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Submerged Citric Acid Fermentation on Orange Peel Autohydrolysate

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The citrus-processing industry generates in the Mediterranean area huge amounts of orange peel as a byproduct from the industrial extraction of citrus juices. To reduce its environmental impact as well as to provide an extra profit, this residue was investigated in this study as an alternative substrate for the fermentative production of citric acid. Orange peel contained 16.9% soluble sugars, 9.21% cellulose, 10.5% hemicellulose, and 42.5% pectin as the most important components. To get solutions rich in soluble and starchy sugars to be used as a carbon source for citric acid fermentation, this raw material was submitted to autohydrolysis, a process that does not make use of any acidic catalyst. Liquors obtained by this process under optimum conditions (temperature of 130 °C and a liquid/solid ratio of 8.0 g/g) contained 38.2 g/L free sugars (8.3 g/L sucrose, 13.7 g/L glucose, and 16.2 g/L fructose) and significant amounts of metals, particularly Mg, Ca, Zn, and K. Without additional nutrients, these liquors were employed for citric acid production by *Aspergillus niger* CECT 2090 (ATCC 9142, NRRL 599). Addition of calcium carbonate enhanced citric acid production because it prevented progressive acidification of the medium. Moreover, the influence of methanol addition on citric acid formation was investigated. Under the best conditions (40 mL of methanol/kg of medium), an effective conversion of sugars into citric acid was ensured (maximum citric acid concentration of 9.2 g/L, volumetric productivity of 0.128 g/(L·h), and yield of product on consumed sugars of 0.53 g/g), hence demonstrating the potential of orange peel wastes as an alternative raw material for citric acid fermentation.

KEYWORDS: Orange peel; autohydrolysis; citric acid; *Aspergillus niger*

INTRODUCTION

World production of citrus fruits has experienced continuous growth in recent decades, Brazil, the Mediterranean countries (particularly Spain and Italy), the United States, and China being the main producing countries, representing more than two-thirds of global production. FAO estimated a total citrus production of more than 105 million tons per year in the period 2000–2004 (*1*). Oranges constitute the bulk of citrus fruit production, accounting for more than half of global production in 2004 (*2*). A large portion of this production is addressed to the industrial extraction of citrus juice, which leads to huge amounts of residues, including peel and segment membranes. The management of these wastes, which produce odor and soil pollution, represents a major problem for the industries involved (*3, 4*). Some attempts were made to use these residues as livestock feed, although their low nutritional value allowed only limited success (*5*). Other applications included the extraction of pectin (*6*), the recovery of essential oils, the production of clouding or thickening agents, and the removal and purification of carotenoids to obtain natural pigments suitable for food or juice coloring (*7*).

This byproduct contains also soluble and other insoluble carbohydrates, which make it an attractive potential feedstock for value-added products, by preliminary chemical or enzymatic hydrolysis and subsequent biological conversion. Soluble sugars contained in orange peel are glucose, fructose, and sucrose, whereas the insoluble polysaccharides of its cell walls are basically cellulose, hemicellulose, and pectin. Hemicellulose is composed principally of xylose units linked together by β-1,4 linkages, but may also contain hexoses and sugar acids (i.e., uronic acid), whereas pectin is mainly constituted by uronic acids and other sugars such as rhamnose and galactose (*8, 9*).

The enzymatic hydrolysis of this residue with crude commercial preparations containing pectinases, cellulases, and also hemicellulases, releases glucose from cellulose, uronic acids from pectin, and arabinose, rhamnose, galactose, and xylose from both pectin and hemicelluloses. Large amounts of glucose

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and fructose entrained in the peel tissues are also released together with some inhibitory compounds, primarily residual limonene, which should be removed before fermentations can proceed (*10*). However, such enzyme mixtures are quite complex and expensive at the moment, because more than a dozen individual enzymatic activities appear to be necessary for complete hydrolysis to monomeric sugars. Furthermore, knowledge of the structure and cross-linking of polysaccharides in cell walls is still incomplete, and there is uncertainty in the transfer of structural information from one plant tissue to another (*8*).

Hydrolysis with dilute mineral acids (prehydrolysis) would offer an alternative to solubilize a large fraction of the citrus peel, providing an insoluble residue made up of cellulose and lignin and releasing solubilized pectin mixed with soluble mono- and oligosaccharides from the peel. However, it releases a variety of sugar degradation products such as furfural and hydroxymethylfurfural (HMF), extractive-derived compounds, phenolics from lignin degradation (acid-soluble lignin), acetic acid, and uronic acids (*11*). The main drawbacks of prehydrolysis are (a) the equipment corrosion caused by the mineral acid, (b) the cost of reagents (the acid used as a catalyst as well as the neutralizing agent), (c) the handling of neutralization sludge, (d) the possibility that the lignin remaining in solid phase undergoes repolymerization reactions, hence limiting its potential for further chemical utilization, and (e) the generation of compounds acting as fermentation inhibitors (*12*).

Alternatively, autohydrolysis in aqueous media, where water and the raw material are the only reagents, shows several advantages for fractionation when compared with prehydrolysis (*13*). This procedure ensures hemicellulose depolymerization, by the catalytic action of hydronium ions mainly from in situ generated compounds (such as acetic, uronic, and phenolic acids), which leads to the release of oligosaccharides, free sugars, and acetic acid as the main reaction products. The mildly acidic conditions used result in liquors with reduced concentration of inhibitors, particularly those from sugar decomposition such as furfural and HMF (*14*). The solid phase from autohydrolysis contains cellulose (which remains practically unsolubilized), lignin, residual hemicelluloses, and residual pectins (*9, 12*).

The hydrolysis liquors obtained after this treatment could be used to make fermentation media suitable for a variety of purposes, including citric acid production. Citric acid is widely used in the food, beverage, pharmaceutical, and cosmetic industries and finds applications in a variety of other industries, from textiles to electroplating (*15*). In addition, production of citric acid could offset the disposal costs of the wastes (*16*). The present study was undertaken to investigate in detail the orange peel composition in order to examine the effect of autohydrolysis conditions on sugars solubilization as well as the suitability of the resulting liquors for citric acid production.

MATERIALS AND METHODS

Raw Material. Samples of Valencia orange (*Citrus sinensis*) peel obtained from a national citrus processing plant were directly milled, without any earlier pretreatment, to a particle size of 2 × 2 cm, dried at 40 °C to a moisture content of 0.07 g/g (about 72 h), and then submitted to a second stage of milling to a particle size of <2 mm. It was homogenized in a single lot to avoid compositional differences and stored at 4 °C in a cold chamber until use.

Characterization of the Raw Material. Samples of the raw material from the homogenized lot were submitted to determination of nitrogen, carbon, hydrogen, and sulfur contents by means of a flash elemental analyzer model 1112 (Thermo Finningan, San Jose, CA), whereas oxygen was calculated as difference from the ash content. All samples were prepared in triplicate.

Protein content was calculated by multiplying the elemental N content by the universal factor of 6.25.

Ash content was determined according to ISO recommended standard 936:1998 (*17*). Ashes were assayed for metal ions (Fe, Mn, Mg, K, Na, Ca, Cu, Zn, Al, Cr, and Ni) by an atomic absorption spectrometer model 220 Fast Sequential (Varian, Palo Alto, CA). To this purpose, 0.15 g of ashes was previously digested with 5 mL of HNO_3 65% (w/w), 1.0 mL of H_2O_2 30% (w/w), and 0.5 mL of HF 40% (w/w) in a Microwave Labstation mls 1200 mega (Milestone, Bergamo, Italy).

The neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined according to the methods of Goering and van Soest (*18*) with the aim to get a rough estimate of the contents of cellulose, hemicellulose, and lignin.

Pectins were determined according to the method of Tibensky et al. (*19*). This method includes acidification with 2.0 M acetic acid to pH 4.0, precipitation with 1% $CuSO_4$, repeated washing of the precipitate with water, dissolution of the pellet in 1.5 M ammonia citrate buffer, pH 9.5, and measurement of absorption at 590 nm after 30 min in 0.25% cuprizon (Merck, Darmstadt, Germany). The amounts of soluble pectins were expressed as percent of the total pectin content in raw material.

Fat from orange peels was extracted by repeated percolation with *n*-hexane under reflux in a Soxhlet (Selecta, Madrid, Spain). Dried samples (5.0 g) were placed in a porous cellulose thimble located in an extraction chamber, which was connected to a flask below containing the solvent (100 mL) and an upper condenser. After heating the flask in an oil bath at 80 °C, the solvent evaporated, entered the condenser, condensed, and trickled into the extraction chamber containing the sample. At the end of the extraction, which lasted for 24 h, the flask containing the solvent and fat was oven-dried to constant weight at 90 °C. The remaining fat was measured by weight and expressed as percent of the raw material.

Orange peel and extracted pectins were submitted to quantitative acid hydrolysis (QAH) according to standard methodology (TAPPI T13m method). The liquors from QAH were filtered through membranes with 0.20 μm pore diameter and assayed for monosaccharides and other monomers by high-performance liquid chromatography (HPLC) on a model 1100 (Agilent, Palo Alto, CA) fitted with a RI detector (as described later). The uronic acid polymers (UAP) in the liquid samples were determined according to the method of Blumenkrantz and Asboe-Hansen (*20*), whereas the solid residue from QAH was labeled as insoluble residue (AIR).

Monosaccharides and other monomers present in these liquors were assayed for sucrose, glucose, fructose, xylose, mannose, rhamnose, galactose, arabinose, acetic acid, furfural, and HMF. Separation was achieved using an ION-300 column (Transgenomic, San Jose, CA) employing water as mobile phase. In HPLC chromatograms, sucrose (S) was eluted first, followed by glucose (G) and fructose (F), whereas xylose (X), mannose (Mn), and galactose (Ga) were eluted together in a third peak (labeled XMG), and arabinose (A) and rhamnose (R) were eluted together in a fourth peak (labeled AR).

To obtain more information on the saccharidic compounds of orange peel, dry samples (5 or 9 g, according to the needs) were extracted with 100 mL of ethanol 96% (v/v) or 90 mL of distilled water, during 24 or 2 h, respectively. In the former case, the above Soxhlet methodology was employed, whereas in the latter a direct extraction was performed. Extracted fractions were assayed for dry matter by oven-drying at 105 °C (ISO method 638:1978), for monosaccharides and other monomers by HPLC, for oligosaccharides as described later, and for uronic acids using the method of Blumenkrantz and Asboe-Hansen (*20*).

Oligosaccharides (OS) were measured according to a method based on acid posthydrolysis of the liquors. For analytical purposes, samples of liquors were subjected to acid posthydrolysis (treatment with 4% sulfuric acid at 121 °C for 45 min), and the reaction products were assayed according to the same HPLC method as for direct monosaccharides and other monomers determination. The increase in the concentrations of monomers caused by posthydrolysis furnished an indirect measure of the OS concentration, expressed as their respective oligosaccharides: glucooligosaccharides, fructooligosaccharides,

xylooligosaccharides, mannooligosaccharides, galactooligosaccharides, arabinooligosaccharides, and rhamnooligosaccharides (*21*). The insoluble fraction was submitted to QAH, and obtained liquors were assayed for monosaccharides and other monomers by HPLC. The results allowed the determination of the contents of glucose polymers [here labeled glucan (Gn), including cellulose, starch, and other glucose-containing polysaccharides], polysaccharides of xylose, mannose and/or galactose (XMGn), polysaccharides of arabinose and/or rhamnose (ARn), and acetyl groups.

Starch was extracted from the dried insoluble residue obtained after ethanol extraction according to the method of Lafta and Lorenzen (*22*). The dried residue was rehydrated in water, heated for 1 h at 90 °C, and incubated with an amyloglucosidase solution (10 units/mL, 20 mM NaF, 100 mM acetate buffer, pH 4.5) for 48 h at 40 °C, and the glucose released was determined colorimetrically in a glucose oxidase coupled reaction.

All of the percentages are referred to a dry solid basis.

Autohydrolysis Treatment. Orange peels were subjected to nonisothermal autohydrolysis in a 600 mL Parr 4842 reactor (Parr Instruments, Moline, IL) under different conditions of temperature (in the range of 100–200 °C), with a liquid/dry solid ratio of 8.0 g/g. After autohydrolysis, the solid and liquid phases were separated by filtration. The liquid phase was assayed for monosaccharides and other monomers by HPLC.

Microorganism and Fermentation Conditions. *Aspergillus niger* CECT-2090 (ATCC 9142, NRRL 599), obtained from the Spanish Collection of Type Cultures (Valencia, Spain), was used in this work. The microorganism was grown on potato dextrose agar slants (Scharlau, Barcelona, Spain) at 33 °C for 5 days. Spore suspensions containing about $0.78–1.09 \times 10^5$ colony-forming units (CFU)/mL were prepared by adding 3.0 mL of sterile distilled water to the slant and shaking vigorously for 1 min, and subsequently used as inoculum. Orange peel hydrolysate (40 g) was dispensed into 100 mL Erlenmeyer flasks and autoclaved at 121 °C for 20 min. Each flask was inoculated with 0.4 mL of the spore inoculum suspension. In selected experiments calcium carbonate was added in excess amount (20 g/L) to guarantee the neutralization of all the citric acid produced, supposing a conversion of 1 mol of citric acid/mol of sugar. Methanol (0, 20, 40, 60, or 80 mL/kg) was added in selected experiments, and the flasks were incubated at 30 °C in an orbital shaker (New Brunswick, Edison, NJ) at 200 rpm.

At given reaction times, samples were withdrawn from the fermentation media, centrifuged, filtered, and analyzed by HPLC for glucose, fructose, sucrose, and citric acid determination.

Spore concentration in the suspensions was determined by colony formation assay (CFU). To this purpose, serial dilutions were made with 0.5% w/w NaCl. One hundred microliters of each dilution was inoculated in triplicate into plates (containing potato dextrose agar) and incubated at 33 °C for 2–4 days. Cell concentration was calculated from plates having a suitable number of colonies (30–300).

All of the experiments were performed in triplicate, and means are indicated in the text. Data were treated by analysis of variance (ANOVA) using the SPSS 14.0 statistical software package, and significant differences were assessed by Tukey's test at $p < 0.05$.

RESULTS AND DISCUSSION

Orange Peel Characterization. The characterization of the orange peel started with the determination of its elemental composition, which resulted to be the following (wt %): C, 45.1; N, 1.04; H, 5.95; S, 0.00; and O, 44.4. Meanwhile, the determination of metals showed the highest contents (mg/kg) for K (8,297), Ca (5,457), Mg (827), and Na (506). All of these metals are usually part of the culture media recommended for the cultivation of several microorganisms. Besides, other fundamental metals were shown to be present in smaller amounts (mg/kg): Zn, 4.95; Mn, 4.60; Fe, 15.1; Al < 105; Ni < 20; Cu, 6.00; Cr < 10. These elements are usually considered to be fundamental in several different fermentation processes. For example, in the field of lactic acid production, Zhou et al. (*23*) recommended the use of K, Mg, Zn, Cu, Fe, and Mn for the cultivation of *Rhizopus oryzae* ATCC 52311, whereas Mercier et al. (*24*) elaborated a fermentation broth containing Na, K, Mg, Mn, and Fe for the fermentation by different *Lactobacillus* strains. According to Kubicek (*25*), the essential metals for the growth of *A. niger* are Fe, Zn, Cu, Mn, and Co, the levels of most of them being fundamental to increase the yields of citric acid in poor broths. Tran et al. (*16*) reported that supplementation of Fe^{2+} between 1 and 10 ppm in the cultivation medium based on pineapple waste in solid-state fermentation by *A. niger* increased citric acid concentration by 22% when compared with the control.

Table 1. Composition of Orange Peels after Extraction with either Ethanol or Water (Percent on Dry Basis)

	ethanol	water
Extractives		
yield (g/g)	0.25	0.45
glucose	3.61	13.1
sucrose	30.1	11.4
fructose	8.52	16.2
glucooligosaccharides	12.0	5.34
uronic acid polymers	0.11	11.8
AIR[a]	25.0	3.50
Composition of the Insoluble Fraction		
yield (g/g)	0.75	0.54
glucans	18.0	16.9
XMGn[b]	11.7	11.5
ARn[c]	9.89	6.46
acetyl groups	1.20	1.00
uronic acid	23.2	23.9
AIR[a]	2.95	9.68

[a] Acid insoluble residue. [b] Polysaccharides made up of xylose, mannose, and/or galactose. [c] Polysaccharides made up of arabinose and/or rhamnose.

The following effort to characterize the orange peel dealt with solubilization with ethanol or water. This step is an important one because soluble sugars in orange peel can account for up to 38–40 wt % (*26*). **Table 1** shows that the extraction with ethanol was able to solubilize 25.0 wt % of the raw material and that 55.8 wt % of this fraction was constituted by fructose, sucrose, glucose, and glucooligosaccharides. According to Grohmann et al. (*8*), there is a considerable variation in the contents of glucose, sucrose, and fructose that undergo relatively rapid change during maturation of the fruit, possibly due to invertase activity. The acid insoluble residue (AIR) obtained after acid posthydrolysis of the ethanol extract, which represented 25.0 wt %, was composed of waxes, fats, and essential oils, among others. Besides, taking into account that there were no other solubilized sugars in the ethanol extract, the uronic acid polymers, glucan, XMGs, and ARn had to be part of more complex structures insoluble in ethanol, such as cellulose, hemicelluloses, and pectins.

On the contrary, the extraction with water at 100 °C for 2 h was able to solubilize up to 45.9 wt % of the orange peel. Once again, fructose, sucrose, glucose, and glucooligosaccharides were the main components of this fraction (46.0 wt %), representing 20.7 wt % of the initial orange peel. This value was about 50% higher than that obtained by ethanol extraction, which demonstrates the better extraction ability of water. Furthermore, the uronic acid polymers (UAP) represented 11.8 wt % of the soluble extract, from pectins soluble in water. No other components such as XMGs or ARn were solubilized. Such a UAP content, which accounted for 5.31 wt % of the raw material

Table 2. Orange Peel Composition (Percent on Dry Basis)

compound	%
soluble sugars	16.9
starch	3.75
fiber	
cellulose	9.21
hemicelluloses	10.5
lignin	0.84
pectins	42.5
ashes	3.50
fats	1.95
protein	6.50
other compounds	4.35

and 12.5 wt % of the total amount of pectins (**Table 2**), represented the high soluble or high methoxyl pectin fraction.

Table 2 shows the overall orange peel composition: soluble sugars, 16.9 wt %; starch, 3.75 wt %; fiber (cellulose, 9.21 wt %; hemicelluloses, 10.5 wt %; lignin 0.84 wt %; and pectins, 42.5 wt %), ashes, 3.50 wt %; fats, 1.95 wt %; and proteins, 6.50 wt %. The small amount not quantified (about 4.35 wt %) likely included other substances of minor concern for this work, principally organic acids such as citric, malic, malonic, and oxalic acids that may represent about 1 wt % of the peel (*27, 28*), and vitamins such as vitamin C, because most of the ascorbic acid of the fruit is in the peel and only about one-fourth appears in the juice. In this way, it was possible to find around 10–20 mg/kg in the albedo and 15–30 mg/kg in the flavedo. Such components, although minor, have a great nourishing value for several microorganisms, hence suggesting that orange peel could have great potential in biotechnological processes. Moreover, high contents in ashes (3.50 wt %), which are a source of trace elements, and proteins (6.50 wt %) were detected. These results are very similar to those obtained by Grohmann et al. (*8*), who found 3.41 wt % of ashes and 6.06 wt % of proteins working with oranges from Florida, and to those reported by Ma et al. (*3*), who found 3.59 wt % of ashes and 5.25 wt % of proteins in oranges from Yucatán (Mexico).

The fat (hexane extract) content of dried orange peel, made up mainly of terpenoids, aldehydes, ketones, and aliphatic esters, was slightly lower (1.95 wt %) than that found by other authors (3.1–4.9%) (*29, 30*). Among them, it is important to point out the limonene, which is assumed to represent approximately 95 wt % of the total. These oils can be easily recovered by extraction with apolar organic solvents such as hexane.

In this study, the most important components were monosaccharides (glucose or fructose), disaccharides (including sucrose), and polysaccharides. Among these polymers, it is necessary to highlight cellulose (9.21%), hemicelluloses (10.5%), and pectins (42.5%) that represented the major fractions.

Another experimental approach, the quantitative acid hydrolysis, allowed determining the carbohydrate composition listed in **Table 3**. It is noteworthy that the aggressiveness of the method led to the well-known degradation of fructose because of its instability in acidic medium, thereby giving a final fructan percentage of zero. The acid insoluble residue (AIR) content (9.66 wt %) was similar to that found by Grohmann et al. (*8*) (8.7 wt %); as expected, it is a quite higher value when compared with the Klason lignin (0.84 wt %) determined according to the fiber detergent analysis (**Table 2**). This can be explained by taking into account the presence of other substances that also have a tendency to precipitate in acidic medium, such as flavonoids and some waxes. The flavonoid content of the peel of mature Valencia orange was in fact estimated at around 2–4 wt % dried solids (*31*).

Table 3. Composition of Orange Peels and Pectins after Quantitative Acid Hydrolysis (Percent on Dry Basis)

compound	orange peel	pectins[a]
polymeric uronic acid	20.5	47.8
glucan	21.6	0.00
XMGn[b]	10.9	11.8
ARn[c]	7.49	3.86
fructan	0.00	0.00
acetyl groups	0.94	1.30
AIR[d]	9.66	2.74
TUC[e]	28.9	32.4

[a] Percentages referred to the dry weight mass of the pectin fraction. [b] Polysaccharides made up of xylose, mannose and/or galactose. [c] Polysaccharides made up of arabinose and/or rhamnose. [d] Acid insoluble residue. [e] Total unidentified compounds.

Taking into account that the percentage of pectins is 42.5 wt % (**Table 2**) and the one of UAP 20.5 wt % (**Table 3**), it is reasonable to infer that pectins should be constituted not only by uronic acids but also by XMGn and ARn. To confirm this hypothesis, extracted dry pectins were analyzed by QAH, the results of which are also listed in **Table 3**.

With regard to the high value of glucan (21.6 wt %) (**Table 3**), it should be stressed that it was significantly higher than the percentage of cellulose (9.21 wt %) quantified according to the method of determination of fiber (**Table 2**). This suggests that glucose was likely not only to be released from the cellulose and starchy fractions, but also, in minor extent, to form part of hemicellulose and even to be present in free form (as monomer or as part of free sucrose).

Autohydrolysis of Orange Peel. It is well-known that the acid prehydrolysis of lignocellulosics (*32*) releases mainly pentoses from the hemicellulosic fraction, which can only hardly and slowly be used as a carbon source for citric acid production. For this reason, autohydrolysis with hot water has been selected in this work to solubilize mainly the simple hexoses (mono- and disaccharides) contained in the raw material. The solubilization of sugars by autohydrolysis in a non-isothermal regimen is a novel technology to processing of orange peel. Two main variables are to be considered for this treatment: the liquid/solid ratio (fixed at 8.0 g/g) and the maximum temperature of treatment (100–200 °C).

Figure 1 shows the composition in fermentable sugars of liquors obtained when orange peel was submitted to autohydrolysis under variable conditions. The ANOVA analysis of the data reflecting total sugars concentration in the autohydrolysates confirmed the significance of the effect exerted by the temperature of operation on the solubilization of sucrose, glucose, fructose, and arabinose (see **Table 4**). A rise in temperature from 100 to 130 °C induced a significant ($p = 0.016$ according to t test) increase in the overall concentration of solubilized sugars up to 38.2 g/L, as the result of increased glucose and fructose levels up to 13.7 and 16.2 g/L, respectively, and a simultaneous decrease in sucrose level up to 8.3 g/L. Over this temperature threshold, glucose and fructose concentrations remained almost constant, whereas that of sucrose progressively decreased, likely because of its hydrolysis catalyzed by acids released by the autohydrolysis. It is noteworthy that, at high temperature, HMF and AR were released even at very low concentrations, but probably other unidentified degradation byproducts were formed. Nevertheless, furfural, the degradation product of xylose, was not detected, because this sugar was not solubilized.

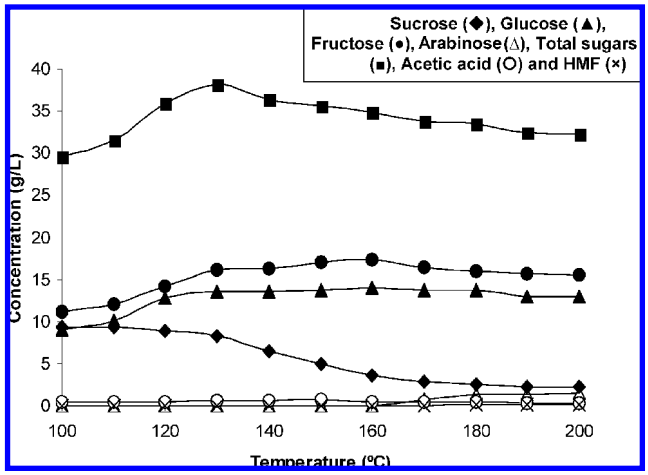

Figure 1. Composition of liquors obtained by orange peel autohydrolysis. Results represent the average of three independent experiments. Standard deviations were below 2.1% of the mean.

Table 4. Concentration (Grams per Liter) of the Total Solubilized Sugars in the Liquors Obtained after Autohydrolysis of Orange Peels at Different Temperatures of Operation[a]

T (°C)	total solubilized sugars
100	29.60a
110	31.59ab
120	35.90bc
130	38.21c
140	36.32bc
150	35.68bc
160	34.91bc
170	33.81abc
180	33.44abc
190	32.51ab
200	32.34ab

[a] Different letters indicate statistically significant differences at $p < 0.05$ according to Tukey's test.

Table 5. Composition of Metals (Milligrams per Kilogram) in Orange Peel Autohydrolysate Liquor

	noncentrifuged hydrolysate	centrifuged hydrolysate
Mg	55.5	56.8
Ca	152	155
Zn	0.59	0.85
Mn	<1.00	<1.00
Na	77.4	55.7
Fe	<3.00	<3.00
Al	<25.0	<25.0
Ni	<5.00	<5.00
Cu	<1.50	<1.50
Cr	<2.50	<2.50
K	806	800

Taking into account these results and the significant differences between total sugars solubilization means according to Tukey's test (**Table 4**), the orange peel liquors obtained by autohydrolysis at 130 °C using a liquid/solid ratio of 8.0 g/g were selected as a culture medium to carry out the biotechnological production of citric acid. To characterize the broth, metal compositions of both raw and centrifuged hydrolysates were determined (**Table 5**). Again, K, Ca, Mg, and Na were present at the highest levels, and no significant difference was observed between hydrolysates centrifuged or not. The concentrations detected for these metals (percentages of solubilizations of 54.3% Mg, 22.5% Ca, 77.4% K, 100% Zn and 100% Na), although lower than those found in the raw orange peel, were still significant and suitable for a medium to be used in subsequent fermentations.

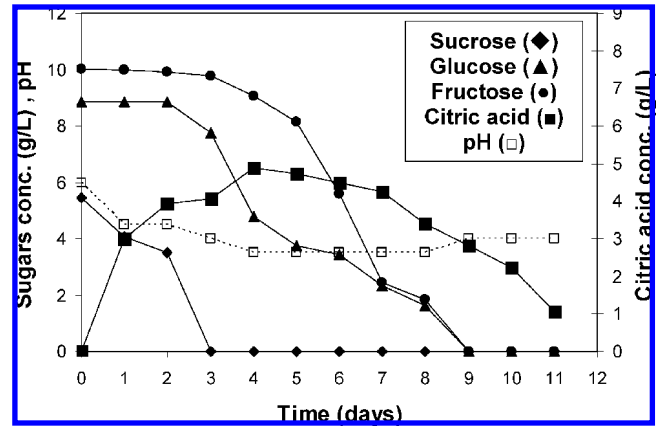

Figure 2. Sugar consumption and citric acid production versus time during citric acid fermentation of orange peel autohydrolysate liquors by *Aspergillus niger*. Results represent the average of three independent experiments. Standard deviations were below 2.4% of the mean.

Citric Acid Fermentation of Orange Peel Autohydrolysate. The medium used throughout this study, obtained by autohydrolysis of orange peel at 130 °C, contained a total sugars concentration of 38.2 g/L with a glucose/fructose/sucrose ratio of 1.0:1.2:0.61. This broth practically lacked furfural and HMF and contained only 0.63 g/L of acetic acid; that is, it had a very low content of the three major inhibitors of the fermentation process (*14*). Having an industrial process in mind, the suitability of *A. niger* CECT 2090 (ATCC 9142, NRRL 599) to ferment sugars to citric acid was assessed. This microorganism was already successfully employed by Aravantinos-Zafiris et al. (*7*) for citric acid fermentation of orange processing wastes.

Figure 2 shows the behavior versus time of sugar consumption, citric acid production, and pH variation, using liquors obtained by autohydrolysis and an initial biomass concentration of 1.09×10^3 CFU/mL. Sucrose was the first sugar to be consumed, followed by glucose and fructose, thus confirming the behavior observed by Hossain et al. (*33*) in synthetic broth containing a mixture of these sugars. From the beginning of the fermentation, this consumption was addressed to the production of citric acid that reached a maximum concentration of only 4.9 g/L after 4 days, and no less than 14 g/L of sugars remained in the medium after this time. The accumulation of citric acid lowered the pH to 3.5, stopped its further formation, and then progressively disappeared. Sugars, entirely consumed after 9 days, were likely destined to both the microbial growth and respiration. Under these conditions, the maximum volumetric productivity and product yield were only 0.051 g/L·h and 0.47 g/g, respectively.

Influence of $CaCO_3$ as Neutralizing Agent on the Citric Acid Fermentation. Several authors investigated the influence of pH on citric acid production. Xu et al. (*34*) reported an optimum value of 4–5, whereas other researchers suggested a range of 5–6 (*33*). Aravantinos-Zafiris et al. (*7*) observed a dramatic increase in citric acid accumulation when the pH was increased from 3 to 4 and an optimum in the range of pH 4–6.

Taking into account that citric acid production can increase considerably the acidity of the fermentation broth, limiting consequently the capacity of the microorganism to ferment all sugars, a batch fermentation was carried out under the same conditions as the previous one but in the presence of $CaCO_3$ in the fermentation broth to neutralize the citric acid released. This

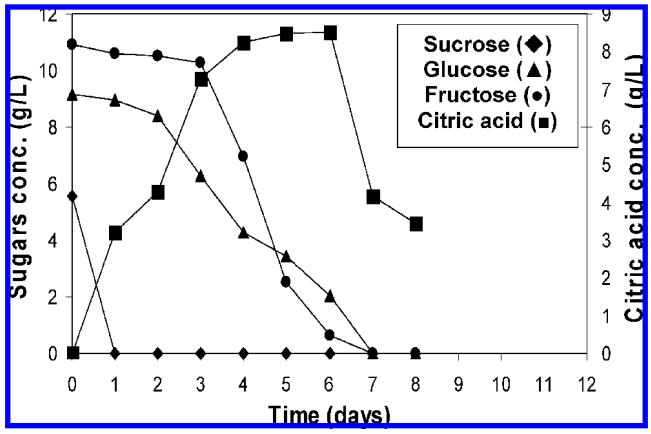

Figure 3. Sugar consumption and citric acid production versus time during citric acid fermentation of orange peel autohydrolysate liquors by *A. niger* in the presence of $CaCO_3$. Results represent the average of three independent experiments. Standard deviations were below 2.4% of the mean.

technique was already used with success by other authors to neutralize the lactic acid generated by different *Lactobacillus* strains (*35, 36*).

As expected, the pH of the fermentation remained constant around 6. In this case, the sugars consumption followed a similar trend, but the concentration of citric acid reached 8.3 g/L after 4 days, whereas 11.3 g/L of residual sugars remained in the medium (**Figure 3**). During the following 2 days, the citric acid concentration grew only slightly, achieving a maximum value of 8.5 g/L, while the unconsumed sugar level lowered to 2.7 g/L, likely due to high biomass growth. Afterward, the citric acid was consumed very quickly as a carbon source owing to the lack of carbohydrates in the medium. Under these conditions, the maximum volumetric productivity increased to 0.086 g/(L·h) and the product yield to 0.57 g/g. These results demonstrate that $CaCO_3$ is necessary to maintain a suitable pH in the fermentation broth.

Influence of Methanol Addition on Citric Acid Fermentation. As is well-known, methanol is able to increase the yield of citric acid production by *A. niger* strains (*7, 37, 38*), the need being to establish its concentration according to conditions. Low methanol concentrations are usually necessary to eliminate the adverse effect of trace metals, whereas high concentrations are used for highly contaminated materials. However, the addition of excess alcohol was shown to be inhibitory when added to synthetic broths (*39*). *A. niger* does not assimilate methanol, and, although its exact role in the stimulation of citric acid production is not yet known, it is believed that methanol increases the permeability of the microorganism cell membrane, thereby making the excretion of citric acid easier (*40*).

Figure 4 shows the time profiles of sugar consumption and citric acid produced using liquors obtained from autohydrolysis of orange peel supplemented with both calcium carbonate and methanol in the concentration range from 0 to 80 mL/kg. All fermentations were carried out with an initial biomass concentration of 0.78–0.81 $\times$ 10^3 CFU/mL and after adaptation of the microorganism in precultivations in the same medium containing methanol.

In all cases, citric acid production increased to a maximum threshold, beyond which it progressively decreased as soon as practically all sugars were consumed. Once again, sucrose was the first sugar to be completely metabolized, followed by glucose and fructose. It is necessary to emphasize that sucrose was completely consumed in all cases, but the rate of this consumption increased with increasing methanol concentration up to 40 mL/kg and decreased beyond this threshold. On the other hand, part of the glucose and fructose remained unconsumed when experiments were conducted with 60 and 80 mL/kg of methanol,

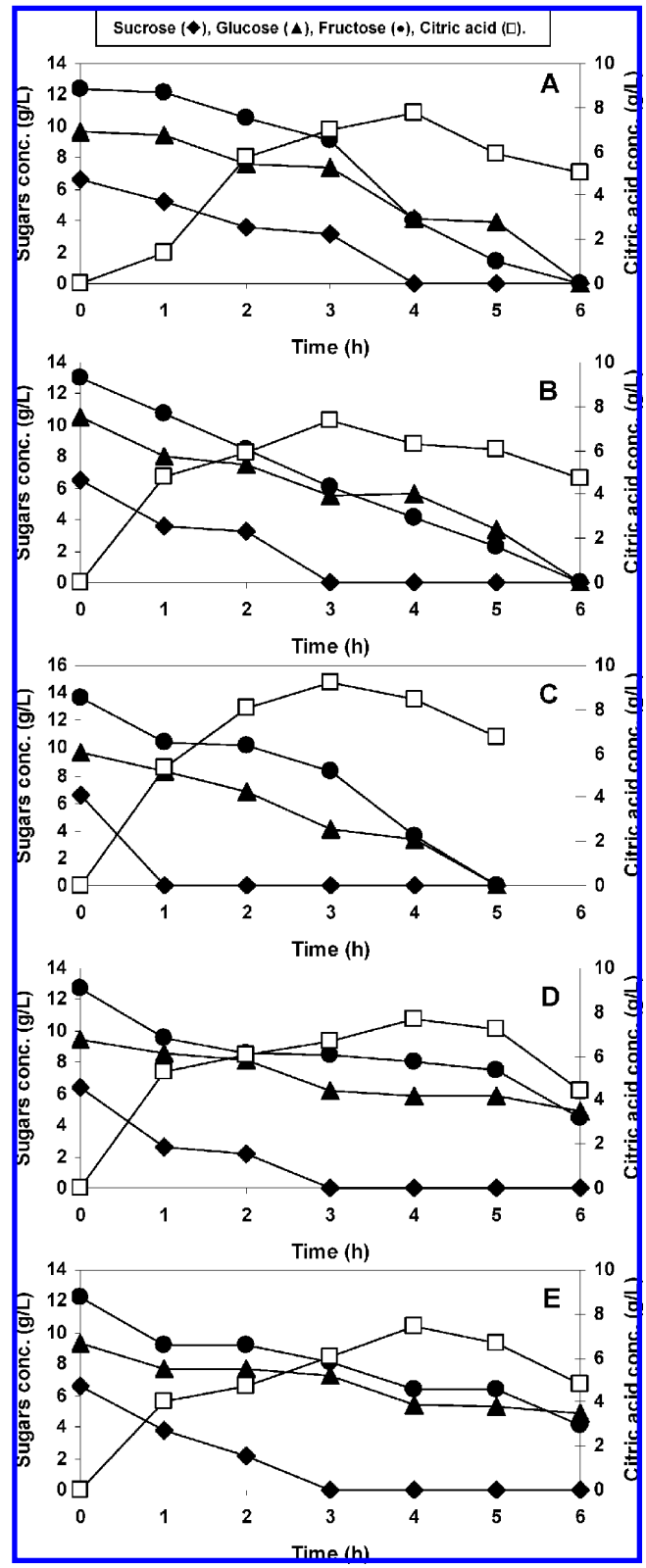

Figure 4. Effect of methanol addition on citric acid production from orange peel autohydrolysate liquors by *A. niger* in the presence of $CaCO_3$. Methanol concentrations: (**A**) 0 mL/kg; (**B**) 20 mL/kg; (**C**) 40 mL/kg; (**D**) 60 mL/kg; (**E**) 80 mL/kg. Results represent the average of three independent experiments. Standard deviations were below 2.1% of the mean.

Table 6. Effect of Methanol Addition on Citric Acid Production from Orange Peel Autohydrolysate Liquors by *Aspergillus niger* in the Presence of $CaCO_3$

methanol (mL/kg)	t [a] (days)	max citric acid[b] (g/L)	glucose (g/L)	fructose (g/L)	residual sugars [c] (g/L)	consumed sugars (g/L)	$Y_{P/S}$ [d] (g/g)	Q_P [e] [g/(L·h)]
0	4	7.7a	4.2	4.0	8.2	20.4	0.38	0.080
20	3	7.4a	5.6	6.1	11.6	18.5	0.40	0.103
40	3	9.2b	4.1	8.3	12.4	17.4	0.53	0.128
60	4	7.7a	5.9	8.0	13.9	14.7	0.52	0.080
80	4	7.5a	5.4	6.4	11.8	16.4	0.45	0.078

[a] Time of maximum citric acid concentration. [b] Letters indicate statistically significant differences at $p < 0.05$ according to Tukey's test. [c] Sucrose was completely consumed in all runs. [d] Yield of citric acid on consumed sugars. [e] Citric acid volumetric productivity calculated at time t.

which clearly demonstrates that high concentrations of this alcohol inhibited the fermentation.

Table 6 summarizes the main results and kinetic and yield parameters of these fermentations. The highest values of citric acid concentration (9.2 g/L), product yield on consumed sugars ($Y_{P/S} = 0.53$ g/g), and productivity ([$Q_P = 0.128$ g/(L·h)] were achieved within 3 days in the presence of 40 mL/kg methanol. Less methanol is usually required to stimulate citric acid release in solid-state fermentations. Tran et al. (*16*), using pineapple waste and *A. niger* ACM 4992 (ATCC 9142), did in fact obtain the highest citric acid yield ($Y_{P/S} = 0.74$ g citric acid/g of consumed sugar) using only 30 mL/kg of methanol. Hang et al. (*37*) reported optimal methanol concentration of only 20 mL/kg in solid-state fermentation of kiwifruit peel by *A. niger* ATCC 9142, obtaining 82 g/L of citric acid after 5 days from 168 g/L of initial sugars [$Q_P = 0.683$ g/(L·h); $Y_{P/S} = 0.60$ g/g). Similar results were reported by Zhang (*41*) for the solid residue of an orange juice factory and by Kang et al. (*42*) for tangerine peel. In the latter case, a semisolid culture was used in the presence also of 0.2% HNO_3 and 0.1% $MgSO_4 \cdot 7H_2O$ (w/w). Flores et al. (*43*) observed maximum citric acid production by *A. niger* [380 g of monohydrate citric acid/kg of dry skin; $Q_P = 0.539$ g/(L·h); $Y_{P/S} = 0.68$ g/g] in solid-state fermentation of prickly pear peel (5 days at 30 °C and 86% humidification), although they needed an inoculum of no less than 175 g biomass/kg of dry skin. Nevertheless, de Lima et al. (*44*), using *A. niger* ATCC 1015 and pineapple waste in solid-state fermentation, achieved the highest production of citric acid by addition of 40 mL/kg methanol as in the present work, obtaining 132 g/kg after 6 days.

These results can be considered quite promising taking into account (a) the high environmental impact and the huge production of this residue that needs disposal or recycling, (b) its relatively low average sugar content ($S_0 = 25.5$ g/L), and (c) the fact that fermentations were only preliminary, needing a rigorous optimization of conditions. Nevertheless, they compare to the results reported by Aravantinos-Zafiris et al. (*7*) for citric acid fermentation from orange processing wastes having almost twice the sugar content ($S_0 = 55$ g/L), using the same fungal strain and the same methanol concentration [citric acid concentration = 30 g/L, $Q_P = 0.104$ g/(L·h), and $Y_{P/S} = 0.63$ g/g].

Finally, despite the relatively low citric acid concentrations achieved in this work due to the low initial sugars concentration, the yields are comparable to or even higher than those obtained for commercial submerged citric acid production using other raw materials such as hydrocarbons, starchy materials, and molasses (*45*). These authors reported a citric acid concentration of 27 g/L with a yield of 45%, using wood hemicellulose and *A. niger* IMI-41874; meanwhile, Adham (*46*) achieved a maximum concentration of 8.6 g/L (9.8% conversion) using beet molasses and *A. niger* A20.

Conclusions. Orange peel is a large volume industrial waste that is underutilized as animal feed or even disposed of, thus causing serious environmental problems. The composition analysis revealed that soluble sugars, cellulose, and pectins are the most outstanding fractions, but proteins and metals play also an important role. A treatment of autohydrolysis at 130 °C and liquid/solid ratio of 8.0 g/g, a novel technology for this material, had a beneficial effect on its hydrolysis, producing liquors rich in soluble sugars, mainly sucrose, glucose, and fructose, which could be utilized for citric acid production by *A. niger*.

The strong pH decrease in the fermentation broth consequent to citric acid release limited its fermentability, making necessary the addition of calcium carbonate to neutralize the product. Furthermore, increasing the concentration of methanol to 40 mL/kg enhanced the production of citric acid from orange peel, but higher levels exerted an inhibitory effect. The experimental data presented in this study showed that fermentation of orange peel autohydrolysate by *A. niger* did not require any supplementation of additional nutrients and that, in the presence of $CaCO_3$ and 40 mL/kg methanol, sugars were quantitatively consumed and citric acid was produced with promising yield, thereby showing the viability of citric acid production from this industrial waste.

Future investigation will be devoted to the optimization of this process.

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