# Using High Pressure Processing for Bioconversion of Hazelnut Shells to Generate Fermentable Sugars

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Development of cost-effective process technologies to produce biofuels and enzymes from lignocellulosic materials has gained importance. However, few studies have considered high pressure processing (HPP) as an emerging technology for the bioconversion of lignocellulosic biomass. This study aimed to determine and optimize the effect of HPP combined with dilute acid and enzymatic saccharification on the production of fermentable sugars from hazelnut shells. Optimization *via* response surface methodology was carried out for acid concentration of 1 to 3% (w/v) for a pretreatment time of 10 to 30 min at a pressure range of 200 to 500 MPa. The combined HPP processes were evaluated in terms of the production of total reducing sugars. The optimized total reducing sugar production was estimated at 473.4 mg/g, with production of 88.4% fermentable sugars under 2.9% $H_2SO_4$ for 10 min at 350 MPa. The results implied a 2.4 fold increase in fermentable sugar production under the optimized conditions using combined HPP. The combined HPP process appeared to significantly lower costs due to the decreases in pressure requirement, liquid consumption, and pretreatment time.

*Keywords: Hazelnut shells; Reducing sugar; Response surface methodology; High pressure processing*

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## INTRODUCTION

Lignocellulosic materials are abundant, inexpensive, and renewable in nature. Agricultural residues and energy crops are common raw materials used for the fermentative production of biofuels, chemicals, and enzymes (Mosier *et al*. 2005). Turkey possesses an untapped stream of agricultural by-products, among which hazelnut shells rank first. The Black Sea region of Turkey meets about 70% of the global hazelnut production (Kilic *et al.* 2008; Ozcimen and Ersoy-Mericboyu 2010). The transformation of hazelnut shells into value-added products poses significant economic and environmental benefits (Atland 2015). Hazelnut shells provide a potential feedstock for the production of ethanol, food enzymes, and organic acids, since they have high contents of carbohydrates (25% cellulose and 28% hemicellulose) and 40% lignin. However, their substantial lignin content inhibits cellulolytic enzymes from producing fermentable sugars, thus reducing their enzymatic saccharification efficiency (Mosier *et al*. 2005). Therefore, the bioconversion of lignocellulosic materials necessitates meeting the main challenge of selecting a suitable pretreatment (Demirbas 2006). Among the chemical pretreatments most studied, dilute acid has been most commonly used for the industrial applications (Silverstein *et al*. 2007; Arslan and Saracoglu 2010; Uzuner and Cekmecelioglu 2014; Uzuner *et al*. 2017). However, there remains significant room for improving the production of fermentable sugars from lignocellulosic materials in a cost-effective way that raises enzymatic saccharification efficiency (Ramon *et al*. 2015).

High pressure processing (HPP) is an emerging technology used to inactivate pathogens, and it has not been used as a pretreatment method in the fermentable sugar production. When compared to thermal treatments, HHP is applied uniformly over the treated materials regardless of their size and shape (Valdez-Fragoso *et al*. 2011). Castanon-Rodriguez *et al*. (2013) recovered very high glucose yield (25 g/L) and removed very high lignin (54.1%) at 250 MPa using NaOH after comparing effects of HPP (0, 150, and 250 MPa), temperature (25 and 50 °C), time (5 and 10 min), and solution (water, sulfuric and phosphoric acid and NaOH solution) on lignocellulosic fractions, structural changes and crystallinity of sugarcane bagasse. High lignin content of hazelnut shells makes them highly recalcitrant against decomposition. Structural changes such as some cracks and fragments on the surface varied according to pressure level and the chemical added. To improve the biomass digestibility, high pressure was used in this study. Also, given the ability of HPP to break hydrogen bonds (Oliveira *et al*. 2012), the efficacy of HPP combined with dilute acid as a pretreatment method remains to be quantified, in particular, for a substrate such as hazelnut shells with over 30% lignin.

Therefore, the objectives of this study were to evaluate (1) the effects of HPP-assisted dilute acid (HADA) and HADA combined with enzymatic saccharification (HADA-ES) on fermentable sugar production from hazelnut shells and (2) optimize the two processes using response surface methodology.

## EXPERIMENTAL

### Materials

The samples of hazelnut shells were supplied from a local plant in Ordu, Turkey. The samples were dried in a convection oven at 70 °C for 24 h, milled to pass through a 1-mm sieve and stored in plastic bags at room temperature until their further uses. Hazelnut shells sampled in this study contained 24.2 ± 1.0% cellulose, 28.2 ± 0.1% hemicellulose, 679.2 ± 4.4 mg reducing sugar/g dry weight biomass, and 47.2 ± 0.5% lignin.

### Analyses of Solid/liquid Ratio and Liquid Phase Type

Solid/liquid ratio was defined as the ratio of dry weight of hazelnut shells to volume of sulfuric acid. Three different solid/liquid ratios of 1/20, 1/10, and 1/7 (w/v) were used as the pretreatments of HPP in order to compare the resultant reducing sugar yields. Hazelnut shells were pretreated with water, sodium hydroxide (NaOH), and dilute sulphuric acid ($H_2SO_4$) under the constant pressure (500 MPa), chemical concentration (3%), and time (20 min) by the HADA process.

### High Pressure Processing-assisted Dilute Acid (HADA)

The HADA treatments were carried out using a 2 L model (Avure Systems, Columbus, OH). The operating pressure capacity of the equipment was up to 650 MPa. Distilled water was used in a pressure vessel kept at the ambient temperature during the treatments. The time required to reach process conditions was less than 30 s, and so was the release time.

The pretreatments were carried out in a vacuum-sealed polyethylene bag containing 5 g of hazelnut shells in 100 mL of dilute acid solution. Based on preliminary experiments, the ranges in the pretreatment were selected for the variables of sulphuric acid ($H_2SO_4$)

concentration, pressure, and pretreatment time. The milled hazelnut shells were pretreated with 100 mL $H_2SO_4$ solution at concentrations of 1, 2, and 3% (v/v), time (10, 20 and 30 min) and pressure (MPa) of 200, 350, and 500 according to the experimental conditions shown in Table 1. Experiments were carried out in triplicate. Control sample was raw grounded hazelnut shells which was performed without application of HADA and HADA-ES. Carbohydrate content (referred to as reducing sugar yield) of the pretreated samples was also measured as total reducing sugars.

## HADA Combined with Enzymatic Saccharification (HADA-ES)

The HADA combined with enzymatic saccharification of hazelnut shells was performed at 1:20 solid loading (dry basis) in 10 mL volume made up by 50 mM sodium acetate buffer (pH 5.2), and Viscozyme L enzyme cocktail (Sigma-Aldrich, Denmark) containing cellulose and xylanase enzymes. Enzyme activities of cellulase and xylanase were measured as 2250 and 1400 U/mL, respectively at 50 °C and pH 5 for 30 min. The samples were hydrolyzed in 50 mL centrifuge tubes in a shaking water bath (GFL 1086, Germany) at 50 °C and 120 rpm for 24 h. The control group of the samples with the equivalent enzyme loading was also hydrolyzed. After enzymatic saccharification, the samples were centrifuged at 10,000 x *g* for 10 min, and the supernatant was used to estimate fermentable sugar yield by measuring reducing sugar content.

## Analytical Methods

Total-reducing sugars in the control group, and the HADA and HADA-ES treatments were estimated using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). Filtrate from the two-step acid hydrolysis described above was used to determine reducing sugars in the pretreated solids.

$$\text{Reducing sugar (mg/g)} = \frac{reducing\ sugar\ concentration\ \left(\frac{\text{g}}{\text{L}}\right) V(\text{L}) \times DF}{dry\ weight\ of\ pretreated\ biomass\ (\text{g})} \quad (1)$$

where DF is the dilution factor and *V* is the volume of acid to be added (0.087 L). Reducing sugar yield (%, w/w) was also calculated (Eq. 2),

$$Yield = \frac{a}{b} \times 100 \quad (2)$$

where *a* is the amount of sugar concentration in pretreated biomass (mg/g) and *b* is the amount of cellulose and hemicellulose in untreated biomass (mg/g).

Total furans including furfural and 5-hydroxymethyl furfural (HMF) were determined applying the method developed by Xie *et al*. (2011). Furfural and HMF were determined using a HPLC system (Prostar, Varian, CA, USA) equipped with a RI detector. The column was Metacarb 87H column (300 mm × 7.8 mm, Varian, S/N:05517112, Varian), and the operation conditions were set as 35 °C with 0.08 N $H_2SO_4$ as the eluent at a flowing rate of 0.5 mL/min.

## Data Analyses

Statistical analyses were conducted to detect the significant mean differences among the control group, and the HADA and HADA-ES treatments in terms of reducing sugar and to model reducing sugar as a function of dilute acid concentration, pressure, and pretreatment time. Optimization was carried out using the Box-Behnken Design (BBD) as

the response surface methodology with a quadratic model. The BBD matrix was constructed using the three predictors with three levels each thus: dilute acid concentration ($X_1$; 1.0, 2.0, and 3.0%, v/v), pressure ($X_2$; 200, 350, and 500 MPa), and pretreatment time ($X_3$; 10, 20, and 30 min) (Table 1). The levels of these variables were determined using preliminary experiments. The uncoded and coded predictors and the overall BBD are given in Table 1. Reducing sugar was reported by averaging three replicates of each run (total 15 runs). All the statistical analyses were performed using MINITAB 17.0 (Minitab Inc. State College, PA, USA)

The following quadratic equation was used to fit the experimentally collected data,

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (3)$$

where $Y_1$ is the response variable of reducing sugar; the $b$ values are regression slope coefficients; and $X_1$, $X_2$ and $X_3$ are dilute acid concentration, pressure and pretreatment time, respectively. The HADA and HADA-ES processes were optimized using the response optimizer function under DOE-RSM. To validate the models, additional experimental runs under the optimal conditions of fermentable sugar production were carried out in triplicate. Analysis of variance (ANOVA) and regression models were performed at 95% confidence interval ($p$<0.05) to define the significant terms of the predictive model.

Analysis of variance was performed to determine statistically significant effects of the three predictors ($p$<0.05). The multiple comparisons were made using Tukey's test. The coefficient of variation (CV) value was computed to finally verify the predicted model as follows,

$$CV = \frac{s}{\bar{C}} * 100 \quad (4)$$

where σ is sample standard deviation, and $\bar{C}$ is sample mean.

## RESULTS AND DISCUSSION

### Composition of Raw and Pretreated Hazelnut Shells

Raw hazelnut shells in this study contained 9.03±0.46 g/L xylose and 0.22±0.02 g/L arabinose. Glucose was not detected. Pretreated hazelnut shells contained 0.65 ± 0.001 g/L glucose, 0.86 ± 0.01 g/L xylose and 0.05 ± 0.001 g/L arabinose.

### Effects of Solid/liquid Ratio and Liquid Phase Type on Reducing Sugar Yield

To determine the effective pretreatment conditions, the samples were pretreated using the solid/liquid ratios of 1:7 to 1:20 and the liquid phase types of NaOH and dilute $H_2SO_4$. Total reducing sugar yield was estimated at 58, 60 and 61% at the solid/liquid ratios of 1:20, 1:10, and 1:7, respectively (Fig. 1). The solid/liquid ratios did not significantly affect the response of reducing sugar yield ($p$ > 0.05). Therefore, the ratio of 1/20 was chosen as the best ratio for the fermentable sugar production.

As for the selection of the liquid phase type, the highest reducing sugar yield was found as 58.1 ± 1.0% using the dilute acid, whereas the lowest reducing sugar yield was 29.1 ± 0.7% using water (Fig. 2). The dilute acid was more effective for the sugar conversion than were NaOH and water. Thus, the entire pretreatment experiments were performed using the dilute acid at the solid/liquid ratio of 1:20.

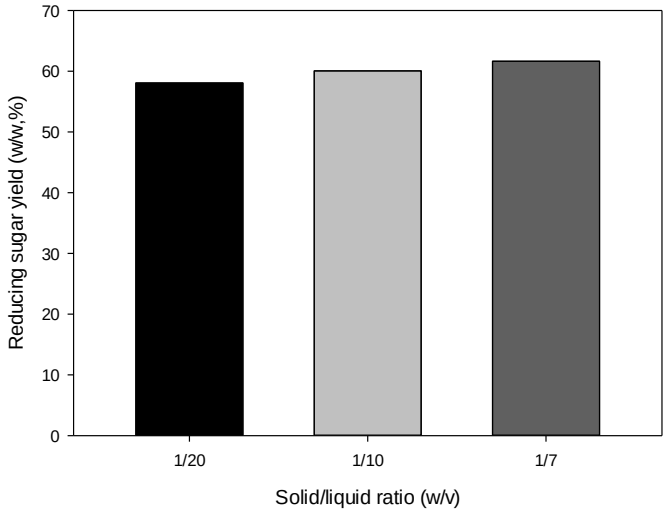


**Fig. 1.** Reducing sugar yield with respect to solid/liquid ratio (3% acid, 500 MPa, & 20 min) (*n* =3)

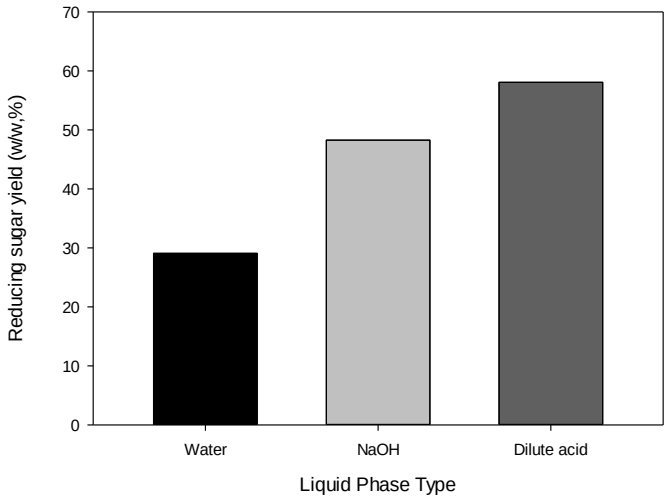


**Fig. 2.** Reducing sugar yield with respect to liquid phase type (3% solvent concentration, 500 MPa, and 20 min at the solid/liquid ratios of 1:20) (*n* =3)

The pretreatments were performed using the solid/liquid ratios of 1:7, 1:10 and 1:20 under the acid concentration of 3% (v/v) and the pressure of 500 MPa for 20 min in order to obtain hydrolysates used for the compositional (glucose, xylose, and arabinose) and inhibitor (HMF and furfural) analyses (Fig. 3). The descending order of the sugar composition of the samples was Xylose>Glucose>Arabinose (Fig. 3). Given the solid/liquid ratio of 1:20, the glucose, xylose, arabinose, HMF and furfural levels were measured as 0.65 ± 0.001 g/L, 0.86 ± 0.01 g/L, 0.05 ± 0.001 g/L, 0.003 ± 0.001 g/L, and 0.14 ± 0.01 g/L, respectively. Mouta *et al*. (2011) found acetic acid, furfural and HMF as 3.19, 0.56, and 0.15 g/L, respectively, in response to the hydrolysis of sugarcane leaf straw using 2.9% $H_2SO_4$ (w/v) at 130 °C for 30 min with the ¼ solid to liquid ratio. The amounts of furfural and HMF found as the inhibitory compounds in this study were lower than those by the previous studies about rice straw (Baek and Kwon 2007) and sugarcane leaf straw (Mouta *et al*. 2011).

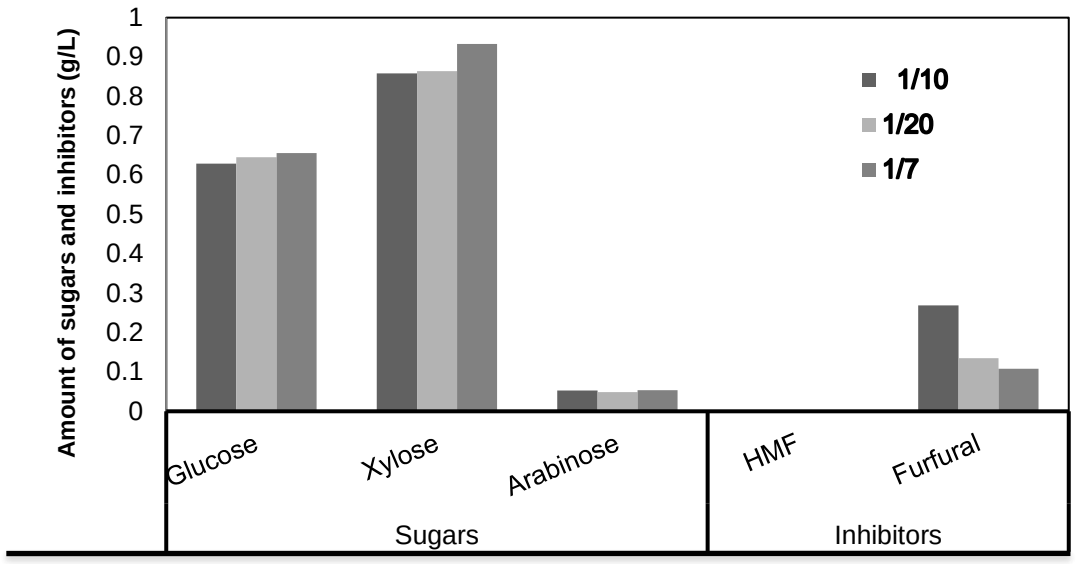


**Fig. 3.** Composition of sugars and inhibitors (g/L) using HPLC at 3% acid, 500 MPa, and 20 min ($n$ = 3)

## Effect of HADA-ES on Reducing Sugar

Total reducing sugar content of the control group was 679.2 ± 4.4 mg/g dry weight biomass, which varied with $H_2SO_4$ concentration, pressure, and pretreatment time. The pretreatment time and the acid concentration-by-pretreatment time interaction had significant effects ($p < 0.05$). Total reducing sugar content significantly increased with the decreased pretreatment time ($p < 0.05$) (Table 1). The highest amount of fermentable sugar after the HADA process was 445.4 mg/g pretreated biomass under 2% $H_2SO_4$ and 500 MPa for 10 min (Table 1).

Enzymatic saccharification of the HADA process was also performed to generate fermentable sugars (Table 1). The total reducing sugars in the hydrolyzate were measured and used to evaluate the overall pretreatment efficacy. Enzymatic saccharification of the control group resulted in 120 ± 10 mg reducing sugar/g dry weight biomass, while the total reducing sugar yield from the HADA samples ranged from 321.2 to 462.5 mg/g. The highest reducing sugar after the HADA-ES process was 462.5 mg/g pretreated biomass under 350 MPa, 3% $H_2SO_4$, and 10 min, with the corresponding enzymatic conversion efficiency of 88.3% (Table 1). This was significantly higher than sugar produced by the control group (120±10 mg reducing sugar/g dry biomass). With the increased pretreatment time during HPP, the amount of fermentable sugars decreased (Table 1, runs 4 & 6). Therefore, the pretreatment time was found significantly influential on the HADA-ES process (Table 2) ($p<0.05$). Also, with the increased acid concentration, fermentable sugars after the HADA-ES process decreased (Table 1, runs 7 & 12).

The theoretical total reducing sugar conversion from the HADA samples ranged from 61.6 to 88.3%. Although the overall total conversion increased slightly with the $H_2SO_4$ concentrations (runs 7 and 12), it decreased with the increased pretreatment time (runs 4 and 6). No significant difference in enzymatic efficiency was observed at the pressure (runs 2 and 10) ($p>0.05$). The HADA process with 3% $H_2SO_4$ concentration provided higher total conversion efficiency than those with 1% and 2% $H_2SO_4$ concentrations ($p<0.05$) (Table 1).

**Table 1.** Tukey's Multiple Comparison of Reducing Sugars from the HADA and HADA-ES Processing

| Levels/run order | $H_2SO_4$ concentration (%, v/v) $X_1$ | Pressure (MPa) $X_2$ | Pretreatment time (min) $X_3$ | HADA (mg/g pretreated biomass) | HADA-ES (mg/g pretreated biomass) | Reducing sugar yield (%) |
|---|---|---|---|---|---|---|
| Low (-1) | 1 | 200 | 10 | | | |
| Middle (0) | 2 | 350 | 20 | | | |
| High (+1) | 3 | 500 | 30 | | | |
| 1 | 2 | 350 | 20 | 425.2±11.3$^{ab}$ | 437.0±4.2$^{a}$ | 84.2±1.1$^{b}$ |
| 2 | 1 | 500 | 20 | 408.5±9.7$^{ab}$ | 428.7±6.6$^{ab}$ | 82.5±1.1$^{bcd}$ |
| 3 | 2 | 200 | 10 | 418.0±5.7$^{a}$ | 435.5±3.6$^{a}$ | 83.5±0.7$^{bc}$ |
| 4 | 2 | 500 | 10 | 445.4±0.7$^{a}$ | 461.0±5.0$^{a}$ | 88.6±0.9$^{a}$ |
| 5 | 2 | 200 | 30 | 320.6±1.0$^{cd}$ | 335.3±1.6$^{c}$ | 64.3±0.5$^{gh}$ |
| 6 | 2 | 500 | 30 | 323.2±0.7$^{cd}$ | 338.9±0.9$^{c}$ | 64.9±0.5$^{g}$ |
| 7 | 3 | 350 | 10 | 437.8±12.4$^{a}$ | 462.5±6.3$^{a}$ | 88.3±0.2$^{a}$ |
| 8 | 3 | 350 | 30 | 352.0±14.0$^{bcd}$ | 367.0±14.1$^{bc}$ | 69.4±0.9$^{f}$ |
| 9 | 3 | 500 | 20 | 394.4±6.5$^{abc}$ | 407.9±6.0$^{ab}$ | 77.6±0.1$^{e}$ |
| 10 | 1 | 200 | 20 | 418.3±10.8$^{ab}$ | 425.2±7.1$^{ab}$ | 81.0±0.2$^{cd}$ |
| 11 | 1 | 350 | 30 | 408.5±0.7$^{ab}$ | 420.4±0.6$^{ab}$ | 80.5±0.4$^{d}$ |
| 12 | 1 | 350 | 10 | 309.5±1.5$^{d}$ | 321.2±1.8$^{c}$ | 61.6±0.4$^{h}$ |
| 13 | 3 | 200 | 20 | 413.6±48.8$^{ab}$ | 429.2±6.0$^{ab}$ | 82.6±1.0$^{bcd}$ |

* Different letters in the same column show statistically significance difference between mean values ($p < 0.05$).

## Optimization of HADA and HADA-ES for Fermentable Sugar Production

The efficacy of the HADA process was based on the structural and chemical compositions of the biomass feedstock. The selection of the most suitable pretreatment conditions was focused on the following aspects: high economic feasibility, low inhibitors, and high total reducing sugar after hydrolysis. Response surface methodology is a frequently used technique for modeling and determining optimal process conditions. The BBD experiments were thus conducted to determine reducing sugar under the changing $H_2SO_4$ concentration, pressure and pretreatment time (Table 2) in order to be able to identify the best pretreatment conditions in the ranges tested. A second-order polynomial equation was developed to identify the relationship reducing sugar ($Y_1$) as a function of $H_2SO_4$ concentration ($X_1$), pressure ($X_2$) and pretreatment time ($X_3$). Since the original reducing sugar values were not normally distributed, Johnson transformation was applied to the original data in order to ensure the normality assumption. According to the revised ANOVA results, insignificant terms were excluded, and the equations (Eq. 5) were re-written thus:

$$Y_1 = 0.623 - 0.973\ X_3 - 0.759\ X_1^2 - 0.650\ X_3^2 - 1.311\ X_1 * X_3 \quad (5)$$

Equation 5 was found to well represent the data with an adjusted $R^2$ value of 0.89 and $R^2_{pred}$ of 0.84. The insignificant lack-of-fit values for total reducing sugar ($p>0.05$) also proved that the model fit the experimental data well. The validation results for fermentable sugar production at the optimal conditions indicated that the experimental reducing sugar

yield of 443.1 mg/g pretreated biomass was slightly lower than the BBD-derived prediction of 473.4 mg/g pretreated biomass. Also, a low value of coefficient of variation (4.7%) satisfies the adequacy of the model.

Only the processing time showed significant effects ($p$<0.05) on total reducing sugars from the HADA-ES process. The time-by-acid concentration interaction showed significant effects ($p$<0.05) (Table 2).

**Table 2.** Results of BBD-derived Regression Model for HADA-ES Processing ($R^2_{adj}$ = 0.863; and $R^2_{pred}$ = 0.837)

| Term | Coefficient | *P*-value |
|---|---|---|
| Regression | | 0.001 |
| Linear | | 0.001 |
| Square | | 0.001 |
| Interaction | | 0.001 |
| Lack-of-fit | | 0.728 |
| Constant | 0.623 | 0.001 |
| Time (min) | -0.973 | 0.001 |
| (Acid concentration)$^2$ | -0.759 | 0.001 |
| (Time)$^2$ | -0.650 | 0.001 |
| Acid concentration*Time | -1.311 | 0.001 |

The experimental and predicted values for total reducing sugar were well correlated ($r$ = 0.99) (data not shown). The best solution obtained for the response optimization is represented in Fig. 4. Therefore, the optimum HADA-ES conditions for total reducing sugar (473.4 mg sugar/g biomass) belonged to the 2.9% $H_2SO_4$ concentration and 10 min pretreatment time (Fig. 4). A 2.4 fold increase in fermentable sugar production using combined HPP was achieved by RSM optimization compared to the control sample. In this study, the HADA and HADA(-ES) process provided high fermentable sugar yield with lower pressure requirement, liquid consumption and pretreatment time.

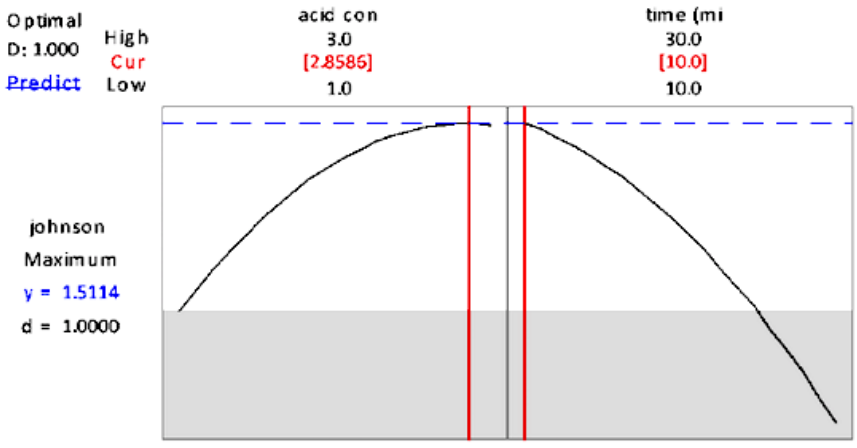


**Fig. 4**. Best global solution for response optimization

The lowest sugar conversion was obtained with the alkaline pretreatment (Table 3). Tutt *et al*. (2012) reported that alkaline pretreatment method lowered hydrolysis efficiency relative to the dilute acid pretreatment methods. Castanon-Rodriguez *et al*. (2015) reported that HPP-assisted alkaline treatment of sugarcane bagasse resulted in overall saccharification yield of about 48.9%. Though the treatment conditions were somewhat different, and lignin content of sugarcane bagasse was less than that of hazelnut shells, the HADA-ES in this study resulted in a higher overall saccharification yield (88.4%) than did HPP-assisted alkaline treatment of sugarcane bagasse by Castanon-Rodriguez *et al*. (2015) (Table 3). The HADA-ES processing of hazelnut shells was demonstrated to able to enhance fermentable sugar production.

**Table 3.** Saccharification Yields for Various Agricultural By-products Using Different Pretreatment Methods and Hydrolysis in Related Literature

| Pretreatment method | Pretreatment conditions | Hydrolysis conditions | Raw material | Overall fermentable sugar yield (%) | Reference |
|---|---|---|---|---|---|
| Dilute acid | 130ºC, 3.42 % $H_2SO_4$, 31.7 min | Viscozyme L, 50 ºC, 120 rpm, 24 h | Hazelnut shell | 72.4 | Uzuner and Cekmecelioglu 2014 |
| HHP-assisted alkaline | 450 MPa, 6% NaOH, 50 ºC for 15 min | Cellulase and cellobiase, 50 ºC, 200 rpm, 72 h | Sugarcane bagasse | 48.9 | Castanon-Rodriguez *et al.* 2015 |
| Alkaline | 121ºC, 3% NaOH, 15 % solid/liquid ratio, 60 min | Ctec2 and Htec2,50 ºC, 150 rpm, 72 h | Hazelnut shell | 61.2 | Uzuner *et al.* 2017 |
| HHP-assisted acid | 350 MPa, 3% $H_2SO_4$, 10 min | Viscozyme L, 50 ºC, 120 rpm, 24 h | Hazelnut shell | 88.4 | This study |

## CONCLUSIONS

1. The HADA and HADA-ES processing of hazelnut shells with high lignin content above 30% was compared and optimized.
2. The optimum conditions were found as 2.9% (w/v) acid concentration and 10 min at 350 MPa which in turn revealed 88.4% (w/w) fermentable sugar yield.
3. A 2.4 fold increase in fermentable sugar production using combined HPP was achieved by RSM optimization compared to the control sample.
4. Given the previous results in related literature, the HADA(-ES) processing appeared to be a successful method when compared to the conventional pretreatment methods.

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