0273-2289/02/98-100/0717/$14.00

Ethanol Production from Olive Oil Extraction Residue Pretreated with Hot Water

Ignacio Ballesteros, José Miguel Oliva, Maria José Negro, Paloma Manzanares, and Mercedes Ballesteros*

Centro De Investigaciones Energéticas Medioambientales Y Technológicas, (CIEMAT), Renewable Energies Department, Av. Complutense, No. 22, 28040-Madrid, Spain, E-mail: m.ballesteros@ciemat.es

Abstract

The olive pulp fraction contained in the residue generated in olive oil extraction by a two-step centrifugation process can be upgraded by using the cellulose fraction to produce ethanol and recovering high value phenols (tyrosol and hydroxytyrosol). Olive pulp was pretreated in a laboratory scale stirred autoclave at different temperatures (150–250°C). Pretreatment was evaluated regarding cellulose recovery, enzymatic hydrolysis effectiveness, ethanol production by a simultaneous saccharification and fermentation process (SSF), and phenols recovery in the filtrate. The pretreatment of olive pulp using water at temperatures between 200°C and 250°C enhanced enzymatic hydrolysis. Maximum ethanol production (11.9 g/L) was obtained after pretreating pulp at 210°C in a SSF fed-batch procedure. Maximum hydroxytyrosol recovery was obtained in the liquid fraction when pretreated at 230°C.

Index Entries: Olive oil extraction by-products; pretreatment; simultaneous saccharification and fermentation; ethanol; hydroxytyrosol.

Introduction

The recent implementation of a new two-step centrifugation process (without external addition of water) for extracting olive oil has substantially reduced the water consumption, and thereby eliminates the olive mill wastewater *(1)*. However, a high-sugar-content residue, called olive pomace, which is a combination of solid and liquid olive wastes, is still generated. This highly polluting waste contains the pulp, the water content of the olive, and portions of seed husks and olive stones, and it is a handicap to the development and growth of this industry. So, the environmental impact of uncontrolled dumping of olive pomace should be reduced.

*Author to whom all correspondence and reprint requests should be addressed.

Cellulose represents about 20–30% of olive pomace, and it could be an attractive low-cost feed material for biological conversion. Although the feasibility to obtain ethanol from free fermentable sugars and cellulose contained in this residue has been demonstrated in a previous work *(2)*, fermentation inhibition was observed, likely due to the presence of phenolic substances in pulp and seeds which are toxic to microorganisms *(3,4)*.

Currently, the industries that use the two-phase centrifugation process separate the residue into two fractions: the pulp, formed from the olive pulp itself and the vegetation liquors engaged in nutritive and growth function, and the wood-like portion, comprising fragments of the olive stones. The pulp contains the majority of phenolic compounds present in the fruit that are much more soluble in water than in oil. Among these polyphenolic compounds, mainly in the form of glucosides and esters, tyrosol and oleuropein (and its natural derivative hydroxytyrosol) are present in a considerable amount. Hydroxytyrosol originates from the hydrolysis of oleuropein by means of an esterase during the mill process *(5)*, and it is characterized by strong antimicrobial properties *(6)* and a high dietary antioxidant activity *(7)*. Currently there is growing evidence that phenolic compounds present in olive pulp, and especially hydroxytyrosol, are potent inhibitors of free radical generation and are involved on the chemo-prevention of cancer *(8)*. Thus, the removal of these compounds with a high value in the food industry for their antioxidant effect, and with a remarkable inhibitory effect on microbial activity, would be desirable before the cellulosic fraction of olive pulp can be used as substrate for ethanol fermentation.

Steam explosion pretreatment of olive stones has been reported *(9)* as a promising method to increase cellulase accessibility, as well as to recover hydroxytyrosol in the water-soluble fraction after steam pretreatment. However, based on previous results *(2)*, steam explosion is not a suitable pretreatment for olive pulp, since it resulted in low recovery of dry matter, high cellulose solubilization, and did not increase enzymatic hydrolysis. Thus, new efficient pretreatment approaches should be tested to separate tyrosol and hydroxytyrosol from olive pulp without large cellulose solubilization, in order to extend the merit of olive oil extraction residue as feedstock for the food industry and ethanol production.

Because of the high water solubility of typical phenols of the olive, hot water was chosen as pretreatment for this study *(10)*. Olive pulp was pretreated with water at 150–250°C temperature range. Pretreatment was evaluated in terms of (a) water-soluble carbohydrates, noncarbohydrate compounds (furfural and hydroxymethylfurfural), and simple phenolic compounds characteristics of olive fruit (tyrosol and hydroxytyrosol) recovered in the filtrate; (b) cellulose recovery in water-insoluble fiber; and (c) enzymatic hydrolysis effectiveness. Finally, the simultaneous saccharification and fermentation (SSF) bioconversion process to obtain ethanol from pretreated olive pulp was tested.

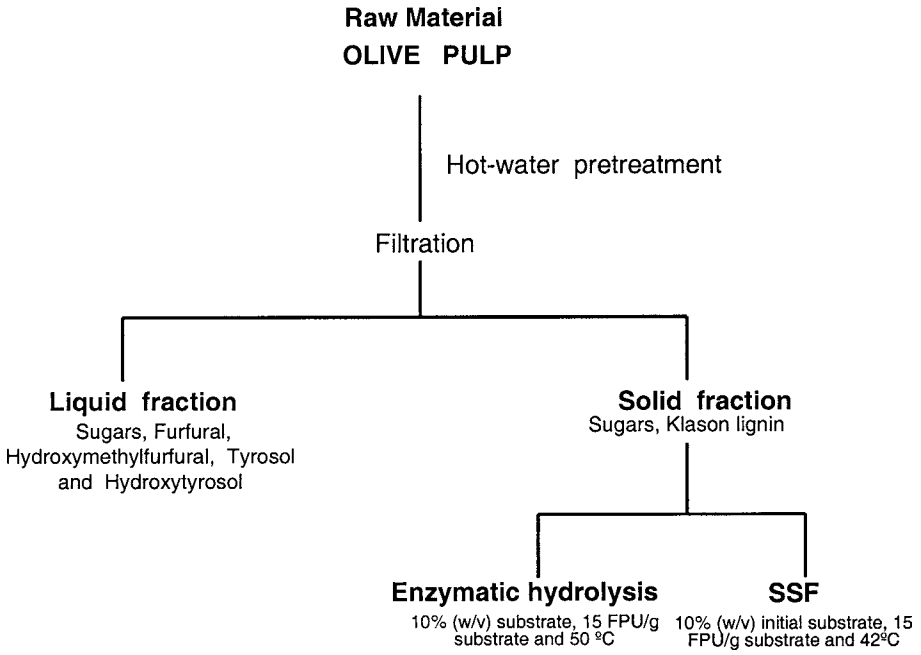

Fig. 1. Scheme of the different steps followed in pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation experiments.

Materials and Methods

Substrates

Olive pulp, generated as a residue from the two-phase centrifugation olive oil production process, was supplied by Oleicoa El Tejar S.C.L. (Córdoba, Spain).

A scheme of the different steps followed in pretreatment, enzymatic hydrolysis, and simultaneous saccharification and fermentation experiments is shown in Fig. 1.

Analytical Procedures

Chemical analysis of the olive pulp was performed according to the following standard methods: ASTM D-1348 *(11)* for moisture content; ASTM D-1102-84 *(12)* for ash content; ASTM D-1111-84 *(13)* for hot water extracts, and ASTM D-1107-87 *(14)* for ethanol/toluene extracts.

Composition of feedstock and water-insoluble fraction after pretreatment was determined by total hydrolysis with H_2SO_4 *(15)*. The sample was first hydrolyzed using 72% (w/w) H_2SO_4 for 60 min and then hydrolyzed a second time with 4% H_2SO_4 for 60 min at 120°C. Sugars were quantified by high-performance liquid chromatography (HPLC) in a 1081B Hewlett Packard (HP) apparatus with refractive index (RI) detector under

the following conditions: column, Aminex HPX-87P (300 mm x 7.6 mm) (BioRad, Hercules, CA); temperature, 85°C; eluent, water at 0.6 mL/min. This analysis gave the hemicellulosic sugars content (expressed as the sum of the xylose + arabinose + galactose + mannose), cellulose (expressed as glucose), and Klason lignin.

The raw material composition was 14.1% glucose, 7.5% hemicellulosic sugars, 28.4% acid insoluble lignin, 2.2% ash, and 47.8 extractives.

Enzymatic activities (filter paper and β-glucosidase) were measured according to the methods described by Ghose *(16)*.

Ethanol was measured by gas chromatography (GC), using a HP 5890 Series II apparatus, with a flame ionization detector and a column of Carbowax 20 M (2 m × 1/8 in.) using helium as carrier gas at 35 mL/min. The GC oven temperature was held at 85°C. The injector and detector temperatures were maintained at 150°C.

Hydroxytyrosol, tyrosol, furfural and hydroxymethylfurfural content were analyzed by HPLC (1100 Hewlett-Packard), equipped with a 1040A diode-array detector. Chromatograph separation was achieved with a Bio-Rad Aminex HPX-87H stainless steel (300 mm × 7.6 mm) column. The gradient elution was 82% H_2SO_4 (5 m*M*) and 18% acetonitrile. Column temperature was 55ºC and the flow rate was 0.3 mL/min. Phenolic compounds were identified by their retention time and absorption spectra in the 200–320 nm range. Tyrosol was purchased from Sigma (St. Louis MO), and oleuropein was provided by Extrasynthese (Genay, France). Hydroxytyrosol was obtained from oleuropein by acid hydrolysis *(17)*.

Pretreatment Reactor and Procedure

Washed feedstocks were pretreated at selected temperatures in a laboratory scale-stirred autoclave (Model EZE-Seal, Autoclave Engineers, Erie, PA). The laboratory-scale autoclave and the typical temperature profiles from room temperature to the final set point temperature are shown in Fig. 2. The stainless-steel Hastelloy-C reactor has a total volume of 500 mL, with an electric heater and magnetic agitation. The temperature/speed controller is a combination of furnace power control (± 1% of range) and motor speed control with tachometer. Cooling water was circulated through a serpentine coil to cool the reactor content at the end of each run.

Olive pulp was pretreated at different temperatures. The amount of dry pulp loaded corresponded to 40 g, and water was added at a 1/10 (w/w) solid/liquid ratio. The working volume of the pretreatment vessel was 400 mL. The pretreatment agitator was set at 600 rpm, and the reactor contents were initially at room temperature. The heater temperature was set at 150, 180, 210, 220, 230, 240, or 250°C. The heating rate was between 2.5 and 3 ºC/min. Pretreatment time (4 min) was initiated when the selected pretreatment temperature was reached. After treatment, the heater was turned off, the reactor was removed from the heating jacket, and cooling

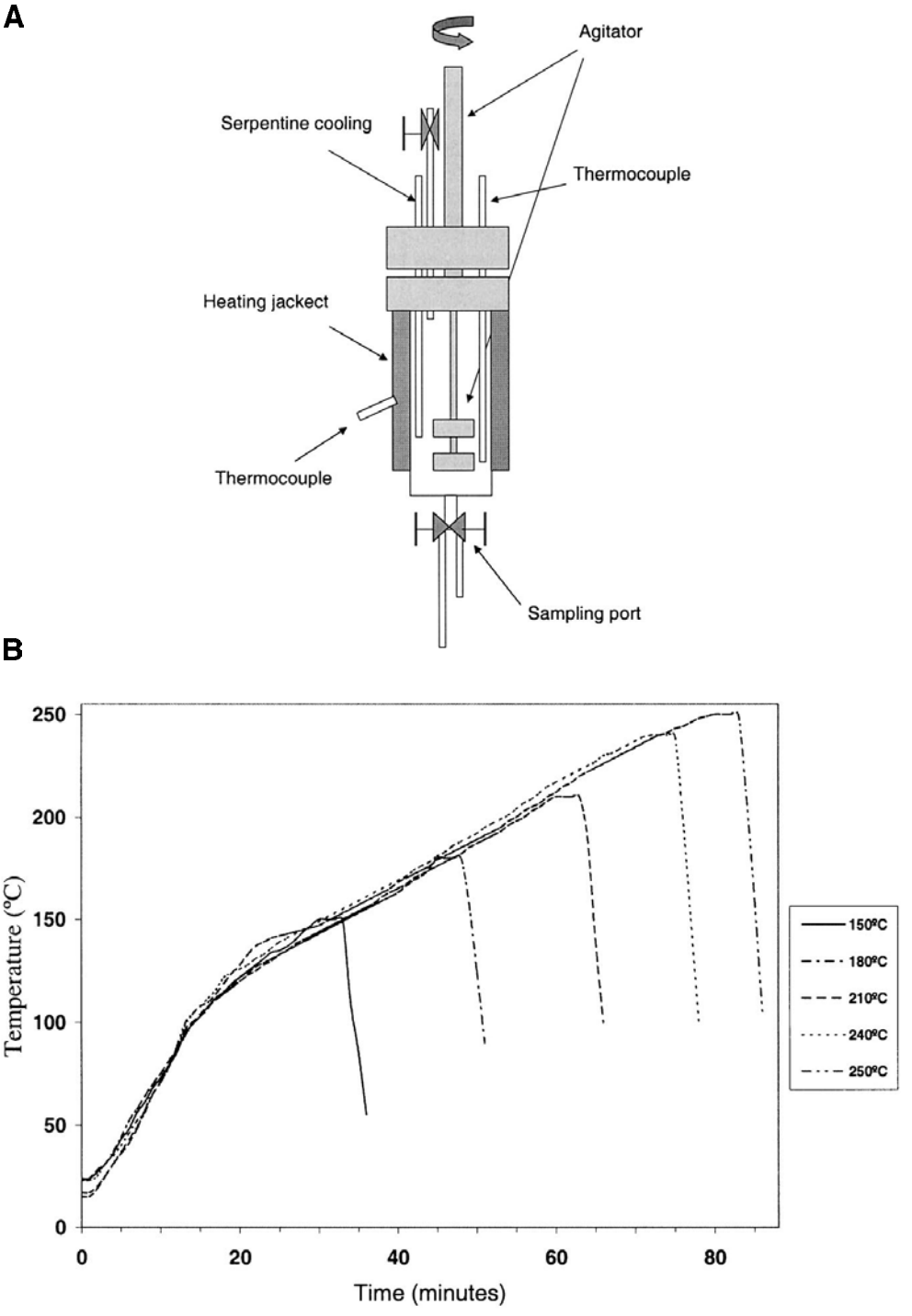

Fig. 2. Laboratory-scale stirred autoclave **(A)** and heating up profile of the olive pulp and water mixture from room temperature to the final set-point temperature **(B)**.

water was charged through the serpentine coil. The content of the reactor cooled down to 130°C in approx 2 min. The reactor was kept sealed, and the slurry agitated until the reactor was cooled to about 40°C, and sampled.

The wet material was filtered for solid recovery. The water-insoluble fraction was analyzed for hemicellulosic sugars, glucose, and acid-insoluble lignin content, and used as substrate in enzymatic hydrolysis and simultaneous saccharification and fermentation tests. The sugars, furfural, hydroxymethylfurfural, and hydroxytyrosol content of the filtrate were also analyzed as described above.

Microorganisms and Growth Conditions

Kluyveromyces marxianus CECT 10875, a thermotolerant mutant yeast strain obtained in our laboratory *(18)*, was used in SSF experiments. Active cultures for inoculation were prepared by growing the organism on a rotary shaker at 150 rpm for 16 h at 42°C in a growth medium containing: 5 g/L of yeast extract (Difco), 5 g/L of peptone (Oxoid), 2 g/L of NH_4Cl, 1 g/L of KH_2PO_4, 0.3 g/L of $MgSO_4 \cdot 7H_2O$, and 30 g/L of glucose.

Enzymatic Hydrolysis and Simultaneous Saccharification and Fermentation (SSF) Tests

The washed feedstocks were enzymatically hydrolyzed to determine sugar yield. Enzymatic hydrolysis was performed in 0.1 *M* sodium acetate buffer (pH 4.8), at 10% (w/v) substrate concentration and 15 FPU/g substrate enzyme loading, at 50°C for 72 h. The enzyme preparation Celluclast 1.5L [50 filter paper units (FPU)/mL and 12.6 International Units (IU/mL) of β-glucosidase] was a gift from NOVO Nordisk (Bagsvaerd, Denmark).

SSF experiments were carried out in 100 mL Erlenmeyer flasks, each containing 50 mL of the fermentation medium (without glucose) as described above, and were agitated at 150 rpm. The solid fraction of pretreated olive pulp at different concentrations [10 and 20% (w/v)] was used as substrate. The cellulolytic complex (Celluclast 1.5L) at 15 FPU/g substrate loading was added.

In the SSF experiments, flasks were inoculated with 10% (v/v) yeast cultures and periodically analyzed for ethanol and glucose. SSF assays were conducted at 42°C for 72 h. A fed-batch variation of the described SSF process was performed, in which pretreated olive pulp was fed in a discrete and successive charges. Fed-batch SSF experiments were initiated as described above using 10% pretreated olive pulp substrate concentration, and 15 FPU/g substrate enzyme loading. A charge of fresh substrate was added 24 h after the onset of SSF. The mixture was incubated for an additional 24 h. Then a new charge of fresh substrate was added again and the mixture was incubated for a further 24 h. Supplementation of enzyme to maintain the initial enzyme loading of 15 FPU/g of substrate was performed at the same time as fresh substrate was added.

Table 1
Composition (%) of Fibrous Residue of Olive Pulp Resulting from Hot Water Treatment at Different Temperature Conditions[a]

Pretreatment conditions	Total gravimetric recovery	Hemicellulosic sugars	Glucose	Klason	Ash
150°C	65.5	9.8 (6.4)	13.1 (8.6)	47.3 (31.0)	3.0
180°C	62.2	9.7 (6.1)	14.5 (9.2)	55.2 (34.3)	3.2
200°C	59.7	1.7 (1.0)	16.7 (10.0)	62.3 (37.1)	3.7
210°C	58.8	1.4 (0.8)	17.8 (10.5)	62.4 (36.7)	4.1
220°C	57.7	0.5 (0.3)	14.1 (8.1)	64.8 (37.4)	3.5
230°C	59.5	nd	13.4 (8.0)	64.2 (38.1)	4.1
240°C	53.6	nd	11.7 (6.3)	67.5 (36.2)	4.1
250°C	52.2	nd	10.3 (5.4)	68.9 (36.0)	4.7

[a] Data are expressed in parentheses as a percentage based on dry weight of raw material. nd, non detected.

SSF results are reported in percentage of the theoretical yield. The theoretical SSF yield was calculated by assuming that all the potential glucose in the starting pretreated material is available for fermentation, and 0.51 of glucose fermentation yield.

Results

Eight pretreatment conditions for olive pulp were evaluated. The target set-point temperatures were 150, 180, 200, 210, 220, 230, 240, and 250°C. Heating up of the olive pulp and water mixture from room temperature to the final set-point temperature took between 35 and 80 min (Fig. 2B). Cooling from the final set-point temperature to below 130°C took approx 2 min. The pH was not controlled during pretreatment. The untreated sample had a pH of 5.1, and the final pH range for the pretreatments varied from 4.9 at 150°C to a minimum value of 3.9 at 210°C. As expected, at higher pretreatment severities lower final pH values were obtained, reaching a minimum value at 210°C (final pH at 180, and 200°C pretreatment temperature was 4.4 and 4.0, respectively). From 210 to 250°C no significant variation in pH values (4, 4.1, and 4.2 pH at 220, 230, and 250°C, respectively) was obtained.

The composition of the solid portion olive pulp resulting from the pretreatment is shown in Table 1. Solubilization of the solids was extensive, and almost 40% of the dry matter was extracted, leaving around 60% of the original dry matter as fibrous residue. The percentage of material recovery for the eight pretreatments ranged between 52 and 65% of the original material, and a linear correlation between the final set point pretreatment temperature and the quantity of solid material solubilized can be established.

Table 2
Composition of Liquid Fraction (g/100 g Raw Material) Resulting from Hot-Water Treatment at Different Temperature Conditions

Pretreatment conditions	Hemicellulosic sugars	Glucose	Furfural	HMF	Hydroxy-tyrosol	Tyrosol
150°C	nd[a]	4.7	0.001	0.01	0.19	nd
180°C	nd	3.6	0.026	0.20	0.40	0.05
200°C	0.8	1.5	0.29	0.60	0.97	0.002
210°C	0.6	0.7	0.88	0.75	1.20	0.18
220°C	nd	0.1	0.87	0.70	1.43	0.35
230°C	nd	0.05	0.34	0.50	1.77	0.46
240°C	nd	nd	0.37	0.31	1.48	0.25
250°C	nd	nd	0.19	0.15	1.66	0.54

[a]nd, non detected.

Hemicellulose was clearly the major constituent extracted. Only pretreatments in which significant hemicellulose remained in the solid were pretreatments at 150 and 180°C. The solubilization of cellulose ranged from 25 to 62% of the total cellulose content in the original solid olive pulp for the pretreatment conditions tested. The remaining solid at lowest pretreatment temperature (150°C) contained lower cellulose (13.1%) compared to the untreated olive pulp. At temperatures from 180 to 200°C, the aqueous pretreatment of olive pulp caused an increase in the cellulose content of the remaining solids. The amount of cellulose ranged from 14.5% for the 180°C pretreatment to 17.8% for 210°C pretreatment of the total remaining solids, compared to 14.1% cellulose for untreated olive pulp. Experiments at higher temperatures (230, 240, and 250°C) caused an increase in cellulose solubilization, and the pretreated residue contained lower cellulose than raw material. Klason lignin content of the solid residue after pretreatment, increased with the temperature.

Table 2 shows the composition (g/100 g original olive pulp) of liquid fraction for each pretreatment condition. There was practically no hemicellulosic sugars recovery in the liquid fraction. For pretreatments at temperatures of 150°C and 180°C, the liquid fraction contained 4.7 and 3.6 g of glucose/100 g raw material, respectively. At higher temperatures the amount of glucose in the media decreased, and the glucose in aqueous extract vanished at the highest severities. Furfural and hydroxymethylfurfural (HMF) increased at higher pretreatment temperatures in the interval 150–210°C. Maximum furfural and HMF amount of 0.88–0.87 and 0.75–0.70 g/100 g raw material were obtained at 210–220°C, respectively. At this temperature interval, a correlation between pentoses and hexoses degradation during pretreatment (Fig. 3) and the quantity of furfural and HMF in the liquid fraction (Table 2) was observed. However, the furfural and HMF amounts in the liquid fraction were much lower

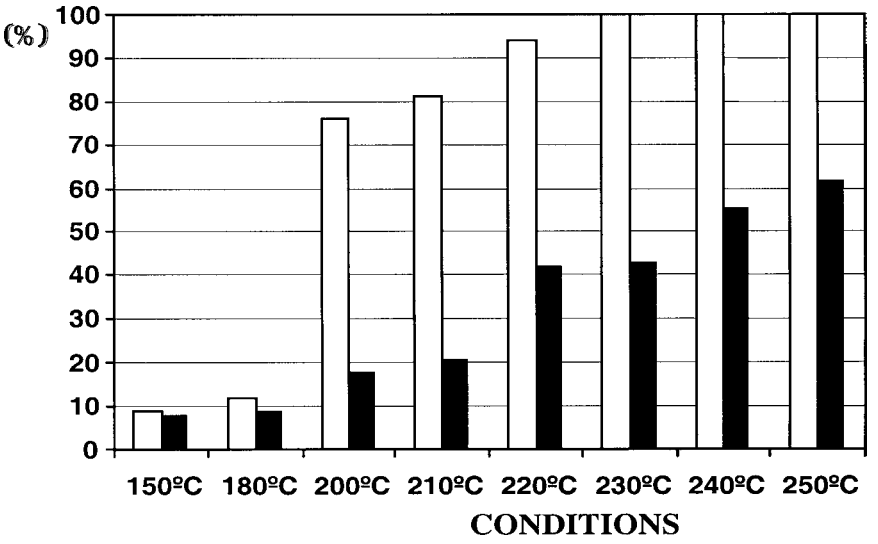

Fig. 3. Hemicellulosic sugars (□) and glucose (■) degradation (percentage based on dry weight raw material) during pretreatment

than the loss observed in pentoses and hexoses in the solid fraction, and therefore a mass balance cannot be performed. This indicated that furfural and HMF were converted to other degradation products, and this fact was harsher at higher temperature conditions. It can be responsible for the lower amounts of furfural and HMF found in the liquid fraction at temperature range from 220 to 250°C.

HPLC analysis of liquid fraction provided detection and quantification of phenolic compounds released during hot water pretreatment (Table 2). Tyrosol and hydroxytyrosol were extracted during pretreatment, and the degree of extraction of these phenols was correlated with pretreatment severity. The monomeric phenols tyrosol and hydroxytyrosol increased with pretreatment temperature, reaching a maximum value at 230°C, and above this temperature the content decreased slightly.

Enzymatic Hydrolysis

In order to establish the effect of pretreatment on the susceptibility to enzymatic attack of the cellulose from the olive pulp, samples of the feedstock, with and without pretreatment, were submitted to enzymatic hydrolysis tests using the commercial cellulolytic complex Celluclast 1.5L.

Enzymatic hydrolysis yields (expressed as a percentage of the glucose produced in the enzymatic hydrolysis divided by the potential glucose in the pretreated material) at 10% substrate concentration (w/v) and 15 FPU/g substrate enzyme loading are shown in Fig. 4. The quantity of sugars produced in samples without treatment was about 40% of the initial material. In the case of pulp subjected to the hot water pretreatment

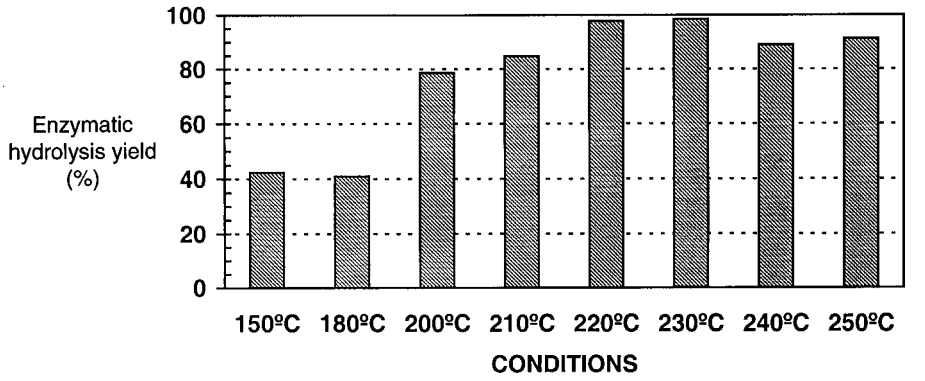

Fig. 4. Enzymatic hydrolysis yields (%) of the water insoluble fraction of olive pulp for different pretreatment conditions. Results are expressed as glucose obtained in the enzymatic hydrolysis divided by potential glucose in pretreated substrate.

at 150°C and 180°C, the amount of sugars resulting from saccharification of the residues were not different from the sugar yield of the pulp without treatment. However, at higher severity pretreatment conditions enzymatic hydrolysis yield is dramatically increased. Yields of 85% at 210°C and about 100% at 220–230°C were obtained. A slight decrease (about 8%) in the enzymatic hydrolysis yield was observed when the highest temperatures (240–250°C) were assayed.

Simultaneous Saccharification and Fermentation (SSF) Tests

Results of the SSF tests for hot-water-treated pulp at different pretreatment conditions and initial substrate concentration of 10% (w/v) are shown in Fig. 5. SSF yield increases as pretreatment temperature rises up to a maximum value of 80% (of theoretical) at 210°C, and remains stable at highest temperatures. On the other hand, ethanol concentration decreases gradually from that temperature as pretreatment severity levels out. Reliable results could not be obtained because of the difficulty of keeping solids in suspension in the SSF, when using initial substrate concentrations of 20% (data not shown). In the experiments where fermentation took place, fermentation onset was delayed 24 h in comparison to usual SSF time-course with *K. marxianus* CECT 10875.

Results of ethanol production and remaining glucose in fed-batch SSF experiments by adding substrate three times at 24-h intervals are shown in Fig. 6. During the first stage of SSF (48 h from SSF onset), there was a continuous increase in the ethanol content while the free glucose in the medium remained very low (lower than 0.5 g/L). At the end on this stage, maxima ethanol concentrations between 8 and 12 g/L were obtained. The end of the first stage was characterized by cessation of ethanol production. From here on, ethanol concentration remained more or less constant, but glucose content started to increase, indicating continuance of cellulolytic

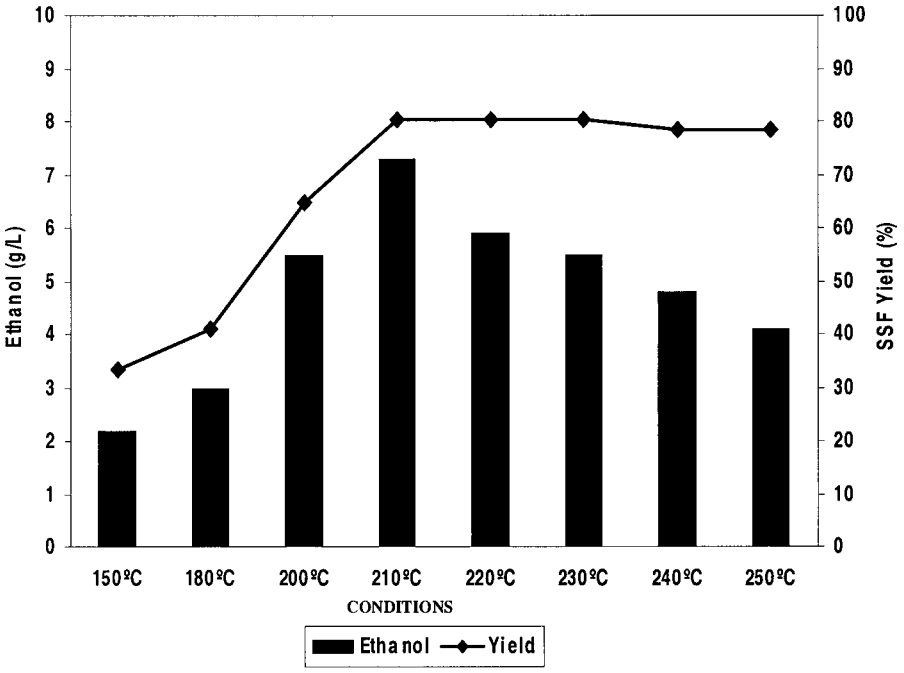

Fig. 5 Ethanol (g/L) and simultaneous saccharification and fermentation (SSF) yield (% of the theoretical) from olive pulp residue pretreated at different conditions. Substrate: 10% (w/v); enzyme loading: 15 FPU/g substrate. SSF results are reported in percentage of the theoretical yield. The theoretical SSF yield was calculated by assuming that all the potential glucose in the starting pretreated material is available for fermentation, and 0.51 of glucose fermentation yield.

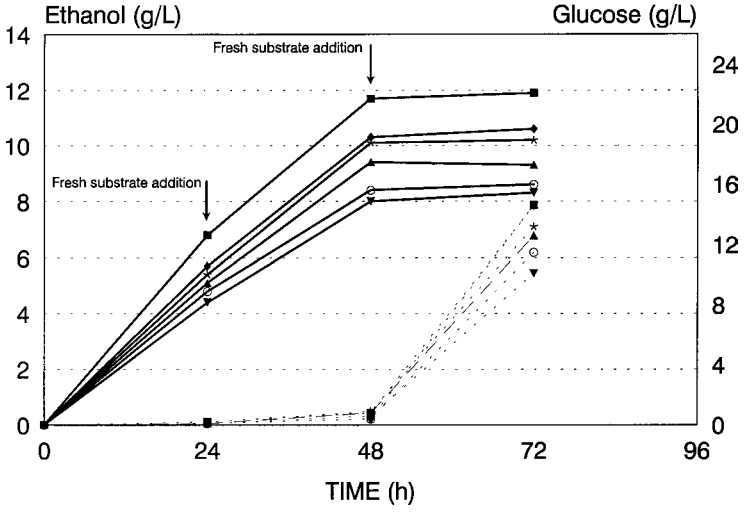

Fig. 6. Ethanol production (—) and remaining glucose (- - -) in fed batch SSF process from olive pulp residue pretreated at different conditions: 200ºC (*), 210ºC (■), 220ºC (◆), 230ºC (▲), 240ºC (○) and 250ºC (▼).

Table 3
SSF Yield for Fed-Batch Mode Operation Experiments at 20% Substrate (w/v) Concentration[a]

Pretreatment conditions	SSF yield	% (of theoretical yield)
200°C	0.30	59
210°C	0.33	65
220°C	0.36	71
230°C	0.35	69
240°C	0.36	71
250°C	0.39	76

[a] Supplementation of enzyme to maintain the initial 15 FPU/g of substrate was performed at the same time as fresh substrate was added.

SSF results are reported in percentage of the theoretical yield. The theoretical SSF yield was calculated by assuming that all the potential glucose in the starting pretreated material is available for fermentation, and 0.51 of glucose fermentation yield.

activity. The high free glucose concentration in the media indicates absence of glucose fermentative activity. In Table 3, SSF yields of these experiments at 48 h are given. As can be observed, the fed batch addition resulted in yields close to 70% of the theoretical at 20% (w/v) substrate concentration.

Discussion

This work was conducted to extend the merit of the pulp fraction contained in the residue generated in olive oil extraction by the two-step centrifugation process.

In previous work using this substrate, steam explosion pretreatment was not suitable for ethanol production, because it produced a high cellulose solubilization, but did not increase enzymatic hydrolysis *(2)*. In the present study hot-water pretreatment was tested in order both to improve cellulose conversion to ethanol and to recover the antioxidant phenols (mainly hydroxytyrosol) contained in such fractions.

After hot water pretreatment of olive pulp residue, percentages of water insoluble fiber recovery were in the range of those obtained for lignocellulosic materials as corn fiber *(10)* and alfalfa fiber *(19)* when using aqueous pretreatments. Hemicellulose is the component most severely solubilized by this kind of pretreatment, which is in agreement with the results of hemicellulose extraction in lignocellulosic materials reported by van Walsum et al. *(20)* for bagasse and aspen and Koegel et al. *(19)* for alfalfa fiber after hydrothermal pretreatments. It is worth mentioning that the dramatic increase in solubilization occurred from 180 to 200°C. From a temperature of 220°C hemicellulose is completely solubilized. Unfortunately, this solubilization is not associated with hemicellulose-derived-sugars recovery in the liquid fraction (Table 2), thus avoiding a potential use of this fraction for ethanol production.

On the other hand, the Klason lignin fraction is increased after pretreatment in comparison to initial material. At temperatures above 150°C, recovery values exceed initial concentration in 20–32%. To explain this fact it must be considered that, owing to the nature of the procedure employed, acid-insoluble components other than Klason lignin may be determined together with true lignin. It would be in agreement with the reported presence of highly polymerized phenolics glycosides in olive pulp *(21)*. The formation of high-molecular-weight products that may be determined as acid-insoluble lignin has been described in steam explosion pretreatment of lignocellulosic materials *(22)*. It is suggested that these substances result from condensation reactions of tannins and flavonoids with sugar degradation products (furfural and hydroxymethylfurfural). Flavonoids as luteinin and apigenin have been described in olive leaves, fruits, and oils *(23)*. They may be involved in the formation of condensed molecules after hot water pretreatment contributing to the increase in "Klason" lignin content. The results of "Klason" lignin concentration differ somewhat from those reported by van Walsum et al. *(20)* for bagasse and aspen, who found 37–65% extraction of the lignin after liquid hot-water pretreatment.

Hot-water pretreatment for olive pulp residue was aimed, on the one hand, to obtain a cellulose rich fraction in the water-insoluble fiber. Results demonstrate that this was achieved at pretreatment temperatures of 200–210°C. Under these conditions the remaining cellulose is more readily hydrolyzed to glucose upon subsequent enzymatic hydrolysis. A two-fold higher enzymatic hydrolysis yield compared to untreated olive pulp was obtained, thus demonstrating improved cellulose accessibility as a consequence of almost complete hemicellulose removal in the pretreated pulp. At higher temperatures of 220–230°C, about 100% enzymatic hydrolysis is obtained, although it must be considered that under these conditions cellulose degradation was considerably high (42–43% cellulose loss during pretreatment).

Hot-water pretreatment of olive pulp solubilized both phenolic monomeric compounds tyrosol and hydroxytyrosol. Hydroxytyrosol in liquid fraction comes from the oleuropein of olive pulp, and during pretreatment this phenol glucoside is chemically hydrolyzed and solubilized. Solubilization of such phenols during steaming of olive stones has been observed previously by other authors *(24)*. Hydroxytyrosol is characterized by strong antimicrobial properties, and it has an antioxidant activity with a high value in food industry. So, its extraction by a hot water pretreatment before the cellulosic fraction of olive pulp was used for enzymatic hydrolysis and ethanol fermentation clearly contributes to the upgrading of this residue.

Yields obtained in the SSF tests with 10% (w/v) substrate (Fig. 5) were in the range of those obtained from other lignocellulosic materials using Celluclast 1.5L and *K. marxianus (25, 26)*. In a previous work *(2)* SSF yields close to 67% of theoretical using nonpretreated olive pulp as substrate. Higher SSF yields (about 80%) have been achieve in the experiments with

hot-water-pretreated olive pulp. The increase in SSF yields for pretreated olive pulp is due to higher enzymatic hydrolysis yield (90%) and higher fermentation efficiency. Thus, hot-water pretreatment has two effects: to increase the cellulose accesibility of olive pulp to enzymatic attack and to remove phenolic compounds tyrosol and hydroxytyrosol that are considered to be fermentation inhibitors.

The SSF fed-batch operation mode tested was expected to allow achieving those high SSF yields together with glucose concentrations adequate for fermentation. Results from fed-batch experiments (Fig. 6) show the possibility of using substrate concentrations up to 20% (w/v) with SSF yields close to 70% of theoretical. At increasing substrate concentration up to 30% (w/v), ethanol concentration in the media remains constant. This inhibition affects the fermentation step and not the enzymatic hydrolysis, since free glucose is found in fermentation media while ethanol production comes to an end. As it was anticipated in previous work *(2)*, the presence of substances as phenols and tannins in olive pulp may promote the formation of unknown toxic compounds during pretreatment, which somehow affect the yeast fermentation capacity. Even for a fed-batch addition pattern, 30% concentration of this type of substrate generates an adverse environment for yeast growth since as SSF proceeds and cellulose fraction is hydrolyzated, an "insoluble-acid-compounds" rich substrate is produced which affects negatively yeast performance.

In order to increase our understanding of this fact, an initial approach was performed by exhaustively extracting a sample of pretreated substrate with ethyl acetate (in a standard soxhlet apparatus). Preliminary results (data not shown) indicate that SSF in such extracted substrate is remarkably improved in comparison to unextracted substrate. It was expected that by ethyl acetate extraction a series of oligomeric substances from lignin *(27)*, and other condensation and degradation products derived from hemicelluloses *(28)*, were extracted. Preliminary results obtained in pulp olive substrate after extraction procedures confirm the presence of these undesirable condensed substances interfering yeast-fermenting capacity. Further experiments would be needed to complete this research.

Briefly, if hot-water pretreatment is to be used for upgrading olive pulp residue, 210°C temperature would be the most suitable condition since it allows the best SSF yield of 65% of the theoretical (12 g ethanol/L using 20% substrate at fed-batch procedure) while hydroxytyrosol is extracted in a considerable quantity. Although 230°C is the optimum temperature for hydroxytyrosol recovery in liquid fraction, it causes extensive cellulose degradation (43% of cellulose content in raw material) that results in lower ethanol production. From an economic point of view, the recovery of hydroxytyrosol as byproduct during ethanol production process from olive pulp would be desirable to improve the profitability of the overall process.

Acknowledgment

The authors wish to acknowledge CICYT (Comisión Interministerial de Ciencia y Tecnología) for its financial support (Project reference 1FD97-0449-C02-02).

References

1. Alba, J. (1994) *Fruticultura Professional.* **62,** 85–95
2. Ballesteros, I., Oliva, J. M., Sáez, F., and Ballesteros, M. (2001) *Appl. Biochem. Biotechnol.* **91–93,** 237–252.
3. Moreno, E., Pérez, J., Ramos Cormenzana, A., and Martínez J. (1987) *Microbios,* **51,** 169–174.
4. Martínez Nieto, L., Garrido Hoyos, S. E., Camacho Rubio, F., Garcia Pareja, M. P., and Ramos Comerzana, A. (1993) *Bioresour. Technol.* **43,** 215–219.
5. Amiot, M. J., Fleuriet, A., and Macheix, J. J. (1989) *Phytochemistry* **28,** 67–69.
6. Ruíz Barba, J. L., Brenes-Balbuena, M., Jiménez-Díaz, P., García-García, P., and Garrido-Fernández, A. (1993) *J. Appl. Bacteriol.* **74,** 15–19.
7. Tsimidou, M., Papadopoulos, G., and Boskou, D. (1992) *Food Chem.* **44,** 53–60.
8. Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Spiegelhalder, B., and Bartsch, H. (2000) *Eur. J. Cancer* **36(10),** 1235–1247.
9. Fernández Bolaños, J., Felizón, B., Heredia, A., Guillen, R., and Jimenez, A. (1999) *Bioresour. Technol.* **68,** 1–12
10. Weil, J. R., Sarikaya, A., Rau, S., Goetz, J., Ladish, M., Brewer, M., Hendrickson, R., and Ladish M. R. (1998) *Appl. Microbiol. Biotechnol.* **73,** 1–17.
11. ASTM-D 1348 (1995), *Annual Book of ASTM Standards*, vol. 04.10, American Society for Testing Materials, Philadelphia, PA.
12. ASTM-D 1102-84 (1995), *Annual Book of ASTM Standards*, vol. 04.10, American Society for Testing Materials, Philadelphia, PA.
13. ASTM-D 1111-84 (1995), *Annual Book of ASTM Standards*, vol. 04.10, American Society for Testing Materials,.Philadelphia, PA.
14. ASTM-D 1107-87 (1995), *Annual Book of ASTM Standards*, vol. 04.10, American Society for Testing Materials, Philadelphia, PA.
15. Puls, J., Poutanen, K., Körner, H. V., and Viikari, L. (1985) *Appl. Microbiol. Biotechnol.* **22,** 416–423.
16. Ghose, T. K. (1987) *Pure Appl. Chem.* **59(2),** 257–268.
17. Graciani, E. and Vázquez, A. (1980) *Grasas y Aceites* **31,** 237–243.
18. Ballesteros, I., Oliva, J. M., Ballesteros, M., and Carrasco, J. (1993) *Appl. Biochem. Biotechnol.* **39–40,** 201–211.
19. Koegel, R. G., Sreenath, H. K., and Straub, R. J. (1999) *Appl. Biochem. Biotechnol.* **77–79,** 105–115.
20. Van Walsum, G. P, Allen, S. G., Spenser, M. J., Laser, M. S., Antal, M. J., and Lynd, L.R. (1996) *Appl. Biochem. Biotechnol.* **57–58,** 157–170.
21. Maestro Durán, R. Borja Padilla, R., Martín Martín, A., Fiestas Ros de Ursinos, J. A., and Alba Mendoza, J. (1991) *Grasas y Aceites* **42(4),** 271–276.
22. Lomax, T. D., Mackie, K. L Meder, R., Croucher M., and Burton, R. J. (1994) *J. Wood Chem. Technol.* **14,** 539–561.
23. Brenes, M., García A., García J., Rios J. J., and Garrido, A. (1999) *J. Agric. Food Chem.* **47,** 3535–3540.
24. Fernández-Bolaños, J., Felizón B., Brenes M., Guillén R., and Heredia A. (1998) *JAOCS* **75,** 1643–1649.

25. Ballesteros, I., Ballesteros, M., Oliva, J. M., and Carrasco, J. E. (1994), in *Biomass for Energy, Environn. Agric. Ind.*, Chartier, Ph., Beenackers, A.A.C.M., and Grassi, G. eds., Pergamon, pp. 1953–1958.
26. Ballesteros, I., Oliva, J. M., Carrasco, J. E., Cabañas, A., Navarro, A. A. and Ballesteros, M. (1998) *Appl. Biochem. Biotechnol.* **70–72,** 369–381.
27. Hishiyama, S. and Sudo, K. (1992) *Mokuzai Gakkaishi* **38,** 944–949.
28. Kuznetsov, B. N.; Efrenov, A. A., Levdanskii, V. A., Kuznetsova, S. A., Polezhayeva, N. I., Shikina, T. A., and Krotova, I. V. (1996) *Bioresour. Technol.* **58,** 181–188.