & Parajó, 2006), however large amounts remains unused as lignocellulosic waste or used as animal feed (Torre, Aliakbarian, Rivas, Domínguez & Converti, 2008), in spite of. Ferulic acid (4-hydroxy-3-methoxycinnamic acid; FA) and *p*-coumaric acid (4-hydroxycinnamic acid; *p*-CA) are the major phenolic compounds identified in the cross-linking of primary and secondary cell walls of graminaceous plants, and in particular of cereals (Pan, Bolton & Leary, 1998), representing together up to 1.5% by weight of cereal cell walls (Gasson et al., 1998). FA and *p*-CA are found covalently linked to polysaccharides by ester bonds and to components of lignin by ester or ether bonds (Scalbert, Monties, Lallemand, Guittet, & Rolando, 1985). A considerable proportion of *p*-CA is known to be esterified with lignin, while ferulic acid is extensively etherified with lignin (Sun, Xiao, & Lawther, 1998). The differences in stability of the ester and ether bonds allow a separation of ester and ether linked FA and *p*-CA (Xu, Sun, Sun, Liu, He, & Fan, 2005). Once released, phenolic acids become substrates of phenolic acid decarboxylase (PAD) enzymes, which catalyse the formation of the corresponding 4-vinyl derivatives (Cavin, Andioc, Etievant, & Diviés, 1993), thus, nowadays, corn cobs has gained importance as raw material for obtaining added-value products (Rivas, Torres, Domínguez, Perego, Converti, & Parajó, 2003).

These 4-vinyl derivatives (4-VD) produced by PAD enzymes have important consequences in the aroma of wine and other fermented foods and beverages and are therefore approved as food additives (Rodríguez, de las Rivas, Muñoz, & Mancheño, 2007). Consequently, these vinyl derivatives are of importance in the flavoring and polymer industries (Matte, Grosse, Bergeron, Abokitse, & Lau, 2010). The activity of PAD can be considered to be a biological response of lactic acid bacteria to the chemical stress induced by phenolic acids at low pH, mainly *p*-coumaric, ferulic and caffeic acids (Rodríguez et al., 2007). However, although it is clear the ability of *L. plantarum* to decarboxylate *p*-CA and CA into 4VP and 4VC respectively, some controversial results have been observed about the decarboxylation of FA into 4VG by *L. plantarum* strains (Barthelmebs, Diviès, & Cavin, 2001; Bloem, Bertrand, Lonvaud-Funel, & de Revel, 2006; Cavin et al., 1993; Couto, Campos, Figueiredo, & Hogg,

2006; Rodríguez, Landete, de las Rivas, & Muñoz, 2008; Van Beek & Priest, 2000). To solve this question, the *L. plantarum* PAD enzyme was biochemically characterized (Rodríguez et al., 2008b). Their kinetic parameters revealed that at high substrate concentration, p-CA and CA were much more efficiently decarboxylated than FA.

Consequently, this article deals with the ability of recombinant *Escherichia coli* overproducing the PAD enzyme from *Lactobacillus plantarum* CECT 748^{T} to decarboxylate *p*-CA, CA, and FA into their corresponding 4-vinyl derivatives (4-VD): 4-vinyl phenol (4-VP), 4-vinyl catechol (4-VC), and 4-vinyl guaiacol (4-VG), respectively, using synthetic media or solutions obtained after hydrolysis of corn cob.

2. Materials and methods

2.1. Chemicals and reagents

Three phenolic acids, ferulic acid (128708), *p*-coumaric acid (C9008) and caffeic acid (C0625) and the corresponding 4 vinyl phenol derivatives, 4-vinyl guaiacol (W267511) and 4-vinyl phenol (W373923), respectively, were purchased to Sigma-Aldrich Química, S. A. (Madrid, Spain) as standards for compound identification by HPLC. 4-vinyl catechol was measured as equivalent in 4-vinyl phenol. Tryptone (403682), NaCl (131659), yeast extract (403687), and methanol HPLC grade (221091) were supplied by Panreac Química Sau (Barcelona, Spain), meanwhile ampicillin (A9518) and isopropylthiogalactoside (IPTG) were supplied by Sigma-Aldrich Química, S. A. (Madrid, Spain).

2.2. Raw material and chemical characterisation

Corn cobs employed in this work were donated by farmers in the area of Ourense (Galicia, Spain). First of all, corn cobs were shelled to remove the kernels. The solid waste obtained was milled to a particle size smaller than 1 mm. Moisture of samples and solid fractions after hydrolysis treatments were characterised by quantitative acid hydrolysis in a two-stage acid treatment (the first stage with 72 wt % sulfuric acid at 30 ºC for 1 h, the second stage after dilution of the media to 4 wt % sulfuric acid at 121 ºC for 1 h) (Bustos, Moldes, Cruz, & Domínguez, 2004). The solid residue after hydrolysis

was considered as Klason lignin meanwhile hydrolysates were analysed by HPLC as described below.

2.3. Fractionation of the material

Materials were fractionated by two different procedures: alkaline hydrolysis or sequential stages of acid hydrolysis (pre-hydrolysis) and alkaline hydrolysis.

Pre-hydrolysis of corn cobs was carried out with diluted sulfuric acid (3%) during 15 min in Erlenmeyer flasks inside autoclave at 130 ºC with a liquid/solid ratio of 8 g/g following the procedure described by Bustos et al., (2004). The hemicellulosic fraction solubilised in the liquid was separated by vacuum filtration and the solid dried at 45 ºC for analysis by quantitative acid hydrolysis.

Raw corn cob or solids from the acid treatment were hydrolysed with solutions of NaOH (0.5 N) at room temperature in Erlenmeyer flasks at 150 rpm, using a liquid/solid ratio of 0.084 g solid/g NaOH solution (Torre et al., 2008; Max, Salgado, Cortés, & Domínguez, 2010). After 6 h, solids were separated by vacuum filtration, washed with water, and air dried for quantitative acid hydrolysis. Meanwhile liquors were neutralised with H_2SO_4 to pH 7, filtrated by vacuum again and stored at 4ºC for analysis by HPLC.

2.4. Experimental designs

Initially, a quarter fraction factorial design (2^{5-2}) was chosen to screen the relative influence in the 4-vinyl derivatives (VD) production and their possible interactions in the experimental domain. The independent factors considered were: agitation (or shaking speed), ampicillin concentration (selective pressure to maintain the plasmid into the *E. coli* cells), substrate concentration, working volume, and the presence of IPTG (inductor of the PAD gene expression). Three center points were added to the eight experiments to ensure enough degrees of freedom for error evaluation. The order of experiments was carried out in randomize order to avoid protection against the effects of lurking variables. Each factor was evaluated in two ranges (low, high) with limits selected based on preliminary experiments (data not shown). **Table 1** lists all the experiments contained in the design matrix. The analysis of data was performed by Statgraphics Plus 5.1.

Considering the results of the quarter fraction factorial design, a full factorial design (3^{2-0}) was planned to optimise the VD production. This design allows estimating the significance of the parameters and their interaction using Student's *t*-test. The

interrelationship between dependent and operational variables was established by Eq.1, a model which includes linear, interaction and quadratic terms:

$$y = b_0 + b_1 \cdot x_1 + b_2 \cdot x_1^2 + b_3 \cdot x_2 + b_4 \cdot x_2^2 + b_5 \cdot x_1 \cdot x_2 + b_6 \cdot x_1 \cdot x_2^2 + b_7 \cdot x_1^2 \cdot x_2 + b_8 \cdot x_1^2 \cdot x_2^2$$

Where y represents the dependent variables, b denotes the regression coefficients (calculated from experimental data by multiple regression using the least-squares method), and x denotes the independent variables. **Table 2** shows the experiments carried out including three center points tested for error evaluation. In this case, data analysis was performed by Statistica version 5.0 (Statsoft, USA) software.

2.5. Microorganism and culture conditions

The cloning and overexpression of the *pad* (or *pdc*) gene from *L. plantarum* have been previously described (Rodriguez et al., 2007). Briefly, the *pad* gene (coding for phenolic acid decarboxylase) from *L. plantarum* CECT 748^T was PCR-amplified and inserted into the pURI3 vector by using a restriction enzyme- and ligation-independent cloning strategy.

The microorganism was maintained in cryovials at -80 ºC using 20% glycerol as cryoprotector. This microorganism was cultivated to prepare the inoculum in 25 mL of sterile Luria-Bertani (LB) medium containing 10 g tryptone/L, 10 g NaCl/L, 5 g yeast extract/L and 100 mg ampicillin/L, during 20 h in Erlenmeyer flasks of 250 mL at 37 ºC and 150 rpm in orbital shakers. pH was adjusted to 7 and sterilised in autoclave (121 ºC, 15 min).

Fermentations were performed under the conditions described in each design using the same growth medium. Ampicillin, IPTG and substrate (caffeic, *p*-CA and FA) were added in different concentrations after sterilisation by ultrafiltration using 0.22 µm membranes (Nalgene) according to the experimental model 1. Each experiment was inoculated with 4% of an overnight *E. coli* culture.

Fermentations with hydrolysates were performed with hydrolysates obtained after alkaline hydrolysis of corn cobs (H-AHCC) or hydrolysates obtained after alkaline hydrolysis of the solid residue coming from pre-hydrolysis (H-AHSRPH), using two systems of sterilisation: ultrafiltation by 0.22 µm filters or sterilisation in autoclave (100 ºC, 60 min). The following experiments were assayed:

F1: H-AHCC sterilised by ultrafiltration.

F2: H-AHCC sterilised in autoclave.

F3: H-AHSRPH sterilised by ultrafiltration.

F4: H-AHSRPH sterilised in autoclave.

Liquors were adjusted to pH 7 with solutions of NaOH (5N) and HCl (0.5 M), and incubated in 250 mL Erlenmeyer flasks containing 25 mL of working volume, at 37 ºC in orbital shaker with the nutrients of the medium growth. 100 mg ampicillin/L was added to each experiment before inoculation. Each experiment was inoculated with 4% of the *E. coli* culture. Culture conditions optimised in both designs were employed for fermentation.

Finally, the process was scaled up in a 2L Bioreactor (Biostat, B. Braun Biotech International, Germany) (F5) using H-AHSRPH supplemented with the nutrients of the medium growth after sterilisation by ultrafiltration. Culture conditions were 70 rpm, 37 ºC and 1.2 L of working volume, similarly to the anaerobic conditions obtained in design 2.

Samples collected during kinetics were centrifuged at 6000 rpm for 10 min and analysed by HPLC.

2.6. Analytical determinations

Samples, containing the phenolic compounds, were filtered through 0.2 μm-pore membranes (Sartorius, Goettingen, Germany) in order to analyse the compounds by High Performance Liquid Chromatograph (HPLC), in a chromatograph Agilent model 1200 (Agilent, Palo Alto, CA, USA), using an UV detector (at 276 nm) and an quaternary pump. Separation was achieved at 35°C using a Zorbax SB-Aq reverse-phase column (Agilent, Palo Alto, CA, USA) with a guard column and a linear gradient run in 35 min from 100 to 52 % of A at a flow rate of 1 ml/min consisting of two solvents: solvent A (2.5% formic acid in water, vol./vol.) and solvent B (100% methanol).

3. Results and discussion

3.1. Preliminary experiments

Recombinant *E. coli* cells overproducing *L. plantarum* PAD were evaluated for their ability to degrade three phenolic acids: FA, *p*-CA, and CA. Assays were performed with the three phenolic acids separately and all together, in test tubes and Erlenmeyer flasks. The results indicated that CA (100 mg/L) was completely degraded after 24h but 4-VC was not quantified after 48 h, meanwhile *p*-CA (140 mg/L) was almost consumed (78.8%) after 24h to produce 137.3 mg/L of 4-VP after 48h. However, FA (150 mg/L) was not metabolised, and consequently 4-VG was not produced. The behavior was similar when the three phenolic acids were added simultaneously in both test tubes and Erlenmeyer flasks. These results are in agreement with those previously reported by Rodríguez, Landete, Curiel, de las Rivas, Mancheño, & Muñoz, (2008) using purified PAD enzyme from *L. plantarum* CECT 748^{T} at higher substrate concentrations, where *p*-CA and CA were more efficiently degraded than FA.

3.2. Screening of growth conditions using a quarter fractional factorial design

VD production by recombinant *E. coli* cells was studied using two consecutive factorial designs. Initially, several factors namely agitation (or shaking speed), ampicillin concentration, substrate concentration, working volume and presence of IPTG were selected to build a quarter fractional factorial design (2^{5-2}). This experimental procedure allows screening a wide number of factors with influence on the production of VD. Fractional factorial designs have been used frequently to observe the effect of several variables such as the alachlor solid-phase microextraction from water samples (González-Barreiro, Lores, Casais, & Cela, 2000) or the fermentative hydrogen production (Nath & Das, 2011), since the effects of several factors on a response can be studied under an economical and practical condition (Nath et al., 2011). Agitation and volume of medium were tested to study the influence of aeration in fermentation; meanwhile ampicillin concentration and IPTG presence were assessed because of their effects on plasmid maintenance and *pad* expression induction, respectively, in the recombinant *E. coli* used. The absence or reduction in the concentration used of ampicillin and IPTG in the growth media will significantly reduce the economical costs of the procedure. FA, CA, and *p*-CA were added as substrates since these phenolic acids could be potentially decarboxylated to 4-VD by *L. plantarum* PAD enzyme.

Table 3 shows the maxima concentration of 4-VD achieved, the percentage of phenolic acids consumption as well as the fermentative parameters calculated for all the

experimental runs of the first design. As it was expected from the preliminary experiments, FA was scarcely consumed (2.87-26.77%) meanwhile *p*-CA and CA were extensively metabolised. The analysis of the results showed that no significant interactions among factors were apparent, being consequently excluded from the analysis. Thus, the individual effect of each variable on the production of 4-VD can be observed. Pareto´s graphics were plotted in **Fig. 1** to represent the estimated effects of the studied variables on the global volumetric productivities *(Q*) and product yields (*Y)* of 4-VP, 4-VC, 4-VG and the total 4-VD. In these figures, each bar is proportional to the estimated effect, and the vertical line is used to evaluate which effects are statistically significant at 90% confidence level. Variables concerning aeration: agitation (-) and working volume (-) were the most decisive variables affecting the yield of 4-VP; meanwhile substrate concentration (-) was the only significant variable in the yields of 4-VC and 4-VG. Agitation (-) and substrate concentration (-) were consequently the most significant variables in the yield of total 4-VD. Conversely, substrate concentration (+), IPTG presence (-) and agitation (-) were statistically significant at 90% confidence level for Q_{VP}. The productivities of 4-VC and 4-VG did not show significant variables, meanwhile the productivity of total 4-VD was positively influenced by concentration of substrate, similarly to 4-VP which can be explained considering that this is the principal product obtained.

Fig. 2 plots the main effects influencing yields (Y_{VP}, Y_{VC}, Y_{VG} and Y_{VD}) and global volumetric productivities ($Q_{4\text{-}VP}$, $Q_{4\text{-}VC}$, $Q_{4\text{-}VG}$, Q_{VD}). This figure allows evaluating the effect of each factor in the studied variables. Each factor varies from its lowest level to its highest level, while all the other factors remain constant at their central values. As can be seen, shaking speed was the most influential factor in *Y* and *Q*. Ampicillin concentration and the presence of IPTG presence showed a lower influence on the 4-VD production. Ampicillin is necessary to maintain a selective pressure to keep the plasmid into the *E. coli* cells. Cells without plasmid could not growth in a media containing ampicillin; however, as the plasmid used has a high copy number, the ampicillin concentration could be reduced without plasmid loss. Similarly, the plasmid used is a T7 gene expression system plasmid on which the basal level of T7 RNA polymerase activity in the uninduced state provides significant transcription of the target gene (Tabor S & Richardson CC 1985, A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes, Proceedings of the National Academy of Sciences USA 82:1074-1078), therefore due to this basal

expression, the gene expression inducer (IPTG) could be omitted from the growth media. Thus, ampicillin could be used at the lowest concentration assayed, meanwhile IPTG was omitted. Agitation and volume of work are two variables that affect the oxygen mass transfer coefficient ($K_L \cdot a$) (Wang & Zhang, 2007) which is a tool for evaluating the effect of oxygen in fermentation processes (Elibol & Ozer, 2000). Shaking speed and agitation are consequently two important variables for obtaining good yields, being a critical factor if the process is scaled-up to a production size (Masurekar, 2008). **Table 4** shows the ANOVA analysis. ANOVA table divides the variability in Y_{VP} and Y_{VC} in different separate segments for each effect, then test the statistical significance of each effect by comparing the average square against an estimate of experimental error. In variable Y_{VP}, only one effect (shaking speed) showed a *p*-value lower than 0.05, indicating that this effect is significantly different from zero at the 95.0% level of confidence, meanwhile variable Y_{VC} showed two effects statistically significant at the 95.0% confidence level. The goodness of the models was checked by the analysis of the determination coefficient (R^2). This value indicates that the models, as fitted, explain 91.78% and 93.53% of the variability in Y_{VP} and Y_{VC} respectively. The adjusted R^2 statistic, which is more suitable for comparing models with different numbers of independent variables, was 81.50% for $Y_{4\text{-}VP}$ and 85.45% for $Y_{4\text{-}VC}$. The standard error of the estimate showed the standard deviation of the residuals was 0.0529386 for $Y_{4\text{-}VP}$ and 0.134815 for $Y_{4\text{-}VC}$. Finally, the mean absolute error (MAE), which is the average value of the residuals, was 0.0234 for Y_{VP} and 0.0079 for $Y_{4\text{-}VC}$.

3.3. Optimisation of growth conditions using a $3^{(2-0)}$ full factorial design.

Considering the results achieved in the first design, the variables concerning agitation (shaking speed and volume of work) were chosen to optimise the production of 4-VP, the main 4-VD quantified in the process, meanwhile the remaining variables previously studied were fixed. Thus, according to the previous discussion, the lowest concentration of ampicillin assayed in the first design (100 mg/L) was added to the culture medium. Although the model predicted an optimum using the highest amount of substrate, in order to avoid possible toxicities of products, this second design was studied using an intermediate value of substrate concentration (350 mg/L). Conversely, IPTG was omitted because no beneficial effect was seen in the production of VD.

The use of experimental designs to optimise the conditions of fermentation is a widely employed tool (Rodríguez, Salgado, Cortés, & Domínguez, 2011). In the current work, the independent variables considered and their variation ranges were: shaking speed (50-150 rpm) and volume of work (50-150 mL), in fermentations carried out in 250 mL Erlenmeyer flasks. The standardised (coded) adimensional variables employed, having variations limits (-1,1), were defined as x_1 (coded shaking speed) and x_2 (coded volume of work). The correspondence between coded and uncoded variables was established by linear equations deduced from their respective variation limits:

$$x_1 = ([\text{Agitation}] - 100)/50 \quad (2)$$

$$x_2 = (\text{volume of work} - 100)/50 \quad (3)$$

The dependent variables studied were: y_1 (CA_{cons}, mg/L), y_2 (p-CA_{cons}, mg/L), y_3 (FA_{cons}, mg/L), y_4 (T_{cons}, mg/L), y_5 (*4-VC*, mg/L), y_6 (*4-VP*, mg/L), y_7 (*4-VG*, mg/L), y_8 (*4-VD*, mg/L), y_9 ($Y_{4\text{-}VC}$, mg/mg), y_{10} ($Y_{4\text{-}VP}$, mg/mg), y_{11} ($Y_{4\text{-}VG}$, mg/ mg), y_{12} ($Y_{4\text{-}VD}$, mg/mg), y_{13} ($Q_{4\text{-}VC}$, mg/L·h), y_{14} ($Q_{4\text{-}VP}$, mg/L·h), y_{15} ($Q_{4\text{-}VG}$, mg/L·h) and y_{16} ($Q_{4\text{-}VD}$, mg/L·h). **Table 5** shows the experimental data obtained for variables y_1 to y_{16}, meanwhile **Table 6** lists the regression coefficients and their statistical significance. Finally, **Table 7** compiles the statistical parameters (R^2, R^2 adjusted, $F_{exp.}$ and the significance level based on the F test), measuring the correlation and the statistical significance of the models, respectively. The statistical parameters reveal the goodness of the sixteenth models studied, since the determination coefficients (R^2) were 0.97607 or higher, showing that 97.6% of the variability in the response could be explained by the model, allowing a close reproduction of the experimental data. Besides, the good significance of the models was supported by the values achieved with the adjusted determination coefficients (adjusted R^2) which were higher than 0.98524, excepting variables y_7 and y_{15} (R^2 = 88033), corresponding to the concentration and global volumetric productivity of 4-VG, respectively. These worse results could be attributed to the poor results achieved during the conversion of FA into 4-VG. Finally, the level of significance level based on the F test was higher than 95.9 in all the sixteenth models considered in this study.

Fig. 3 shows a three dimensional representation of the response surface for the predicted dependence of y_1, y_2, y_3 and y_4 on the agitation and volume of work as well as

a two-dimensional contour plots generated by the model. The response surface shows an optimum value at the highest volume of work and intermediate shaking speed in 4-VP production. In all cases, the highest volume of work was optimum to produce 4-VD, but in 4-VC and 4-VG, the production was increased by low agitation. From the coefficients of **Table 6**, and using the "solver" application of Microsoft Excell, the maximum 4-VD concentration predicted for the model (y_8 = 648.1 mg L^{-1}) was achieved when x_1 = -0.62 (69.04 rpm) and x_2 = 1.00 (150 mL), which are almost the most anaerobic conditions studied, with agitation enough to mix the culture. Under these conditions 389.1 mg/L 4-VP, 237.1 mg/L 4-VC and 23.5 mg/L 4-VG were produced. These results confirm the preference of *E. coli* cells producing PAD to mainly degrade *p*-CA and CA to 4-VP and 4-VC, respectively, meanwhile FA was hardly decarboxilated to 4-VG.

3.5 Characterisation of raw material and solid residues after hydrolysis treatments

Once optimised the decarboxylation of synthetic phenolic acids to their corresponding vinyl derivatives by the recombinant *E. coli* containing PAD, the production of 4-VD was evaluated by liquors obtained after alkaline hydrolysis of corn cob or sequential pre-hydrolysis and alkaline hydrolysis of corn cobs. Previously, the material was milled and screened for their characterisation by quantitative acid hydrolysis. **Figure 4** shows the chemical composition of the material employed in this work, the experimental data of weight loss and composition of the corresponding dry solid residues obtained after alkaline hydrolysis, acid pre-treatment (pre-hydrolysis) or sequential pre-hydrolysis/alkaline hydrolysis expressed as grams of cellulose (glucan), hemicelluloses (xylan + arabinan), lignin (Klason), acetyl groups and other (including proteins, ashes and extractives) per 100 g of dry solid. Corn cobs were composed by almost equal amounts of cellulose (26.2 ± 1.1 g/100 g dry corn cob), hemicelluloses (29.0 ± 0.5 g/100 g dry corn cob) and lignin (23.5 ± 1.5 g/100 g dry corn cob).

The alkaline hydrolysis of the raw material scarcely affected the composition of the solid residue, with the exception of acetyl groups that were completely removed. The percentage of cellulose removed was 36.4% (w/w), meanwhile hemicelluloses (10.0%, w/w), lignin (17.6%, w/w), and other compounds (18.3%, w/w) remained almost unaffected.

The acid hydrolysis pre-treatment was efficient in releasing hemicelluloses (84.4%, w/w) and acetyl groups (100%, w/w). Additionally, the group denoted as "other compounds" was almost completely removed (98.3%, w/w) from the raw material. Because of their branched, amorphous nature, hemicelluloses are relatively easy to be hydrolysed (Hamelinck, Van Hooijdonk, & Faaij, 2005). Particularly, in contrast to cellulose, the polymers present in hemicelluloses are easily hydrolysable (Kumar, Barrett, Delwiche, & Stroeve, 2009). These hemicellusoles are hydrolysed into fermentable sugars which can be biotransformated into other products such as xylitol (Cruz , Domínguez, Domínguez, & Parajó, 2000) or lactic acid (Moldes, Torrado, Converti, & Domínguez, 2006). This pre-treatment also improves the subsequence enzymatic hydrolysis of the remaining solid residue (Taherzadeh & Karimi, 2008). It is also remarkable the amount of lignin solubilised during this step (55.8%, w/w). Consequently, the residue obtained after pre-hydrolysis, with 56.6% (w/w) weight loss, was enriched in cellulose (62.3 ± 1.2 g/100 g dry solid), and consequently impoverished in hemicelluloses (11.2 ± 0.3 g/100 g dry solid), meanwhile the percentage of lignin remained similar (25.8 ± 0.1 g/100 g dry solid). No acetyl groups were quantified and the amount of other compounds was negligible (0.71 ± 0.01 g/100 g dry solid).

Conversely to the observed with the direct alkaline hydrolysis of corn cobs, the preliminary acidic treatment resulted efficient to break the structure by alkaline hydrolysis, considering that 86.5% (w/w) of the lignin was solubilised during the alkaline hydrolysis of pretreated materials. Lignin is a very complex molecule constructed of phenylpropane units linked in a three-dimensional structure which is particularly difficult to biodegrade (Taherzadeh et al., 2008). The solid residue obtained was rich in glucan (83.6 ± 0.2 g/100 g dry solid), which could be easily hydrolysed into glucose by enzymatic hydrolysis.

3.6 Extraction of phenolic compounds from corncobs.

Both raw corn cobs and the solid residue obtained after acid hydrolysis were subjected to alkaline hydrolysis at room temperature during 6 h following the conditions optimised by Torre et al. (2008) to release hydroxy-cinnamic acids resulting from breaking the ester bonds in the lignin/phenolics-polysaccharide complexes (Kondo, Ohshita, & Kyuma, 1992). **Figure 4** summarises the amount of *p*-CA and FA

solubilised during the process. Conversely, CA, the third phenolic compound evaluated for conversion when using synthetic media, could not be quantified. However, other compounds, such as vanillic acid, benzoic acid, vanillin, and syringic acid were present, although in lower concentrations. Xu et al., (2005) also observed small amounts of gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillic acid, vanillin, syringic acid, and syringaldehyde but not observed the presence of CA. In our case, the liquors resulting from the alkaline hydrolysis of the solid residue obtained after pre-hydrolysis (H-AHSRPH) had a higher content of *p*-CA (2526.5 mg/L ± 1.1%) and lower content of FA (798.8 mg/L ± 2.3%) than the liquors obtained after direct alkaline hydrolysis of corn cobs (H-AHCC), with a content of 1478.5 mg *p*-CA/L ± 1.2% and 1145.1 mg FA/L ± 2.1%. The concentration of hydroxy-cinnamic acids in H-AHSRPH was 26.7% higher than that obtained with H-AHCC, probably because the ferulated-hemicellulosic structure detected in most cereals including corn cobs (Saulnier & Thibault, 1999) can be better destroyed since the pre-hydrolysis can solubilise most of the hemicellulose fraction (see **Figure 4**).

The results obtained were similar to those reported by Torre et al. (2008) who obtained 2156 mg of *p*-CA/L and 1171 mg of FA/L in the liquors from alkaline hydrolysis of corn cobs. The high solubility of cereal lignin in alkaline solution might originate either from alkali cleavage of FA crosslink between lignin and hemicelluloses or from modification of lignin polyelectrolyte properties induced by free carboxyl groups of phenolic acids ethers (Xu et al., 2005).

The considerable decrease observed in the concentration of FA in H-AHSRPH can be explained considering that during acid hydrolysis, the FA located in the cell wall is released, since it appears both in esterified bonds to arabinose in hemicelluloses and in etherified linkages with lignin, being consequently liberated along with the arabinoxylan during solubilisation of hemicelluloses (Mussatto, Dragone, & Roberto, 2007). In fact, the liquors obtained after pre-hydrolysis has 138.0 mg FA/L ± 1.7%. Conversely, during the acid pre-treatment only slight amounts of *p*-CA were released because this compound is found more extensively esterified to lignin in the cell wall (Xu et al., 2005).

3.7 Fermentation of alkaline hydrolysate of corn cobs.

Hydrolysates obtained after alkaline hydrolysis of corn cobs (H-AHCC) and hydrolysates obtained after pre-hydrolysis/alkaline hydrolysis (H-AHSRPH) were

initially tested for the conversion of FA and *p*-CA into their corresponding 4-VD by recombinant *E. coli* cells producing *L. plantarum* PAD. Fermentations were carried out in Erlenmeyer flasks using the optima conditions achieved in design 1 (100 mg ampicillin/L and absence of IPTG) and 2 (69.04 rpm and 150 mL working volume). Both hydrolysates were sterilised under two different methods: ultrafiltation by 0.22 µm filters or sterilisation in autoclave (100 ºC, 60 min). **Table 8** shows the concentration of FA, *p*-CA, 4-VP and 4-VG, volumetric rate of substrate consumption, and the global volumetric productivities and yields of products, meanwhile **Figure 5** shows the course with time for these fermentations.

In both kind of hydrolysates, although no reduction of phenolic acids was observed when the liquors were sterilised by autoclaving, these compounds were hardly metabolised since only 463.2 mg/L of phenolic compounds (FA and *p*-CA) in H-AHCC (Q_S = 15.440 mg/L·h) and 545.7 mg/L in H-AHSRPH (Q_S = 18.190 mg/L·h) were consumed after 30h. On the other hand, using hydrolysates sterilised by ultrafiltration led to the consumption of 1018.8 mg/L of phenolic compounds in H-AHCC (Q_S = 33.960 mg/L·h) and 1257.1 mg/L in H-AHSRPH (Q_S = 41.903 mg/L·h). The harmful effect of hydrolysate exposure to high temperatures evidences or assumes the formation of toxic compounds (Rivas, Domínguez, Domínguez, & Parajó, 2002). The increment in the concentration of 4-VP was also considerable, 4-fold increase using H-AHCC hydrolysates, from 201.8 mg/L ($Q_{4\text{-}VP}$ = 6.728 mg/L·h, $Y_{4\text{-}VP}$ = 0.54 mg/mg) after autoclaving up to 801.4 mg/L ($Q_{4\text{-}VP}$ = 26.712 mg/L·h, $Y_{4\text{-}VP}$ = 0.84 mg/mg) in membrane-sterilised media. Meanwhile using H-AHSRPH, the increment was slightly higher (4.5-fold increase), from 221.6 mg/L ($Q_{4\text{-}VP}$ = 7.386 mg/L·h, $Y_{4\text{-}VP}$ = 0.54 mg/mg) after autoclaving up to 993.9 mg/L ($Q_{4\text{-}VP}$ = 33.128 mg/L·h, $Y_{4\text{-}VP}$ = 0.89 mg/mg) after sterilisation by ultrafiltration.

Comparing the fermentation of both hydrolysates sterilised by the same technique (see **Table 8**), H-AHSRPH provided slightly better results in terms of final concentrations of 4-VP and global volumetric productivities, which can be explained considering the higher amount of initial concentration of *p*-CA. However, the product yields were identical (0.54 mg/mg after autoclaving and 0.84-0.89 mg/mg after ultrafiltration), reflecting that both hydrolysates could be used as feedstock for the production of 4-VD. Nevertheless, these yields are considerably lower than those achieved using synthetic media, where the maximum value ($Y_{4\text{-}VP}$ = 1.46 mg/mg) was

reached in the experiment 5 of the first design corresponding to 500 mg/L of substrate (CA, *p*-CA and FA).

Since there was no lag phase, and consequently there was no inhibition by the presence of other phenolic compounds present in the hydrolysate, the inhibition could only be attributed to the production of 4-VP. This fact was supported taking into account that hydrolysates showed high substrate concentrations, in comparison to the previous synthetic media, but is not possible to exceed a threshold of 993.9 mg/L of 4-VP. Similarly, Ben-Bassat et al., (2007) during biotransformations to produce 4-VP employing whole cells with *para*-hydroxycinnamic acid decarboxylase activity, suffered long reaction times, low yields, and product concentrations due to the toxicity of 4-VP. To overcome this drawback, these authors suggest the use of two-phase biotransformation phase, which offer the prospect of increased mass transfer, alleviation of possible substrate and product toxicity, and hence, enhanced biotransformations and simplified product recovery (Yang, Wang, Lorrain, Rho, Abokitse, & Lau, 2009).

Similarly to the observed using the synthetic media, the microorganism showed only specificity towards the decarboxylation of *p*-CA to 4-VP, since FA was hardly consumed and consequently 4-VG was scarcely produced, yielding in all cases concentrations lower than 25.6 mg/L.

3.8 Scale-up of the process

Once the decarboxylation was studied in Erlenmeyer flasks, a final fermentation was tested in a 2 L bioreactor to scale up the process to larger sizes. Although new engineering variables have to be considered, aeration and agitation are critical for assuring adequate supply of oxygen to the growing culture (Masurekar, 2008). Therefore, 1200 mL of hydrolysates obtained after sequential stages of prehydrolysis/alkaline hydrolysis, without nutrients addition and sterilised by ultrafiltration (F5 in **Table 8**) was employed. The behavior was similar to F3 using the same hydrolysates and sterilisation procedure. After 30 h of fermentation, 878.4 mg 4-VP/L was achieved, yielding 0.85 mg/mg and a volumetric productivity of 29.279 mg/L·h. Additionaly, in relation to 4-VG, this product was scarcely produced, not exceeding a threshold of 21.3 mg 4-VG/L after 30 hours. These results corroborated the selectivity of recombinant *E. coli* cells producing *L. plantarum* PAD towards the

decarboxylation of *p*-CA, and the need of studding the simultaneous production and recovery of 4-VP to avoid product inhibition.

Acknowledgments

We are grateful for the financial support of this work to the Xunta de Galicia (project 09TAL13383PR).

References

Barthelmebs, L., Diviès, C., & Cavin, J.F. (2001). Expression in *Escherichia coli* of native chimeric phenolic acid decarboxylases with modified enzymatic activities and methods for screening recombinant *E. coli* strains expressing these enzymes. *Applied and Environmental Microbiology*, 67, 1063–1069.

Ben-Bassat,A., Breinig, S., Crum, G.A., Huang, L., La Bonte Altenbaugh, A., Rizzo, N., Trotman, R.J., Vannelli, T., Sariaslani, F. S., & Haynie, S. L. (2007). Preparation of 4-vinylphenol using pHCA decarboxylase in a two-solvent medium. *Organic Process Research & Development*, 11, 278□-285.

Bloem, A., Bertrand, A., Lonvaud-Funel, A., & de Revel, G. (2006). Vanillin production from simple phenols by wine-associated lactic acid bacteria. *Letters in Applied Microbiology*, 44, 62–67.

Bustos, G., Moldes, A. B., Cruz, J. M., & Domínguez, J. M. (2004). Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by *Lactobacillus pentosus*, *Journal of the Science of Food and Agriculture*, 84, 2105–2112.

Cavin, J.F., Andioc, V., Etievant, P. X., & Diviès, C. (1993). Ability of wine lactic acid bacteria to metabolize phenol carboxylic acids. *American Journal of Enology and Viticulture*, 44, 76–80.

Cavin, J. F., Barthelmebs, L., & Diviès, C. (1997). Molecular characterization of an inducible *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*: gene cloning, transcriptional analysis, overexpression in *Escherichia coli*, purification, and characterization. *Applied and Environmental Microbiology*, 63, 1939–1944.

Couto, J. A., Campos, F. M., Figueiredo, A. R., & Hogg, T. A. (2006). Ability of lactic acid bacteria to produce volatile phenols. *American Journal of Enology and Viticulture*, 57, 166–171.

Cruz, J. M., Domínguez, J. M., Domínguez, H.,& Parajó, J. C. (2000). Preparation of fermentation media from agricultural wastes and their bioconversion into xylitol. *Food Biotechnology*. 14, 79-97.

Elibol, M., & Ozer, D. (2000). Influence of oxygen transfer on lipase production by *Rhizopus arrhizus*. *Process Biochemistry*, 36, 325–329.

Gasson, M.J., Kitamura, Y., Mclauchlan, W.R., Narbad, A., Parr, A.J., Parsons, E.L.H., Payne, J., Rhodes, M.J.C., & Walton, N.J. (1998). Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. *Journal of Biological Chemistry*, 273, 4163–4170.

González-Barreiro, C., Lores, M., Casais, M.C. & Cela, R. (2000). Optimisation of alachlor solid-phase microextraction from water samples using experimental design. *Journal of Chromatography A*, 896, 373–379.

Hamelinck, C. N., van Hooijdonk, G. & Faaij, A. P. C. (2005). Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy,* 28, 384–410.

Kondo, T., Ohshita, T., & Kyuma, T. (1992). Comparison of characteristics of soluble lignins from untreated and ammonia-treated wheat straw. *Animal Feed Science and Technology*, 39 , 253–263.

Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for pretreament of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research,* 48, 3713-3729.

Masurekar, P. S. (2008). Nutritional and engineering aspects of microbial process development. *Progress in Drug Research*, 65, 292-328.

Matte, A., Grosse, S., Bergeron, H., Abokitse, K., & Lau, P. C. K. (2010). Structural analysis of *Bacillus pumilus* phenolic acid decarboxylase, a lipocalin-fold enzyme. *Acta Crystallographica*, 66, 1407-1414.

Max, B., Salgado, J.M., Cortés, S., & Domínguez, J.M. (2010). Extraction of phenolic acids by alkaline hydrolysis from the solid residue obtained after prehydrolysis of trimming vine shoots. *Journal of Agricultural and Food Chemistry,* 58, 1909–1917.

Moldes, A. B., Torrado, A., Converti, A., & Domínguez, J. M. (2006). Complete bioconversion of hemicellulosic sugars from agriculture residues into lactic acid by *Lactobacillus pentosus. Applied Biochemistry and Biotechnology*. 135(3), 219-227.

Mussatto, S., Dragone, G., & Roberto, I. (2007). Ferulic and *p*-coumaric acids extraction by alkaline hydrolysis of brewer's spent grain. *Industrial Crops and Products* , 25, 231–237.

Nath, K., & Das, D. (2011). Modeling and optimisation fermentative hydrogen production. *Bioresource Technology* , 102, 8569-8581.

Pan, G.X., Bolton, J.L., & Leary, G.J. (1998). Determination of ferulic and *p*-coumaric acids in wheat straw and the amounts released by mild acid and alkaline peroxide treatment. *Journal of Agricultural and Food Chemistry*, 46, 5283–5288.

Rivas, B., Domínguez, J.M., Domínguez, H.,& Parajó, J.C. (2002). Bioconversion of posthydrolysed autohydrolysis liquors: an alternative for xylitol production from corn cobs. *Enzyme and Microbial Technology*, 31, 431–438.

Rivas, B., Torres, P., Domínguez, J. M., Perego, P., Converti, A.,& Parajó, J. C.(2003). Carbon material and bioenergetic balances of xylitol production from corncobs by *Debaryomyces hansenii*. *Biotechnology Progress*, *19*, 706-713.

Rivas, B., Torre, P., Domínguez, J.M., Converti, A., & Parajó, J.C. (2006). Purification of xylitol obtained by fermentation of corncob hydrolysates. *Journal of Agricultural and Food Chemistry*, 54, 4430-4435.

Rodríguez, H., de las Rivas, B., Muñoz, R. & Mancheño, J.M. (2007). Overexpression, purification, crystallization and preliminary structural studies of *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*. *Acta Crystallographica*, 63, 300–303.

Rodríguez, H., Landete, J. M., de las Rivas, B.,& Muñoz, R. (2008). Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748^{T}. *Food Chemistry*, 107, 1393–1398.

Rodríguez, H., Landete, J. M., Curiel, J. A., de las Rivas, B., Mancheño, J. M., & Muñoz, R. (2008b). Characterization of the *p*-coumaric acid decarboxylase from *Lactobacillus plantarum* CECT 748^{T}. *Journal of Agricultural and Food Chemistry*, *56*, 3068–3072.

Rodríguez, N., Salgado, J. M., Cortés, S., & Domínguez, J.M. (2011). Antimicrobial activity of D-3-phenyllactic acid produced by fed-batch process against *Salmonella enterica*. *Food Control*, 25, 274-284.

Saulnier, L.,& Thibault, J. (1999). Ferulic acid and diferulic acids as components of sugarbeet pectins and maize bran heteroxylans, *Journal of the Science of Food and Agriculture*, 79, 396– 402.

Scalbert, A., Monties, B., Lallemand, J.Y., Guittet, E., & Rolando, C. (1985). Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochemistry*, 24, 1359-1362.

Sun, R.C., Xiao, B.,& Lawther, J.M. (1998). Fractional and structural characterization of ball-milled and enzyme lignins from wheat straw. *Journal of Applied Polymer Science*, 68, 1633-1641.

Taherzadeh, M. J., & Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. *International Journal of Molecular Sciences*, 9, 1621-1651.

Torre, P., Aliakbarian, B., Rivas, B., Domínguez, J.M., & Converti, A. (2008). Release of ferulic acid from corn cobs by alkaline hydrolysis. *Biochemical Engineering Journal*, 40, 500–506.

Van Beek, S., & Priest, F. G. (2000). Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whisky fermentation. *Applied and Environmental Microbiology*, 66, 5322–5328.

Wang, Y.H., & Zhang, X. (2007). Influence of agitation and aeration on growth and antibiotic production by *Xenorhabdus nematophila*. *World Journal of Microbiology and Biotechnology*, 23, 221-227.

Xu, F., Sun, R., Sun, J., Liu, C., He, B., & Fan, J.. (2005). Determination of cell wall ferulic and *p*-coumaric acids in sugarcane bagasse. *Analytica Chimica Acta*, 552(1-2), 207-217.

Yang, J., Wang, S., Lorrain, M.J., Rho, D., Abokitse, K. & Lau, P.C.K.(2009). Bioproduction of lauryl lactone and 4-vinyl guaiacol as value-added chemicals in two-phase biotransformation systems. *Applied Microbiology and Biotechnology*, 84, 867–876.

Figure legends

Fig. 1. Pareto's graphics measuring the influence of the independent variables studied in the quarter fractional design (2^{5-2}) on (a) $Y_{4\text{-}VP}$, (b) $Y_{4\text{-}VC}$, (c) $Y_{4\text{-}VG}$, (d) $Y_{4\text{-}VD}$, (e) $Q_{4\text{-}VP}$, (f) $Q_{4\text{-}VC}$, (g) $Q_{4\text{-}VG}$, and (h) $Q_{4\text{-}VD}$.

Fig. 2. Influence of the independent variables studied in the quarter fractional design (2^{5-2}) on (a) $Y_{4\text{-}VP}$, (b) $Y_{4\text{-}VC}$, (c) $Y_{4\text{-}VG}$, (d) $Y_{4\text{-}VD}$, (e) $Q_{4\text{-}VP}$, (f) $Q_{4\text{-}VC}$, (g) $Q_{4\text{-}VG}$, and (h) $Q_{4\text{-}VD}$.

Fig. 3. Dependence of (a) 4-VP concentration, (b) 4-VC concentration, (c) 4-VG concentration, and (d) 4-VD concentration on x_1 (coded agitation) and x_2 (coded volume of work).

Fig. 4. Scheme for the release of phenolic acids by alkaline hydrolysis or sequential pre-hydrolysis-alkaline hydrolysis and composition of raw material and solid residues.

Fig. 5. Course with time for vinyl derivatives production using a) alkaline hydrolysate sterilised by ultrafiltration; b) alkaline hydrolysate sterilised in autoclave, c) alkaline hydrolysate of solid after acid hydrolysis sterilised by ultrafiltration, d) alkaline hydrolysate of solid after acid hydrolysis sterilised in autoclave, and e) alkaline hydrolysate obtained from the solid after acid hydrolysis after sterilisation by ultrafiltration in bioreactor. (♦) *p*-CA, (●) FA, (◊) 4-VP, (○) 4-VG.

Table 1. Design matrix to study the VD production by recombinant *E. coli* cells using a quarter fractional design (2^{5-2}).

Run	Shaking (rpm)	Ampicillin (mg/L)	Substrate* (mg/L)	Volume (mL)	IPTG (mM)
1	150	200	500	100	0.4
2	150	200	150	100	0
3	150	100	500	25	0.4
4	150	100	150	25	0
5	70	200	500	25	0
6	70	200	150	25	0.4
7	70	100	500	100	0
8	70	100	150	100	0.4
9.1	110	150	325	62.5	0.2
9.2	110	150	325	62.5	0.2
9.3	110	150	325	62.5	0.2

**caffeic acid, ferulic acid and p-coumaric acid*

Table 2. Design matrix to optimise the 4-VD production by recombinant *E. coli* cells overproducing *L. plantarum* PAD using a full factorial design (3^{2-0})

Runs	Shaking speed (rpm)	Volume (mL)
1	50	50
2	50	100
3	50	150
4	100	50
5.1	100	100
5.2	100	100
5.3	100	100
6	100	150
7	150	50
8	150	100
9	150	150

Table 3. Response values (percentage of phenolic acids consumption and maxima concentration of 4-VD achieved) and fermentative parameters calculated in the screening design (2^{5-2}).

Run	$p\text{-}CA_{cons}$ (%)	FA_{cons} (%)	CA_{cons} (%)	$4\text{-}VP_{max}$ (mg/L)	$4\text{-}VG_{max}$ (mg/L)	$4\text{-}VC_{max}$ (mg VP equiv/L)	T_f (h)	$Y_{4\text{-}VP}$ (mg/mg)	$Y_{4\text{-}VG}$ (mg/mg)	$Y_{4\text{-}VC}$ (mg/mg)	$Q_{4\text{-}VP}$ (mg/L·h)	$Q_{4\text{-}VG}$ (mg/L·h)	$Q_{4\text{-}VC}$ (mg/L·h)
1	67.2	12.4	74.7	357.1	0.0	67.6	72	1.08	0.00	0.13	4.960	0.000	1.808
2	100.0	3.2	100.0	179.2	8.2	179.2	24	1.16	1.49	0.77	7.465	0.343	4.723
3	93.3	9.8	96.5	488.7	0.0	282.8	48	1.07	0.00	0.38	10.181	0.000	6.969
4	100.0	23.5	100.0	174.1	19.9	165.2	24	1.17	0.51	0.74	7.256	0.830	4.274
5	100.0	7.3	92.6	548.9	12.5	402.7	24	1.46	0.35	0.61	22.870	0.521	9.875
6	100.0	13.2	100.0	179.5	26.9	206.9	24	1.36	1.19	1.07	7.479	1.121	5.511
7	95.7	12.1	93.2	504.0	11.8	342.6	24	1.24	0.19	0.51	21.000	0.493	8.793
8	100.0	26.8	100.0	162.3	38.4	275.6	24	1.20	0.69	1.41	6.760	1.600	7.312
9.1	52.3	2.9	36.8	222.8	0.0	149.8	48	1.32	0.00	0.70	4.642	0.000	1.745
9.2	54.7	6.8	41.0	229.8	0.0	138.3	48	1.26	0.00	0.63	4.788	0.000	1.814
9.3	63.5	4.9	41.3	224.6	0.0	85.7	48	0.85	0.00	0.61	4.68	0.000	1.790

Nomenclature: $p\text{-}CA_{cons}$: *p*-coumaric acid consumed; FA_{cons}: ferulic acid consumed; CA_{cons}: caffeic acid consumed; $4\text{-}VP_{max}$: maxima concentration of 4-vinyl phenol; $4\text{-}VG_{max}$: maxima concentration of 4-vinyl guaiacol; $4\text{-}VC_{max}$: maxima concentration of 4-vinyl catechol; T_f: time of fermentation to achieve the maxima concentration of 4-VP; $Q_{4\text{-}VP}$: global volumetric productivity of 4-VP; $Q_{4\text{-}VG}$: global volumetric productivity of 4-VG; $Q_{4\text{-}VC}$: global volumetric productivity of 4-VC; $Y_{4\text{-}VP}$: yield of 4-VP; $Y_{4\text{-}VG}$: yield of 4-VG; $Y_{4\text{-}VC}$: yield of 4-VC.

Table 4. ANOVA analysis for variables $Y_{4\text{-}VP}$ and $Y_{4\text{-}VC}$ in the screening design (2^{5-2}).

a) Dependent variable: $Y_{4\text{-}VP}$

Source	**SS**	**d.f.**	**MS**	***F*-ratio**	***p*-value**
Shaking speed	0.07605	1	0.07605	27.14	0.0065
Ampicillin	0.01805	1	0.01805	6.44	0.0641
Substrate	0.0002	1	0.0002	0.07	0.8026
Volume of work	0.01805	1	0.01805	6.44	0.0641
IPTG	0.0128	1	0.0128	4.57	0.0994
Residual	0.01121	4	0.0028025		
Total (Corr.)	0.13636	9			

R^2 = 91.78%

R^2 (adjusted for d.f.) = 81.50%

Standard Error of Est. = 0.0529386

Mean absolute error = 0.0234

b) Dependent variable: $Y_{4\text{-}VC}$

Source	**SS**	**d.f.**	**MS**	***F*-ratio**	***p*-value**
Shaking speed	0.31205	1	0.31205	17.17	0.0143
Ampicillin	0.02645	1	0.02645	1.46	0.2941
Substrate	0.6962	1	0.6962	38.31	0.0035
Volume of work	0.00005	1	0.00005	0.00	0.9607
IPTG	0.0162	1	0.0162	0.89	0.3986
Residual	0.0727	4	0.018175		
Total (Corr.)	1.12365	9			

R^2 = 93.53%

R^2 (adjusted for d.f.) = 85.44%

Standard Error of Est. = 0.134815

Mean absolute error = 0.079

Table 5. Fermentative parameters of experiments after 24 h to optimise the VD production by recombinant *E. coli* cells using a full factorial design (3^{2-0}).

	Substrate consumed				Production				Yield (*Y*)				Volumetric productivity (*Q*)			
Runs	y_1 $CA_{cons.}$ (mg/L)	y_2 $p\text{-}CA_{cons.}$ (mg/L)	y_3 $FA_{cons.}$ (mg/L)	y_4 $T_{cons.}$ (mg/L)	y_5 *4-VC* (mg/L)	y_6 *4-VP* (mg/L)	y_7 *4-VG* (g/L)	y_8 *4-VD* (mg/L)	y_9 $Y_{4\text{-}VC}$ (mg/mg)	y_{10} $Y_{4\text{-}VP}$ (mg/mg)	y_{11} $Y_{4\text{-}VG}$ (mg/mg)	y_{12} $Y_{4\text{-}VD}$ (mg/mg)	y_{13} $Q_{4\text{-}VC}$ (mg/L·h)	y_{14} $Q_{4\text{-}VP}$ (mg/L·h)	y_{15} $Q_{4\text{-}VG}$ (mg/L·h)	y_{16} $Q_{4\text{-}VD}$ (mg/L·h)
1	294.7	305.5	20.0	620.1	236.1	237.6	8.9	482.6	0.80	0.78	0.45	0.78	9.836	9.900	0.370	20.106
2	301.9	339.8	16.3	658.0	288.8	289.3	16.2	594.4	0.96	0.85	1.00	0.90	12.035	12.053	0.677	24.764
3	289.3	307.9	34.8	632.0	252.8	357.7	24.0	634.5	0.87	1.16	0.69	1.00	10.531	14.906	1.002	26.438
4	303.6	303.8	22.7	630.1	173.2	225.5	11.5	410.1	0.57	0.74	0.50	0.65	7.214	9.397	0.477	17.089
5.1	358.2	313.6	95.9	767.8	159.7	376.6	6.0	542.3	0.45	1.20	0.06	0.71	6.655	15.690	0.251	22.596
5.2	357.4	313.0	98.4	768.7	166.1	370.1	8.0	544.2	0.46	1.18	0.08	0.71	6.920	15.419	0.335	22.673
5.3	360.9	310.1	96.4	767.4	161.9	380.7	10.0	552.6	0.45	1.23	0.10	0.72	6.747	15.862	0.415	23.024
6	343.9	362.1	168.1	874.0	191.2	396.1	19.9	607.2	0.56	1.09	0.12	0.69	7.968	16.503	0.830	25.301
7	310.4	327.8	54.3	692.6	165.8	263.5	9.5	438.8	0.53	0.80	0.17	0.63	6.906	10.981	0.395	18.282
8	324.9	274.5	59.3	658. 1	138.3	296.3	9.3	444.0	0.43	1.08	0.16	0.67	5.762	12.347	0.389	18.498
9	322.9	266.1	119.0	708.0	63.52	292.2	7.3	363.0	0.20	1.10	0.06	0.51	2.647	12.174	0.303	15.124

Nomenclature: CA_{cons}: caffeic acid consumed; $p\text{-}CA_{cons}$: *p*-coumaric acid consumed; FA_{cons}: ferulic acid consumed; T_{cons}: total amount of caffeic acid, *p*-coumaric acid and ferulic acid consumed; *4-VC*: concentration of 4-vinyl catechol; *4-VP*: concentration of 4-vinyl phenol; *4-VG*: concentration of 4-vinyl guaiacol; *4-VD*: concentration of vinyl derivatives (4-vinyl catechol, 4-vinyl phenol and 4-vinyl guaiacol); $Y_{4\text{-}VC}$: yield of 4-VC; $Y_{4\text{-}VP}$: yield of 4-VP; $Y_{4\text{-}VG}$: yield of 4-VG; $Y_{4\text{-}VD}$: yield of 4-VC, 4-VP and 4-VG; $Q_{4\text{-}VC}$: global volumetric productivity of 4-VC; $Q_{4\text{-}VP}$: global volumetric productivity of 4-VP; $Q_{4\text{-}VG}$: global volumetric productivity of 4-VG; $Q_{4\text{-}VD}$: global volumetric productivity of 4-VD.

Table 6. Regression coefficients and significance level for dependent variables in the full factorial design (3^{2-0}).

	b_0	b_1	b_{11}	b_2	b_{22}	b_{12}	b_{122}	b_{112}	b_{1122}
y_1	358.818627*	11.481381*	-45.4162651*	20.1452013*	-35.0913341*	4.48101798*	0.84697585	-18.3646997*	26.011157*
y_2	312.22058*	-32.6469748*	-5.06093064***	29.128711*	20.7277881*	-16.0380186*	27.7841777*	-43.9570192*	-26.0511176*
y_3	96.9026137*	21.5178083*	-59.1074049*	72.6888884*	-1.50533654	12.4505895*	8.12755263*	-52.8010868*	20.7479851*
y_4	767.94*	0.35	-109.58*	121.965*	-15.865*	0.895	36.76*	-115.125*	20.7*
y_5	375.768605*	3.53158842	-82.9635129*	85.2626009*	-64.9707404*	-22.8726584*	-13.4331345***	-48.0652733*	59.9272587*
y_6	162.576994*	-75.2763613*	50.976415*	9.04205702**	19.6104544*	-29.7265281*	10.389197***	-30.4285887*	-53.643468*
y_7	8.00392036*	-3.45181986	4.78829219***	4.2342579***	7.68623288**	-4.34101494*	-0.58771908	-0.99812503	-8.05208264***
y_8	546.349519*	-75.1965927*	-27.1988057*	98.5389158*	-37.6740532*	-56.9402014*	-3.63165658	-79.491987*	-1.76829192
y_9	1.20361021*	0.11410615*	-0.23821546*	0.17576204*	-0.28551548*	-0.02248171	-0.12353384*	-0.00620918	0.28047363*
y_{10}	0.45310962*	-0.26551009*	0.23802056*	-0.0071177	0.11011393*	-0.10247455*	0.0295037***	-0.0590502*	-0.19988484*
y_{11}	0.08257217*	-0.42023688*	0.49514099*	-0.19297084*	0.22893556*	-0.08958617*	0.19526243*	0.22581933*	-0.46375491*
y_{12}	767.941821*	0.35221449	-109.584601*	121.962801*	-15.8688826*	0.89358889	36.7587062*	-115.122806*	20.7080245*
y_{13}	15.6570252*	0.14714952	-3.45681304*	3.55260837*	-2.70711418*	-0.95302743*	-0.55971394***	-2.00271972*	2.49696911*
y_{14}	6.77404143*	-3.13651505*	2.12401729*	0.37675238**	0.81710226*	-1.23860534*	0.43288321**	-1.26785786*	-2.2351445*
y_{15}	0.33349668*	-0.14382583	0.19951217	0.17642741***	0.3202597**	-0.18087562*	-0.0244883	-0.04158854	-0.33550344***
y_{16}	22.7645633*	-3.13319136*	-1.13328357*	4.10578816*	-1.56975222*	-2.37250839*	-0.15131902	-3.31216613*	-0.07367883

*Significant coefficients at the 99% confidence level

**Significant coefficients at the 95% confidence level

***Significant coefficients at the 90% confidence level

Table 7. Statistical parameters (R^2 and F) measuring the correlation and significance of the models

Variable	R^2	corrected R^2	$F_{exp.}$	significance level (based on the F test)
y_1	0.99909	0.99544	9851.06573	99.218
y_2	0.99904	0.99522	9404.25226	99.200
y_3	0.99986	0.99929	63271.6369	99.692
y_4	0.99999	0.99993	619560.63	99.901
y_5	0.99848	0.99239	5900.45	98.990
y_6	0.99944	0.99719	15999.54	99.387
y_7	0.97607	0.88033	367.05	95.951
y_8	0.99923	0.99614	11653.05	99.281
y_9	0.99705	0.98524	3039.73	98.593
y_{10}	0.99959	0.99793	21756.05	99.474
y_{11}	0.99914	0.99572	10497.96	99.243
y_{12}	0.99935	0.99676	13876.45	99.341
y_{13}	0.99848	0.99239	5900.45	98.990
y_{14}	0.99944	0.99719	15999.54	99.387
y_{15}	0.97607	0.88033	367.045	95.951
y_{16}	0.99923	0.99614	11653.05	99.281

Table 8. Vinyl derivatives production in fermentations carried out with alkaline hydrolysate of corn cob or alkaline hydrolysate of the solid residue coming from acid hydrolysis, after sterilisation by ultrafiltration or autoclave, in fermentations performed in shaker or bioreactor.

				p-CA			FA			Substrate		4-VP				4-VG			
		Sterilis.	Type of hydrolysate	*p*-CA_{t0} (mg/L)	*p*-CA_{tf} (mg/L)	*p*-CA_{cons} (mg/L)	FA_{t0} (mg/L)	FA_{tf} (mg/L)	$FA_{cons.}$ (mg/L)	T_{cons} (mg/L)	Q_S (mg/L·h)	4-VP_{t0} (mg/L)	4-VP_{tf} (mg/L)	$Y_{4\text{-}VP}$ (mg/mg)	$Q_{4\text{-}VP}$ (mg/L·h)	4-VG_{t0} (mg/L)	4-VG_{tf} (mg/L)	$Y_{4\text{-}VG}$ (mg/mg)	$Q_{4\text{-}VG}$ (mg/L·h)
F1	Shaker	UF	H-AHCC	1482.9	533.3	949.6	1045.6	976.3	69.3	1018.8	33.960	0.0	801.4	0.84	26.712	0.0	24.9	0.36	0.830
F2	Shaker	AC	H-AHCC	1447.4	1073.4	374.0	1105.7	1016.5	89.2	463.2	15.440	0.0	201.8	0.54	6.728	0.0	20.0	0.22	0.667
F3	Shaker	UF	H-AHSRPH	2222.8	1099.7	1123.1	894.1	760.1	134.0	1257.1	41.903	0.0	993.9	0.89	33.128	0.0	25.6	0.19	0.854
F4	Shaker	AC	H-AHSRPH	2283.1	1872.6	410.5	896.1	760.9	135.2	545.7	18.190	0.0	221.6	0.54	7.386	0.0	20.1	0.15	0.671
F5	Bior.	UF	H-AHSRPH	2124.9	1094.1	1030.8	915.5	897.5	18.0	1048.8	34.960	0.0	878.4	0.85	29.279	0.0	21.3	1.19	0.711

Nomenclature: UF: ultrafiltration; AC: autoclave; H-AHCC: hydrolysate obtained after alkaline hydrolysis of corn cob; H-AHSLPH: hydrolysate obtained after alkaline hydrolysis of the solid residue resulting from pre-hydrolysis; $p\text{-}CA_{cons}$: *p*-coumaric acid consumed; FA_{cons}: ferulic acid consumed; T_{cons}: total amount of *p*-coumaric acid and ferulic acid consumed; Q_S = volumetric rate of substrates (*p*-coumaric acid and ferulic acid) consumption; *4-VP*: concentration of 4-vinyl phenol; *4-VG*: concentration of 4-vinyl guaiacol; $Y_{4\text{-}VP}$: yield of 4-VP; $Q_{4\text{-}VP}$: global volumetric productivity of 4-VP; $Y_{4\text{-}VG}$: yield of 4-VG; $Q_{4\text{-}VG}$: global volumetric productivity of 4-VG; $_{t0}$ = at the beginning of fermentation; tf = at final time of fermentation (30h).

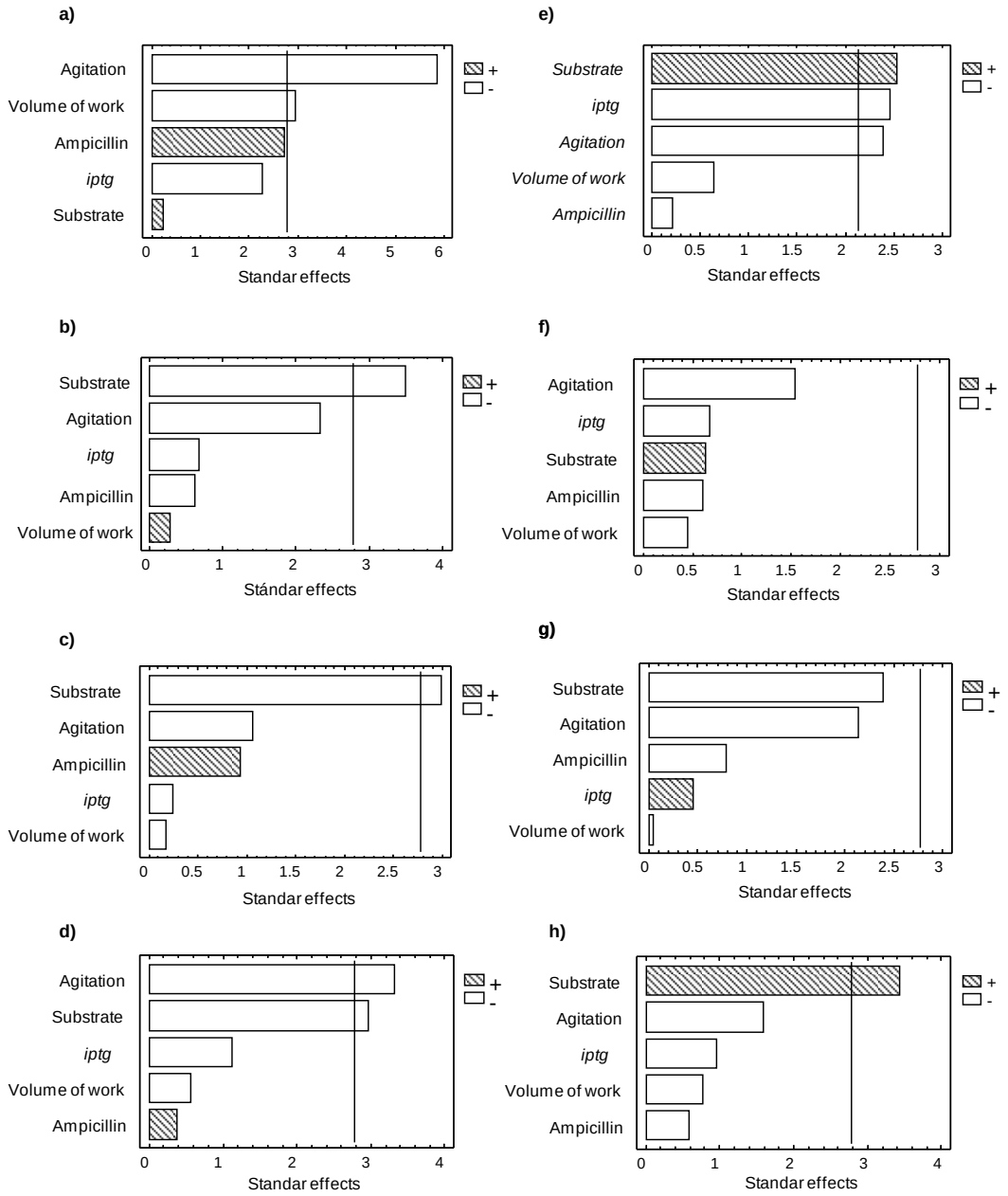
a)
Agitation
Volume of work
Ampicillin
iptg
Substrate
0 1 2 3 4 5 6
Standar effects
+
-
e)
Substrate
iptg
Agitation
Volume of work
Ampicillin
0 0.5 1 1.5 2 2.5 3
Standar effects
+
-
b)
Substrate
Agitation
iptg
Ampicillin
Volume of work
0 1 2 3 4
Stándar effects
+
-
f)
Agitation
iptg
Substrate
Ampicillin
Volume of work
0 0.5 1 1.5 2 2.5 3
Standar effects
+
-
c)
Substrate
Agitation
Ampicillin
iptg
Volume of work
0 0.5 1 1.5 2 2.5 3
Standar effects
+
-
g)
Substrate
Agitation
Ampicillin
iptg
Volume of work
0 0.5 1 1.5 2 2.5 3
Standar effects
+
-
d)
Agitation
Substrate
iptg
Volume of work
Ampicillin
0 1 2 3 4
Standar effects
+
-
h)
Substrate
Agitation
iptg
Volume of work
Ampicillin
0 1 2 3 4
Standar effects
+
-

Figure 1

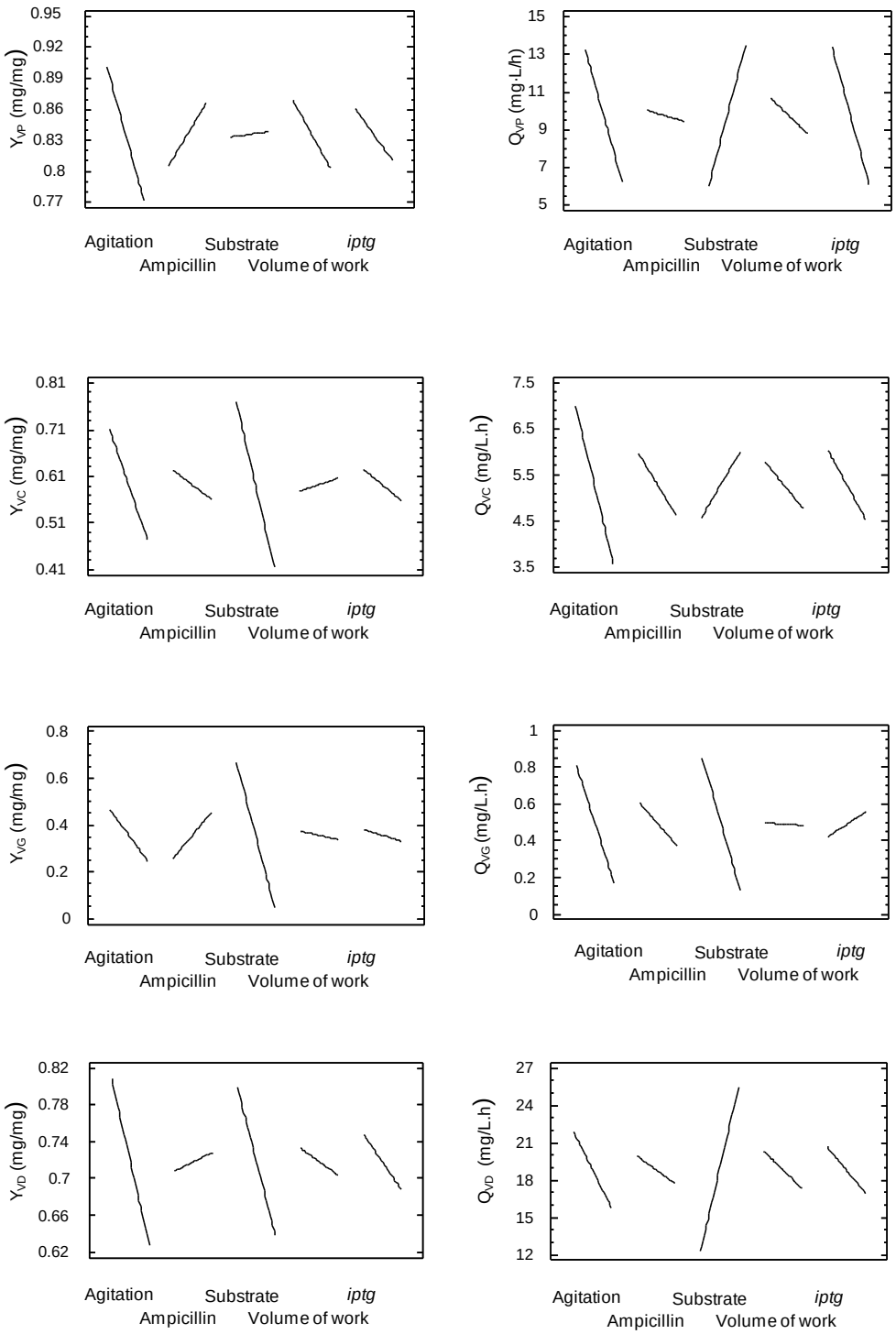

Figure 2

1
2
3
4
5
6
7

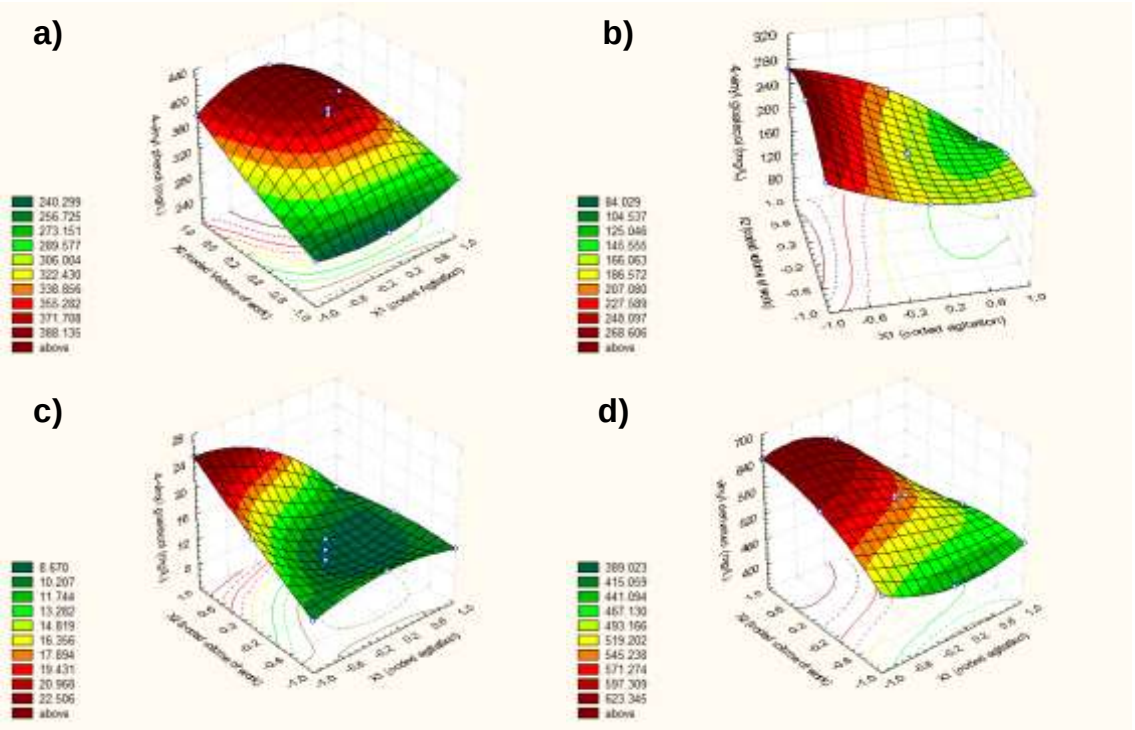
a)
240.239
256.725
273.151
289.577
306.004
322.430
338.856
355.282
371.708
388.135
above
b)
84.029
104.537
125.046
146.555
166.063
186.572
207.080
227.589
248.097
268.606
above
c)
8.670
10.207
11.744
13.282
14.819
16.356
17.894
19.431
20.968
22.506
above
d)
389.023
415.059
441.094
467.130
493.166
519.202
545.238
571.274
597.309
623.345
above

Figure 3

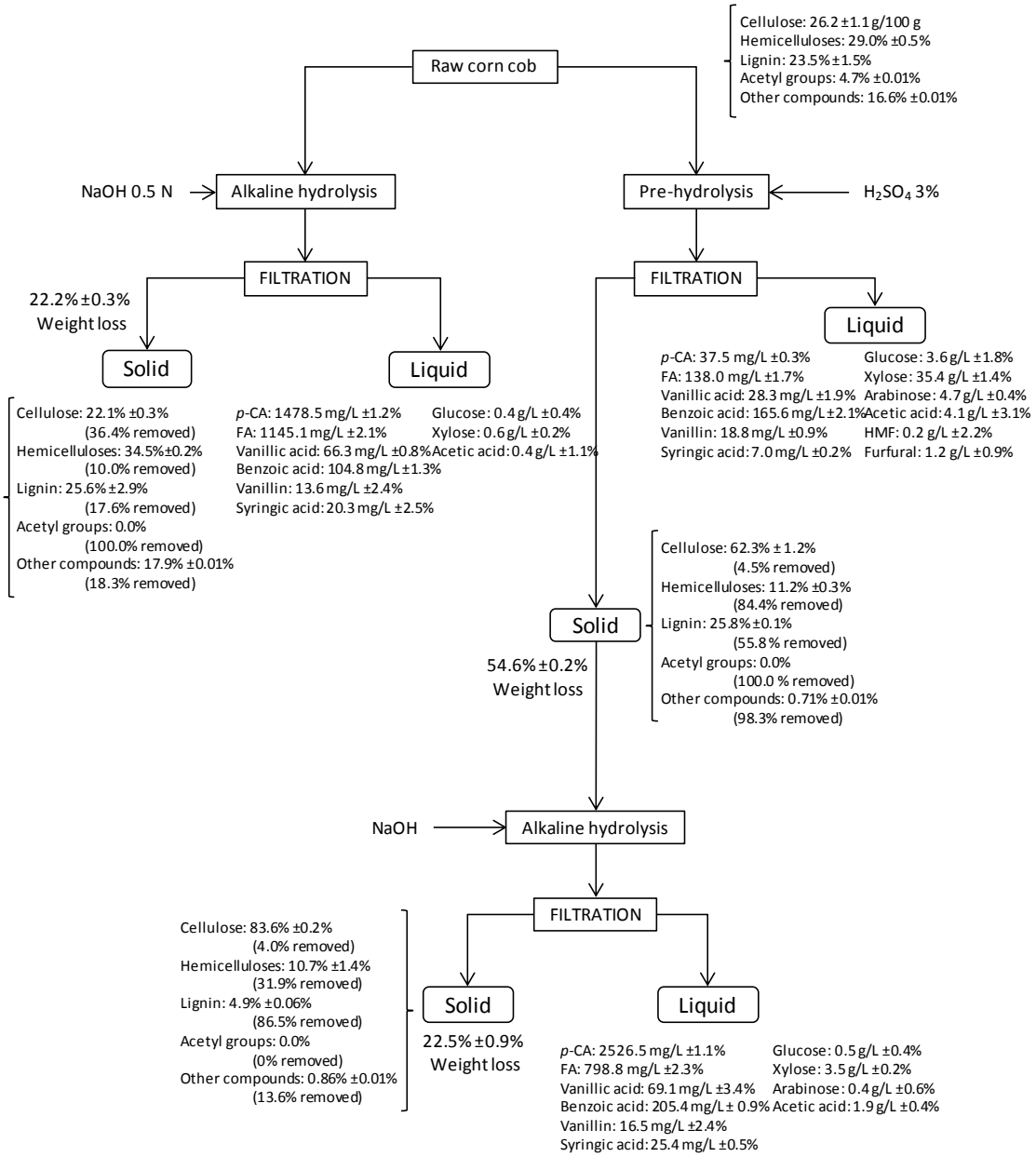
Raw corn cob
Cellulose: 26.2 ±1.1 g/100 g
Hemicelluloses: 29.0% ±0.5%
Lignin: 23.5% ±1.5%
Acetyl groups: 4.7% ±0.01%
Other compounds: 16.6% ±0.01%
NaOH 0.5 N
Alkaline hydrolysis
Pre-hydrolysis
H2SO4 3%
FILTRATION
FILTRATION
22.2% ±0.3%
Weight loss
Solid
Liquid
Liquid
Cellulose: 22.1% ±0.3%
(36.4% removed)
Hemicelluloses: 34.5%±0.2%
(10.0% removed)
Lignin: 25.6% ±2.9%
(17.6% removed)
Acetyl groups: 0.0%
(100.0% removed)
Other compounds: 17.9% ±0.01%
(18.3% removed)
p-CA: 1478.5 mg/L ±1.2%
FA: 1145.1 mg/L ±2.1%
Vanillic acid: 66.3 mg/L ±0.8%
Benzoic acid: 104.8 mg/L ±1.3%
Vanillin: 13.6 mg/L ±2.4%
Syringic acid: 20.3 mg/L ±2.5%
Glucose: 0.4 g/L ±0.4%
Xylose: 0.6 g/L ±0.2%
Acetic acid: 0.4 g/L ±1.1%
p-CA: 37.5 mg/L ±0.3%
FA: 138.0 mg/L ±1.7%
Vanillic acid: 28.3 mg/L ±1.9%
Benzoic acid: 165.6 mg/L ±2.1%
Vanillin: 18.8 mg/L ±0.9%
Syringic acid: 7.0 mg/L ±0.2%
Glucose: 3.6 g/L ±1.8%
Xylose: 35.4 g/L ±1.4%
Arabinose: 4.7 g/L ±0.4%
Acetic acid: 4.1 g/L ±3.1%
HMF: 0.2 g/L ±2.2%
Furfural: 1.2 g/L ±0.9%
Solid
54.6% ±0.2%
Weight loss
Cellulose: 62.3% ±1.2%
(4.5% removed)
Hemicelluloses: 11.2% ±0.3%
(84.4% removed)
Lignin: 25.8% ±0.1%
(55.8% removed)
Acetyl groups: 0.0%
(100.0 % removed)
Other compounds: 0.71% ±0.01%
(98.3% removed)
NaOH
Alkaline hydrolysis
FILTRATION
Cellulose: 83.6% ±0.2%
(4.0% removed)
Hemicelluloses: 10.7% ±1.4%
(31.9% removed)
Lignin: 4.9% ±0.06%
(86.5% removed)
Acetyl groups: 0.0%
(0% removed)
Other compounds: 0.86% ±0.01%
(13.6% removed)
Solid
22.5% ±0.9%
Weight loss
Liquid
p-CA: 2526.5 mg/L ±1.1%
FA: 798.8 mg/L ±2.3%
Vanillic acid: 69.1 mg/L ±3.4%
Benzoic acid: 205.4 mg/L ± 0.9%
Vanillin: 16.5 mg/L ±2.4%
Syringic acid: 25.4 mg/L ±0.5%
Glucose: 0.5 g/L ±0.4%
Xylose: 3.5 g/L ±0.2%
Arabinose: 0.4 g/L ±0.6%
Acetic acid: 1.9 g/L ±0.4%

Figure 4

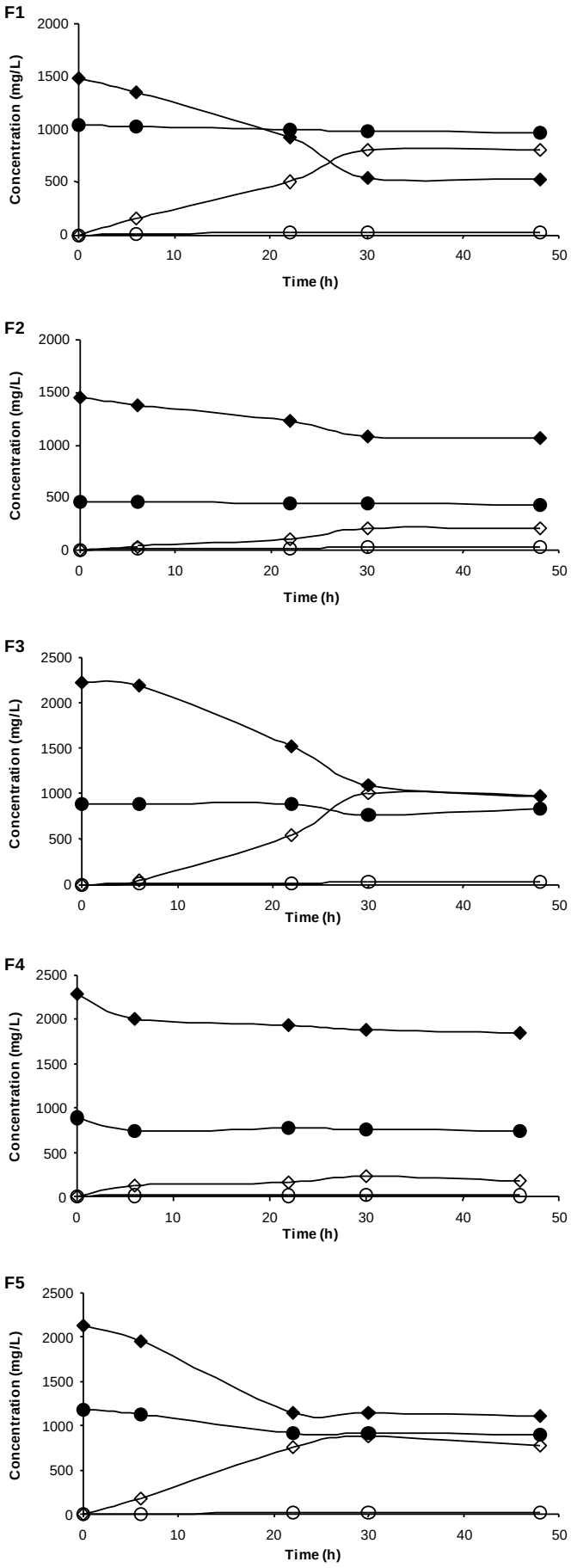
F1
F2
F3
F4
F5
Concentration (mg/L)
Time (h)
0
500
1000
1500
2000
2500
0
10
20
30
40
50

Figure 5

1