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Ethanol Production from Lignocellulosic Byproducts of Olive Oil Extraction

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Abstract

The recent implementation of a new two-step centrifugation process for extracting olive oil in Spain has substantially reduced water consumption, thereby eliminating oil mill wastewater. However, a new high sugar content residue is still generated. In this work the two fractions present in the residue (olive pulp and fragmented stones) were assayed as substrate for ethanol production by the simultaneous saccharification and fermentation (SSF) process. Pretreatment of fragmented olive stones by sulfuric acid–catalyzed steam explosion was the most effective treatment for increasing enzymatic digestibility; however, a pretreatment step was not necessary to bioconvert the olive pulp into ethanol. The olive pulp and fragmented olive stones were tested by the SSF process using a fed-batch procedure. By adding the pulp three times at 24-h intervals, 76% of the theoretical SSF yield was obtained. Experiments with fed-batch pretreated olive stones provided SSF yields significantly lower than those obtained at standard SSF procedure. The preferred SSF conditions to obtain ethanol from olives stones (61% of theoretical yield) were 10% substrate and addition of cellulases at 15 filter paper units/g of substrate.

Index Entries: Ethanol; olive oil extraction byproducts; pretreatment; enzymatic saccharification; fermentation.

Introduction

The olive oil industry represents one of the most important economic agro-food sectors in Spain. On a worldwide scale, Spain is the main producer and exporter of olive oil, with an average of 590,000 t/yr, which makes up >33% of the world's production *(1)*.

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The extraction process of olive oil yields a highly contaminating residue that causes serious environmental concerns, still unsolved in the olive oil–producing countries. The production of oil mill wastewater in the Mediterranean area is estimated to be approx 1.2 million t/yr *(2)*. Traditionally, a three-step centrifugation process performs the continuous extraction of olive oil, in which the addition of water in a proportion of 1:1 is needed, that large amounts of highly contaminated oil mill wastewater are generated *(3)*.

Currently, a new two-step centrifugation process (without exogenous addition of water) to extract the olive oil has been developed that dramatically reduces the oil mill wastewater *(4)*. Implementation of the two-phase centrifugation process by many olive oil–producing Spanish industries has reduced water consumption and, consequently, oil mill wastewater. However, in this new process, a residue is still generated (estimated to be 800 kg/t of olive), containing the pulp, the water content of the olive, and portions of the seed husks and olive stones. This new residue, called pommace, consists of 70% water, 21% organic substances, and 9% mineral substances, respectively *(5)*. The organic fraction comprises 25% sugars, making it a potentially attractive, low-cost feed material for biologic conversion. Upgrading this residue by fractionation, with the possibility of obtaining ethanol from the free fermentable sugars and the cellulose present in the residue, would mean an improvement in the management of the byproducts of the olive oil industry. Currently, the extraction industries that use the two-phase centrifugation process separate the residue into two fractions: the pulp, formed by the olive pulp itself and the vegetation liquors engaged in nutritive and growth function, and the woodlike portion, comprising fragments of the olive stones.

In the present study, the two fractions contained in the pommace (olive pulp and olive stone fragments) were evaluated as a carbon source for the production of ethanol. This article reports the analysis (carbohydrates, lignin, ash, and other components) of these materials. This composition has been used in the calculation of conversion efficiencies. Steam explosion was tested as a pretreatment to enhance enzymatic hydrolysis. Raw materials were hydrolyzed with cellulase enzyme at different substrate and enzyme loadings. Finally, the simultaneous saccharification and fermentation (SSF) bioconversion process of olive pulp and olive stones was tested.

Materials and Methods

Substrates

Olive pulp and olive stone fragments, generated as a residue from the two-phase centrifugation olive oil production process, were supplied by Oleicoa El Tejar S.C.L. (Córdoba, Spain). The substrates were chemically analyzed according to the following standard methods: ASTM D-1348 *(6)* for moisture content, ASTM D-1102-84 *(7)* for ash content, ASTM D-1111-84 *(8)* for hot water extracts, and ASTM D-1107-87 *(9)* for ethanol/toluene extracts. The sample was first hydrolyzed using 72% H_2SO_4 for 60 min and then hydrolyzed a second time with 4% H_2SO_4 for 60 min at 120°C. This analysis gave the hemicellulosic sugars content (expressed as the sum of the xylose + arabinose + galactose + mannose), cellulose (expressed as glucose), and klason lignin.

Pretreatment

Washed feedstocks were pretreated in a steam explosion pilot unit, operated by batches and equipped with a 2-L reaction vessel. The plant description and working methodology were described in a previous article *(10)*. The temperature (210°C) and residence time (4 min) conditions of the biomass pretreatment were selected with regard to the maximum glucose recovery after 72 h of enzymatic hydrolysis. The reactor was filled with 200 g of feedstock per batch and was directly heated to the desired temperature with saturated steam. After the explosion, the material was recovered in a cyclone. The wet material was cooled to about 40°C and then filtered for solid recovery. The water-insoluble fraction was analyzed for xylans, glucans, and lignin content. The carbohydrate content of the filtrate was also analyzed. A variation of the described pretreatment procedure using olive stones was carried out in the presence of an acid catalyst. Two hundred grams of dry material was soaked in 1 L of acid solution (0.5% [w/v] H_2SO_4), and then a vacuum was applied to extract the air from the sample and leave it well impregnated. The vacuum was then released and allowed to stand for 1 h. It was next filtered through a Buchner funnel and rinsed with plenty of water to remove any remaining acid.

Microorganisms and Growth Conditions

Kluyveromyces marxianus CECT 10895, a thermotolerant mutant yeast strain obtained in our laboratory *(11)*, was used in fermentation and SSF experiments. Active cultures for inoculation were prepared by growing the organism on a rotary shaker at 180 rpm for 16 h at 42°C in a growth medium containing the following: 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 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), and substrate concentrations and enzyme loadings were tested at 50°C for 72 h. The enzyme preparation Celluclast 1.5L was a gift from Novo Nordisk (Bagsvaerd, Denmark).

SSF experiments were carried out in 100-mL Erlenmeyer flasks that contained 50 mL of the growth medium as described above and then agitated at 150 rpm. Glucose was substituted by the lignocellulose biomass at different substrate concentrations; the cellulolytic complex (Celluclast

1.5L), at different enzyme loadings, was also added. Enzymatic hydrolysis and SSF assays were conducted at 42°C for 72 h.

In the SSF experiments, flasks were inoculated with 10% (v/v) yeast cultures and periodically checked during the tests for ethanol and glucose. A fed-batch variation of the described SSF process was performed, in which olive pulp and pretreated stones were fed in discrete and successive charges. Fed-batch SSF experiments were initiated as described above, using 15 and 10% olive pulp and olive stone substrate concentrations, respectively, and 15 filter paper units (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 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.

Various fed-batch tests using the previously described variation of SSF process were performed. Substrate charges were carried out at 24-h intervals as follows:

1. 15% pulp as an initial substrate + 5% pulp + 5% pulp.
2. 15% pulp as an initial substrate + 7.5% pulp + 7.5% pulp.
3. 15% pulp as an initial substrate + 10% pulp + 5% olive stones pretreated by steam explosion.
4. 15% pulp as an initial substrate + 10% pulp + 5% olive stone pretreated by steam explosion in the presence of acid.
5. 10% olive stone pretreated by steam explosion as an initial substrate + 15% pulp + 5% pulp.
6. 10% of olive stone pretreated by steam explosion in the presence of acid as an initial substrate + 15% pulp + 5% pulp.
7. 10% olive stone as an initial substrate + 10% olive stone + 10% olive stone, all pretreated by steam explosion.
8. 10% olive stone as an initial substrate + 10% olive stone + 10% olive stone, all pretreated by steam explosion in the presence of acid.

Analytical Procedures

Composition of feedstocks and water-insoluble fraction after pretreatment has been determined by total hydrolysis with H_2SO_4 *(12)*. Enzymatic activities (filter paper and β-glucosidase) were measured according to the methods described by Ghose *(13)*.

Sugars were quantified by high-performance liquid chromatography in a 1081B Hewlett Packard (HP) apparatus with differential refractometer detector under the following conditions: column, AMINEX HPX-87P (Bio-Rad, Hercules, CA); temperature, 85°C; eluent, water at 0.1 mL/min.

Ethanol was measured by gas chromatography, using an HP 5890 Series II apparatus, with flame ionization detector and a column of Carbowax 20 M (2 m × 0.3175 cm) at 95°C. Both injector and detector temperatures were 150°C.

Table 1
Composition of Pulp and Olive Stone Fractions Generated as Residues in Olive Oil Extraction by the Two-Phase Centrifugation Process

Composition	Olive pulp (%)	Olive stone (%)
Extracts	53.3	19.2
Free sugars	6.4	0.5
Hemicellulosic sugars	13.4	23.5
Xylose	8.4	20.6
Arabinose	2.5	1.5
Galactose	1.0	1.0
Mannose	1.5	0.4
Cellulose as glucose	15.8	27.6
Klason lignin	15.2	29.1
Ash	2.3	0.6

Results

Feedstock Composition

Table 1 shows the compositions of the two fractions (pulp and olive stone fragments) that make up the residue from the olive oil extraction by a two-phase centrifugation process. Water extraction dissolved 31.6% of the pulp, of which 6.4% corresponds to free sugars; mainly, glucose. The process using organic solvents extracted 21.7%. The hemicellulose fraction comprises 13.4% of the pulp, xylose being the main sugar (8.4%). Cellulose and lignin represent 15.8 and 15.2%, respectively.

The olive stones basically consist of a strongly lignified secondary wall (29.1%), rich in cellulose (27.6%) and hemicelluloses (23.5%), with low salt content (0.6%). A percentage of 56% (dry wt) of the olive stone consists of carbohydrates, with a cellulose content >27%.

Steam Explosion Pretreatment

The results of the steam explosion pretreatment at 210°C and 4 min for the pulp and the olive stone fractions (with no catalyst and with impregnation by sulfuric acid) are shown in Table 2.

The majority of the pulp was solubilized during pretreatment, and, therefore, only 25% of the initial material was recovered as solids. Although 72% of the hemicellulose and 67% of the cellulose present in the pulp dissolved during pretreatment, practically no free sugars were detected in the filtrate, indicating almost total degradation of the dissolved sugars. Lignin and ash were also solubilized during pretreatment of the pulp by 15 and 56%, respectively.

In the pretreatment of the olive stone fraction as a substrate, about 60% of the material was recovered as solids. Analysis of the results of the com-

Table 2
Composition of Filtrate (g/100 g Raw Material) and Water-Insoluble Fiber (%) of Olive Pulp and Olive Stone Residues at 210°C and 4-min Steam Explosion Pretreatment Conditions[a]

	Olive pulp		Olive stone			
			No catalyst		0.5% (w/v) H_2SO_4	
Composition	Filtrate	Water-insoluble fiber	Filtrate	Water-insoluble fiber	Filtrate	Water-insoluble fiber
Glucose	0.8	20.8 (5.2)	0.21	40.5 (24.3)	0.28	42.3 (23.3)
Hemicellulosic sugars	0	14.7 (3.7)	14.90	9.8 (5.9)	15.9	7.7 (4.2)
Xylose	0	11.9 (3.0)	13.40	9.8 (5.9)	14.2	7.7 (4.2)
Galactose	0	0.9 (0.3)	0.6	0 (0)	0.7	0 (0)
Arabinose	0	1.5 (0.4)	0.9	0 (0)	1.0	0 (0)
Mannose	0	0.4 (0.1)	ND	ND	ND	ND
Lignin	—	39.6 (9.9)	—	45.7 (27.4)	—	44.0 (24.2)
Ash		4.1 (1.0)		0.6 (0.4)		0.5 (0.3)
Solubilization (%)		75		40		45

[a]Data are expressed in parentheses as a percentage based on dry wt of raw material.
ND, No determined.

Table 3
Enzymatic Hydrolysis of Olive Pulp
at Different Substrate and Enzyme Loadings[a]

Substrate (%)	Enzyme loading (FPU/g substrate)	Potential glucose (g/L)	Free glucose (g/L)	Enzymatic hydrolysis yield (%)
5	7.5	7.9	3.0	38.0
	15		3.0	38.0
	30		3.6	45.6
10	7.5	15.8	6.2	39.2
	15		6.4	40.5
	30		7.7	48.7
15	7.5	23.7	9.6	40.5
	15		9.8	41.3
	30		11.6	48.9
20	7.5	31.6	12.2	38.6
	15		12.5	39.6
	30		13.7	43.3

[a]Yield is expressed as glucose obtained in the enzymatic hydrolysis divided by potential glucose in the raw material.

position of the water-insoluble fraction of the olive stones showed that 75% of the hemicellulose and 12% of the cellulose dissolved during pretreatment. When sulfuric acid was used as a catalyst, a slight increase in the fraction of carbohydrate that was solubilized (82 and 16% for the hemicellulose and cellulose, respectively) was observed. Almost all the dissolved glucose was degraded to other products during pretreatment with and without catalyst (94%), and, therefore, no free glucose was found in the liquid fraction. Regarding the hemicellulosic sugars, 24% of the dissolved sugars was degraded during treatment with no impregnation and 26% when sulfuric acid was used. The lignin content of the water-insoluble fraction obtained after pretreatment of the olive stone fragments without a catalyst remained unchanged, whereas some dissolution of the lignin resulted (12.3%) in the sulfuric-acid-catalyzed steam explosion.

Enzymatic Hydrolysis Tests

To establish the effect of steam explosion pretreatment on the susceptibility to enzymatic attack of the cellulose from the pulp and olive stone fragments, samples of these feedstocks, with no pretreatment and exploded, were submitted to enzymatic hydrolysis tests using the commercial cellulolytic complex Celluclast 1.5L.

Enzymatic hydrolysis yields of the exploded pulp decreased when compared with the sample as received (data not shown). Consequently, tests to evaluate the effect of the initial concentrations of the substrate and enzyme (Table 3) were carried out using pulp, which had not been previously pretreated. Enzymatic hydrolysis yields were in the range of 38–49%

Table 4
Enzymatic Hydrolysis of Steam-Exploded Olive Stones
at 10% (w/v) Substrate and 15 and 30 FPU/g Substrate Enzyme Loading

Pretreatment conditions (210°C, 4 min)	Enzyme loading (FPU/g substrate)	Potential glucose (g/L)	Free glucose (g/L)	Enzymatic hydrolysis yield (%)
No catalyst	15	40.5	16.4	40.5
	30		22.4	55.3
0.5% (w/v) H_2SO_4	15	42.3	22.3	52.7
	30		25.8	61.0

for all conditions tested. As can be seen, these values did not vary significantly when the concentration of the substrate was increased. At different enzyme loadings, no variations were observed at 7.5 and 15 FPU/g of substrate. However, an enzyme loading of 30 FPU/g of substrate caused a significant increase in enzymatic yield. Owing to the relatively low cellulose content of the pulp, little glucose was released during enzymatic hydrolysis; thus, a high initial substrate loading (above 15%) was needed to obtain glucose concentrations >10 g/L.

Concerning the stone fraction, there was no enzymatic hydrolysis when the olive stone residue was not pretreated (data not shown). The enzymatic hydrolysis results for the pretreated olive stone fraction at 10% substrate concentration (w/v) and 15 and 30 FPU/g of substrate enzyme loads are shown in Table 4. When steam-exploded olive stone fragments without a catalyst were used as substrate, enzymatic hydrolysis yields of 40 and 55% were obtained at enzyme loadings of 15 and 30 FPU/g of substrate, respectively. These yields increased up to 53 and 61%, respectively, when sulfuric acid was used as a catalyst during pretreatment.

SSF Tests

Results of the SSF tests for the untreated pulp, using different initial substrate concentrations, are shown in Table 5. Yields of the SSF process decreased as the initial substrate concentration increased. Initial substrate loading >25% (w/v) could not be used because of the difficulty of keeping solids in suspension in the SSF media (poor mixing). The best ethanol yield (67% of theoretical) was obtained at 15% substrate concentration.

The results of the SSF experiments, using the pretreated olive stone fraction as substrate, are shown in Table 6. As in the case of the pulp, the ethanol yield of the process decreased as the initial substrate concentration increased. No ethanol production was obtained at 25% initial substrate loading (data not shown). The best ethanol yields in the SSF process (59% of theoretical) were obtained at 10% olive stone steamed with sulfuric acid as a catalyst.

Results of the fed-batch experiments by adding substrate (olive pulp and pretreated olive stones) three times at 24-h intervals are shown in

Table 5
Effect of Initial Substrate Concentration on SSF Yield
from Untreated Olive Pulp (enzyme loading of 15 FPU/g of substrate)

Substrate (%)	Ethanol (g/L)	Potential glucose (g/L)	SSF yield	Theoretical yield (%)
15	8.1	23.7	0.34	66.6
20	10.5	31.6	0.33	64.7
25	11.8	39.5	0.30	58.9

Table 6
Effect of Initial Substrate Concentration on SSF
from Pretreated Olive Stones (enzyme loading of 15 FPU/g of substrate)

Pretreatment conditions (210°C, 4 min)	Substrate (%)	Potential glucose (g/L)	Ethanol (g/L)	SSF yield	Theoretical yield (%)
No catalyst	10	40.5	7.8	0.19	37.2
	20	81.0	14.5	0.18	35.3
0.5% (w/v) H_2SO_4	10	42.3	12.9	0.30	58.8
	20	84.6	17.9	0.21	41.2

Table 7. For assays with pulp, the SSF process is better when fed-batch of pulp is used (76.5% of theoretical) than when the substrate is added all at once (58.9% of theoretical). No significant improvement in SSF yield was achieved when pulp and stones are combined. Moreover, a high free glucose concentration in the media is observed after 72 h from the onset of SSF, indicating continuance of cellulosic activity and cessation of ethanol production. Experiments with fed-batch, pretreated olive stones provide SSF yields significantly lower than those obtained at standard SSF procedure; however, high residual glucose concentration remains unmetabolized in the medium.

For comparing the ethanol production rate of different fed-batch experiments, the ethanol production kinetics in the SSF process are shown in Fig. 1. Ethanol production of fed-batch experiments by adding pulp (Fig. 1A) increased after each addition of substrate, reaching values of 15.5 and 18.6 g/L for 25 and 30% final substrate concentration, respectively. In the SSF assays using pulp and olive stone for the second addition (Fig. 1B), no increase in ethanol production was obtained from 48 to 72 h. The SSF assay with pretreated olive stones as initial substrate combined with a fed batch of pulp (Fig. 1C) gives ethanol concentrations in the range of 15.6–18.5 g/L. For experiments using olive stones as the sole substrate, at fed-batch mode of operation (Fig. 1C), no ethanol production was observed during the last 24 h, when 30% total substrate loading was reached.

Table 7
SSF for Fed-Batch Mode Operation Experiments[a]

Substrate	Substrate loading (%)	Ethanol (g/L)	Potential glucose (g/L)	Residual glucose (g/L)	SSF yield	Theoretical yield (%)
Pulp	25 (15 + 5 + 5)	15.5	39.5	2.1	0.39	76.5
Pulp	30 (15 + 7.5 + 7.5)	18.6	47.4	6.3	0.39	76.5
Pulp + stone	30 (15 + 10 + 5)	13.6	59.7	16.1	0.23	45.1
Pulp + stone[b]	30 (15 + 10 + 5)	15.0	60.6	16.4	0.25	49.0
Stone + pulp	25 (10 + 10 + 5)	15.6	64.2	12.3	0.24	47.1
Stone[b] + pulp	25 (10 + 10 + 5)	18.5	66.0	12.9	0.28	54.9
Stone	30 (10 + 10 + 10)	17.5	121.5	56.2	0.14	28.2
Stone[b]	30 (10 + 10 + 10)	19.7	126.9	63.2	0.16	31.4

[a]Supplementation of enzyme to maintain the initial 15 FPU/g of substrate was performed at the same time as fresh substrate was added.
[b]Olive stones impregnated with 0.5% H_2SO_4 prior to steam explosion pretreatment.

Discussion

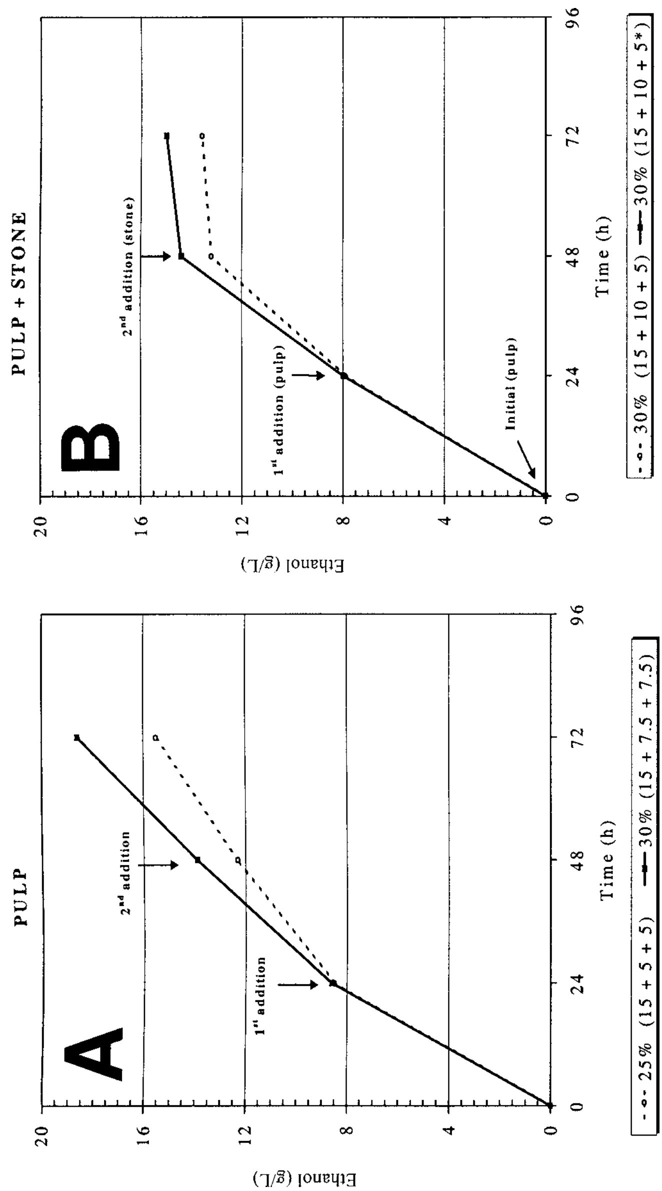
Upgrading of the lignocellulosic residue generated in the production of olive oil by the two-phase centrifugation process (pulp and olive stone fragments) would yield a significant advance in the management of byproducts originating from the olive oil extraction industry. In the present study, the possibility of obtaining ethanol from the fermentable sugars and cellulose present in the residue was investigated.

The results obtained from analysis of the composition of the raw material showed that the main hemicellulosic sugar, both in the pulp and in the olive stone fractions of olives, was xylose, which indicated that the predominant hemicelluloses in these kinds of materials was xylan. However, the xylose content of the olive stone fraction represents nearly 88% of the hemicellulosic sugars, whereas in the pulp it represents about 63%. The lower amount of xylose in the hemicellulosic sugars in the pulp compared with those obtained for stones resulted from the fact that this fraction contained the olive seed, which had an appreciable quantity of hemicelluloses of the arabinane and glucomanane type *(14)*. Although fermentable sugars content of both feedstocks was lower than that from typical lignocellulosic biomass *(15–17)*, the cellulose content in these materials was high enough to be considered as potential substrate for ethanol production.

The percentage of material dissolved during both the aqueous and organic extraction was much greater in the pulp than in the fragmented stone fractions. According to Brenes et al. *(18,19)*, the olive pulp fraction, composed of the rest of the olive skin, the pulp, and the seed, contains a low quantity of true lignin and presents a series of substances, including low molecular weight phenols, which can be extracted using organic solvents. This lignin structure would explain the high percentage value for dissolution (20.1%) during organic extraction of the pulp.

During the pretreatment, a large part of the fraction determined as acid-insoluble lignin contained in the pulp was dissolved. This fact differs from that of the klason lignin present in woody substrates, which basically remains unchanged during steam explosion pretreatment, or even increases, owing to the formation of pseudolignin as a result of the repolymerization of the decomposition products of hemicellulose and lignin *(20,21)*. This different performance of acid-insoluble lignin of olive pulp can be explained by the fact that the olive pulp and seed do not contain true lignin, but highly polymerized phenolic glycosides *(22)*.

The steam explosion process was not suitable as pretreatment for olive pulp, because it produced a high cellulose solubilization and did not increase enzymatic hydrolysis. On the other hand, the nonpretreated pulp was an appropriate substrate for enzymatic hydrolysis, because, under all initial substrate concentrations and 7.5 and 15 FPU/g enzyme loadings, hydrolysis yields in the range of 38–41.5% were obtained. These yields can be considered to be in the range of values obtained for other types of materials with high hemicellulose content. Higher enzymatic hydrolysis


A
PULP
Ethanol (g/L)
Time (h)
1st addition
2nd addition
- o - 25% (15 + 5 + 5) —■— 30% (15 + 7.5 + 7.5)
B
PULP + STONE
Ethanol (g/L)
Time (h)
Initial (pulp)
1st addition (pulp)
2nd addition (stone)
- o - 30% (15 + 10 + 5) —■— 30% (15 + 10 + 5*)

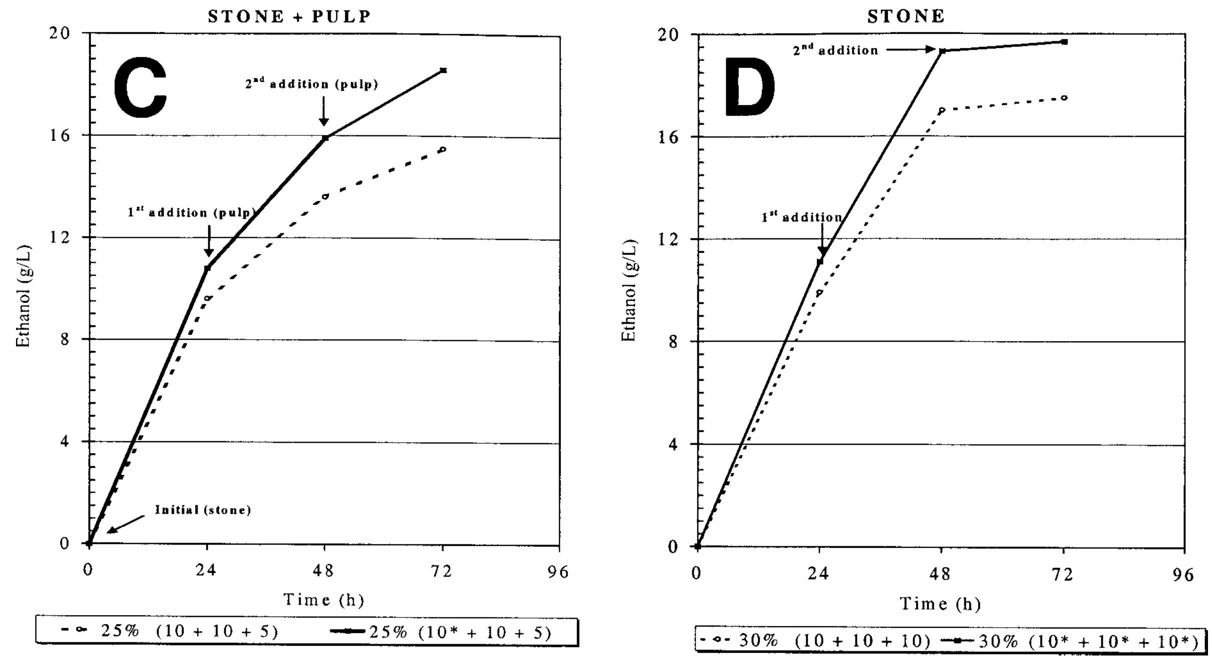


Fig. 1. Ethanol concentration in fed-batch SSF process as a function of time. Supplementation of enzyme to maintain the initial 15 FPU/g substrate was performed at the same time as fresh substrate was added. **(A)** Untreated pulp; **(B)** untreated pulp plus steam-exploded stones; **(C)** steam-exploded stones plus untreated pulp; **(D)** steam-exploded stones. *Olive stone impregnated with 0.5% (w/v) prior to steam explosion pretreatment.

yields were achieved at 30 FPU/g of substrate, but this enzyme loading can be considered too costly.

Enzymatic hydrolysis of the olive stone fragment fraction was favored by sulfuric acid impregnation prior to pretreatment. Under these conditions, with an enzyme loading of 15 FPU/g of substrate, a hydrolysis yield of 53% of theoretical was obtained. In the tests without a catalyst, olive stone portions remained intact after pretreatment, reducing the accessibility of the enzyme to the cellulose of the olive stone. Penetration of the acid inside the structure of the olive stone brings about a better hydrolysis of the acetyl groups of the xylanes *(23)*. During autohydrolysis more acetic acid is formed, which favors the cleavage of a series of bonds. This ensures the cohesion of the material, and during decompression and expansion of the steam, the material is subsequently reduced to smaller particles.

Yields obtained in the SSF tests of nonpretreated pulp (Table 5) were in the range of those obtained from other lignocellulosic materials using Celluclast 15L and *K. marxianus* CECT 10895 *(24,25)*. SSF yields decreased as initial substrate concentration increased. This decrease is owing to lower yields in the fermentation stage and not in enzymatic hydrolysis step, because, as previously stated, hydrolysis yields were not affected by an increased substrate concentration (Table 3). This inhibition may be owing to the presence of phenolic substances in pulp and seed, which at higher concentrations are toxic for microorganisms *(26,27)*. It has been reported *(28,29)* that oil and pulp from the two-phase centrifugation extraction process contain a higher amount of phenolic compounds, mainly in the form of glycosides and esters, than those present when the conventional extraction process is used.

In the SSF tests using the olive stone fraction as substrate, the best yield (59% of theoretical) was obtained using 10% of the pretreated material in the presence of sulfuric acid catalyst. Initial steam-exploded olive stone concentrations of 25% inhibited ethanol production. This shows that toxic compounds present in steam-exploded olive stones have a stronger inhibitory effect on the microorganisms than those present in unpretreated pulp, since initial pulp loads of 25% were feasible for the SSF process. Although the characteristics of olive stone lignin are similar to that of hardwood (syryngil-guayacil type), recently, glucosides of tyrosol and hydroxityrosol have been identified as components of olive stone *(30)*. These polyphenols are well known as part of olive pulp, and they have not been described in any lignocellulosic material. They account for the characteristics of olive stones such as hardness and high reluctance to acid and enzymatic attacks, and, during steam explosion pretreatment, chemical changes (probably hydrolysis) in these compounds can occur that increase the toxicity. This suggestion is under research in our laboratory.

As in pulp, lower initial substrate loads are needed to obtain higher SSF yields, but it means low sugar concentration for fermentation. Consequently, it is necessary to establish SSF conditions that make an adequate substrate concentration in the media (providing a reasonable sugar concentration for fermentation) compatible with high SSF yields. It would then be possible to obtain ethanol concentrations in the SSF process, which would allow later distillation of the ethanol from the fermentation medium to be economically viable. The variation of the SSF process, by fed-batch operation mode, allows achievement of high SSF yields, together with ethanol concentrations suitable for fermentation.

In pulp assays, for the same substrate loading, the SSF process is better when the fed-batch procedure is used (76% of theoretical yield) than when substrate is added all at once (59%). Under these conditions not only were concentrations of ethanol in the medium >18 g/L achieved, but also a significant increase in the SSF yield was observed. The addition of pulp by pulses every 24 h allowed the microorganism to maintain the capacity to ferment at high substrate concentration, thus reducing the inhibitory effect of toxic compounds (phenols and tannins) present in the pulp.

The addition of pretreated olive stones in a sequential model did not permit use of substrate loadings above 20% as occurred in standard SSF. Fermentation ceases when substrate concentration increases up to 30%, confirming the formation of unknown toxic compounds during steam explosion pretreatment.

Considering carbohydrate composition of raw materials and yields for the different SSF conditions studied, it can be concluded that lignocellulosic byproducts from olive oil extraction by the two-phase centrifugation process are suitable feedstocks for ethanol production.

The most suitable scheme to obtain ethanol by the SSF process from pulp would be a fed-batch operation mode using untreated pulp at 15% + 7.5% + 7.5% addition each 24 h. At this condition, 10 kg of pulp will yield 1 L of ethanol.

Owing to olive stone fraction, sulfuric acid impregnation prior to steam explosion pretreatment improves SSF yields. Substrate loading above 20% was not suitable because of strong inhibition of ethanol production. The preferred SSF conditions would be 10% acid steam-exploded olive stones and 15 FPU/g enzyme loading. In these conditions, SSF yields of about 59% of theoretical yield can be obtained; thus, by using 6 kg of acid-exploded stones, 1 L of ethanol could be obtained.

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