JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enzymatic Treatment To Improve Extraction of Capsaicinoids and Carotenoids from Chili (*Capsicum annuum*) Fruits

MANUEL SALGADO-ROMAN, ENRIQUE BOTELLO-ÁLVAREZ, RAMIRO RICO-MARTÍNEZ, HUGO JIMÉNEZ-ISLAS, MARCELA CÁRDENAS-MANRÍQUEZ, AND JOSÉ LUIS NAVARRETE-BOLAÑOS*

Departamento de Ingeniería Química-Bioquímica, Instituto Tecnológico de Celaya, Avenida Tecnológico s/n, C.P. 38010, Celaya, Gto., Mexico

Enzymatic treatments using noncommercial enzymes as a means to the improve the extraction of carotenoids and capsaicinoids from chili fruits are explored in this study. The results show that it is possible to obtain chili fruit powder with a higher concentration of both capsaicinoids and carotenoids than previously reported for similar processes. Furthermore, extraction yields above 96% for carotenoids and 85% for capsaicinoids as separate fractions can be achieved using a sequential and selective two-stage extraction. Evidence is presented demonstrating that the content and extraction yield depend directly on the extent of the enzymatic hydrolysis of chili cell walls, and higher yields are obtained when the sample is completely hydrolyzed. The enzymatic treatment described here is a promising alternative to current industrial practices, and it improves the extraction of carotenoids and capsaicinoids from chili fruits.

KEYWORDS: Improved production; carotenoids; capsaicinoids; *Capsicum annuum*

INTRODUCTION

Capsicum annuum is the most cultivated pepper in the world due to its unique flavor and pungency. Some species (for example, *C. annuum* L.) are widely used to obtain carotenoids (capsanthin, capsorubin, zeaxanthin, violaxanthin, cryptoxanthin, β-carotene, etc.) for applications in the food industry as natural colorants. Other species (*C. pubescen* or *C. chinense*) are used as a source of capsaicinoids (nordihydrocapsaicin, nonivamide, capsaicin, dihydrocapsaicin, etc.) for the pharmaceutical industry due to their biological functions as therapeutic agents (*1, 2*). Some chili fruits (*C. frutescens*, *C. annuum cayenne*, or *C. annuum bydagi*) are a source of both carotenoids and capsaicinoids, which are extracted via a two-tier solid−liquid extraction in a multistage countercurrent system that uses hexane in the first extraction step and ethanol in the second (*3*−*5*). However, the yield extraction depends heavily on the pretreatment of the solid to enhance the mass transfer during the lixiviation process (*6*). Using traditional processing methods, a combination of mechanical operations (drying, cutting, grinding, and pressing) is used as pretreatments to expose and rupture the chili cell membranes to increase the yield. An alternative to rupturing the cell membranes is based upon enzymatic treatments using commercial enzymes with a broad range of activities (*7, 8*). The results of recent studies have demonstrated that enzymatic treatment of chili powder with Viscozyme L (an enzyme preparation from *Aspergillus* with a wide variety of carbohydrase activities) leads to a significant increase in yield (11% for carotenoids and 7% for capsaicinoids). The treatment takes place at 50 °C, requires 7 h of agitation in a rotary shaker at 120 rpm, and uses a chili powder to water ratio of 1:50 (*7*). Similar results were reported using Extrazyme (with a declared activity of 7500 pectinase S units and a multienzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) and Energex (with declared activity for β-glucanase), which led to yield increases of 24 and 32% for carotenoids and capsaicinoids, respectively. In this case, the treatment takes place at 37 °C, requires 12 h, and utilizes a much smaller chili powder to water ratio of 1:1 (*8*). In addition, both methods rely on a selective two-stage extraction process. After treatment with Viscozyme L, lixiviation with industrial ethanol leads to 83% carotenoid recovery and 60% capsaicinoid recovery. With the Extrazyme-Energex pretreatment, recoveries of 95% for carotenoids and 98% for an oleoresin containing up to 1.7% of the capsaicinoids are found. The process reported by Desikacharya et al. (*8*) appears to have clear advantages when compared with the process described by Santamaría et al. (*7*), including a requirement to add less water, and improved extraction of chili constituents. However, the process uses a multienzyme preparation that is expensive, requires a longer processing time, and involves the recovery of extraction solvents by fractional distillation for solvent reuse. In addition, the main product is an oleoresin with improved color value but limited application

* Corresponding author [e-mail jlnb@itc.mx; telephone (+52) 461 61 1 75 75; fax (+52) 461 61 1 79 79].

10.1021/jf801823m CCC: $40.75
Published on Web 10/11/2008

due to the presence of capsaicinoids. In a previous study it has been demonstrated that, during its growth, the *Rhizopus nigricans* synthesizes an extracellular enzyme with high cellulase activity. The enzymatic extract obtained from the fungus growth increases the cell-wall permeability of several substrates (here, chili samples), and it facilitates the diffusive processes that are inherent to an extraction process. The on-site cellulase production from *R. nigricans* significantly reduces costs and increases the extraction efficiency as compared with commercial cellulase solutions (*9*−*11*).

Following these ideas, we propose an enzymatic treatment that relies upon enzymatic extracts produced on-site by *R. nigricans* for the extraction of carotenoids and capsaicinoids from chili powder. The viability of this treatment is tested on the basis of extraction yield, process time reduction, and cost reduction due to the use of noncommercial enzyme extracts. In addition, on the basis of the premise that chili cells are surrounded by a matrix of insoluble matter formed mainly by cellulose and small quantities of lignin and cutin, histological studies were performed. The relationship between the enzymatic extract activity and the structural changes in chili cells was characterized using microscopy and studied in relation to the extraction efficiency. Finally, we propose a sequential and selective two-stage extraction process with hexane and ethanol that will enable carotenoids and capsaicinoids to be obtained in separate fractions.

MATERIALS AND METHODS

Raw Material. Two different varieties of *C. annuum* chili (*bydagi* and *cayenne*) were used as plant materials in the experiments. *bydagi* fruits were supplied by AVT Natural Products Ltd. (Aluva, Kerala, India), and *cayenne* fruits were obtained from the Mexican market. During the experiments, the chili fruits of both varieties were cut, and the whole fruit was used with stems and seeds.

Microorganisms. *R. nigricans* fungus, which has previously been isolated and characterized as a cellulase producer (*9*), was used to produce enzymatic extracts.

Enzymatic Extracts. *R. nigricans* was cultured on potato dextrose agar (PDA; Difco Laboratory. Detroit, MI) slants at 28 °C for 24 h. The biomass taken from the slants was transferred to 250 mL Erlenmeyer flasks containing 100 mL of PDA (Difco Laboratory) and incubated on a rotary shaker at 28 °C and 100 rpm (Forma Scientific, model 4520) for 24 h for biomass propagation. These conditions led to morphological development in the form of pellets and high cellulase synthesis (*12*). After propagation, the biomass was separated by filtration on Millipore membranes (0.45 μm pore size), and the supernatant obtained was used as an enzymatic extract. The enzyme concentration was determined using the Bradford method (*13*).

Fermentation System. A stirred tank bioreactor (ADI autoclavable bioreactor, Applikon Dependable Instruments, Schiedam, The Netherlands) of 3 L capacity with a dished bottom was used for enzymatic treatments. The system includes a BioConsole (ADI 1035, Applikon Dependable Instruments), a BioController (ADI 1030, Applikon Dependable Instruments) that allows parameters to be accurately set, and a bidirectional serial communication port for supervisory control and data acquisition (BioXpert software, Applikon Dependable Instruments).

Experimental Design for Optimization of the Enzymatic Treatment. To maximize the yield of extraction of carotenoids and capsaicinoids from chili powder as a result of the enzymatic treatment on a stirred vessel bioreactor, the following variables were considered: X_1, temperature; X_2, agitation. These variables were singled out on the basis of their relationship with the enzymatic activity (temperature), the enzyme−substrate interaction induced by mixing, and the inactivation induced by the shear force over the fungus (agitation). The feasible ranges for these variables were defined as 30−36 °C for X_1 and 100−300 rpm for X_2, on the basis of a preliminary screening design. A central composite design for two variables with axial points ($\pm\alpha_i$) was constructed (**Table 1**) as described by Montgomery (*14*). In this scheme, the variable levels are coded (−1 for the lower level, +1 for the upper level, and 0 for the mean value) on the basis of the following equation: (factor level − mean factor value)/[(factor upper level − factor lower level)/2]. In all experimental assays, the ratio between the chili fruits and enzymatic extract was kept constant as 1:14 (w/v), allowing enzyme saturation of the chili fruits.

Table 1. Central Composite Design To Optimize Enzymatic Treatment

assay	X_1 (temperature)	X_2 (agitation)	Y^a (yield)
1	0	0	5.43
2	−1	−1	4.12
3	1	−1	4.72
4	−1	1	4.68
5	1	1	4.25
6	−1.41	0	3.97
7	1.41	0	4.43
8	0	−1.41	4.37
9	0	1.41	4.24
10	0	0	5.31

[a] Grams of carotenoids per kilogram of powder (dw).

Enzymatic Treatment Kinetics. To evaluate the effect of the enzymatic extract on chili fruits, the enzyme solution was blended with dried chilis in a 1:14 (w/v) ratio and kept on the bioreactor at 33 °C and 196 rpm. Samples were taken every 10 min for kinetic studies and to determine the optimum processing time to maximize carotenoid and capsaicinoid contents in the treated samples.

Chili Powder Analysis. The treated samples were divided into two parts; the first part was filtered to separate the solid and liquid phases. The solid phase, the treated chili, was dehydrated in a vacuum oven (Shel Laboratory, model 1430) to up to 10% (±1) moisture content and milled (0.5-mm sieve) using a Brinkmann mill (Brinkmann, Westbury, NY). The powder obtained was analyzed to determine the concentration of carotenoids according to the modified AOAC 970.64 method (*15*), and the concentration of capsaicinoids was determined according to the ISO 7543-1 method (*16*). The second portion was also filtered, and the phases were separated. The solid phase was prepared for microscopy analysis, and the liquid phase was analyzed to determine the amount of soluble reducing sugars, according to the method of Miller (*17*), to monitor cellulase activity.

Carotenoids Analysis Based on the AOAC (970.64) Method. Powder samples were treated with 60 mL of a mixture of hexane, ethanol, acetone, and toluene (ratio 10:6:7:7) (J. T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ) under mild conditions (56 °C for 45 min). When the extraction was finished, 40 mL of the solvent mixture was added. A sample (10 mL) of the liquid phase was mixed with 20 mL of the solvent mixture and 2 mL of a solution of potassium hydroxide (Sigma Chemical Co., St. Louis, MO) in methanol (40% w/v). The resulting solution was incubated for 20 min at 56 °C for saponification. Next, the solution was mixed with 30 mL of hexane and 38 mL of sodium sulfite (Sigma Chemical Co.) solution (10% w/v). After settling, the immiscible phases were separated. The light phase (that contained the free carotenoids) was analyzed by a UV−vis spectrophotometer at 460 nm, and the concentration of carotenoids was obtained on the basis of the following expression: total carotenoids = $(A_{(460nm)} \times 11.57)$/wt of sample]. The same solution was also used for the HPLC analysis.

Capsaicinoids Analysis Based on the ISO 7543-1 Method. Powder samples were extracted in a Soxhlet system with tetrahydrofurane (J. T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ) under mild temperature conditions (60 °C). When the extraction was finished, the solvent was evaporated and the extract was mixed with activated charcoal in a 10:1 (extract to charcoal) ratio. The resulting solution was mixed with 90 mL of methanol (J. T. Baker) and filtered to separate the charcoal; a colorless liquid that contains the capsaicinoids was obtained. Then, two solutions were prepared, one containing 1 mL of the colorless liquid, 2.7 mL of water, and 2 mL of a 1 N chloridric acid solution (Merck, Darmstadt, Germany); and the other containing 1 mL of the colorless liquid, 2.7 mL of water, and 2 mL of a 1 N sodium hydroxide solution (Sigma Chemical Co.). These solutions were analyzed by UV−vis spectrophotometry. Two blank solutions were prepared for

calibration: a blank acid solution [3 mL of water, 2 mL of chloridric acid (1 N), and 20 mL of methanol] and a blank alkali solution [3 mL of water, 2 mL of sodium hydroxide (1 N), and 20 mL of methanol], that were used to calibrate the spectrophotometer at 248 and 296 nm, respectively. The capsaicinoid solutions were processed through the spectrophotometer and their concentrations obtained on the basis of the expression total capsaicinoids = $(A_{(248nm)} \times 2500)/(314 \times$ wt of sample) or total capsaicinoids = $(A_{(296nm)} \times 2500)/(314 \times$ wt of sample).

Capsaicinoid Analysis. To obtain the capsaicinoid concentration values, we constructed a calibration curve using external standards of capsaicin (98% and 2% of nordihydrocapsaicin; Sigma-Aldrich Chemie GmbH) and dihydrocapsaicin (90%; Sigma-Aldrich Chemie GmbH) based on HPLC analysis. The peaks on the chromatogram were associated with a particular component by retention time comparison, and the component profile was obtained using the relative percentage of HPLC chromatogram area covered for each individual standard.

Sample Preparation for Microscopy Analysis. Chili samples treated at different processing times were washed for 24 h at 4 °C with a solution of formaldehyde (37%), ethanol (70%), and concentrated acetic acid in a ratio of 18:1:1. A second wash using ethanol solutions of 80 and 95% was carried out for 24 h to remove the remaining water from the chili tissue. Once the chili samples were dehydrated, the samples were divided into two groups for embedding. One sample was prepared with histoclear solution, and the second was prepared with paraffin at 60 °C. Both samples were further processed by placing them in histoclear and paraffin solutions in a 4:1 ratio, according to the processing steps that had previously been carried out on them, and kept at 60 °C for 8 h. This was followed by paraffin exchange every 12 h for 4 days. The samples were then embedded, gradually cooled to 4 °C, and cut with a microtome (model Jung RM2035, from Leica GmbH, Wetzlar, Germany) to obtain 8 μm slices.

Staining and Microscopy Analysis. The sectioned samples were placed on microscope slides and washed (two steps, 15 min each) with water and with a 5% solution of periodic acid. The samples were then rehydrated in water for 10 min and dyed using the Amido Black method (*18*) that stains the proteins blue and the cellulose red. Each sample was analyzed on a Leica microscope (model DMRAX2, Leica GmbH).

Carotenoid and Capsaicinoid Extraction in a Continuous Multistage Extraction System. The relevant constituents of the powders obtained from the enzymatic treatments were extracted in a cross-current extraction system using analytical grade hexane (J. T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ) for carotenoid extraction or ethanol (J. T. Baker, Mallinckrodt Baker Inc.) for capsaicinoid extraction. The number of stages that yielded the maximum quantity of carotenoids and capsaicinoids was determined from these experiments. In both cases, when the extraction was concluded, the light phase (hexane or ethanol + carotenoids or capsaicinoids) was separated from the heavy phase (solids) and analyzed according to the previously cited methods for determining the carotenoid or capsaicinoid concentrations.

Sequential and Selective Extraction Process. To obtain separate fractions of carotenoids and capsaicinoids from treated chili fruits, a selective extraction process in a cross-current extraction system was implemented. The process starts with carotenoid extraction using hexane until the carotenoid concentration in chili powder is depleted. This is then followed by capsaicinoid extraction using ethanol until depletion.

High-Performance Liquid Chromatography (HPLC) Analysis. For HPLC analysis, a stand-alone module was used, which was composed of an LC1120 advanced spindle-driven pump, an LC1205 programmable UV−vis detector, and a WinChrom Chromatography Management System for computer control (GBC Scientific Equipment, Dandenong, Victoria, Australia). For carotenoid analysis, samples consisting of 20 μL of saponified pigment extract were injected. The analysis was performed using an adsorbosphere column (HS SI, 5 μm, 4.6 × 250 mm, from Alltech, Grace Co., Deerfield, IL). Solvent elution was operated at 1.0 mL/min with a mixture containing 85.5% hexane, 1.5% 2-propanol, and 13% acetone. The separation was performed at room temperature. The pigments were monitored using a 474 nm detector with 1 s and 2 nm as the time and wavelength resolutions, respectively. For capsaicinoid analysis, samples consisting of 20 μL were prepared according to the method described by Hoffman et al. (*19*). The analysis was performed using a Spherisorb ODS2 column (ODS2, 5 μm, 4.6 × 250 mm, from Waters, Milford, MA). Solvent elution was operated at 1.5 mL/min with a mixture containing 50% acetonitrile and 50% water containing 1.0% acetic acid. The separation was performed at room temperature. The capsaicinoids were monitored using a 280 nm detector with 1 s and 2 nm as the time and wavelength resolutions, respectively. In both analyses, every peak on the chromatogram was associated with a particular component by retention time comparison, and the component profile was obtained using the relative percentage of the HPLC chromatogram area covered for each individual component that was identified.

The experimental results described below were performed in triplicate, and the reported values represent the mean values of these experiments and were compared against the values obtained from control samples (powder without enzymatic treatment).

RESULTS AND DISCUSSION

Optimal Operating Conditions for Enzymatic Treatment. To optimize the variables associated with the enzymatic treatment of chili fruits to maximize the extraction yield, the carotenoid concentration of the treated samples was used as a response function. The results obtained from the central composite design are shown in the last column of **Table 1**, and they were used to describe the relationship that exists among independent variables (X_i) and the response function (Y) by constructing a regression-based polynomial model using least-squares. For this case, the regression model obtained was the following:

$$Y = 5.37 + 0.103X_1 - 0.012X_2 - 0.54X_1^2 - 0.26X_1X_2 - 0.49X_2^2$$

Here, the variables are specified in their coded units. In this equation, it is implicitly assumed that the dependence of the response variable with respect to the factors is nonlinear and that the effects of these factors are additive. Statistical analysis of the model indicates that these assumptions are adequate. The location of the optimum provides a simple example of locating an extreme for a multidimensional system via the derivation of the second-order model and solving the resulting set of linear equations:

$$\frac{\partial[Y]}{\partial X_1} = 0.103 - 1.08X_1 - 0.26X_2 = 0$$

$$\frac{\partial[Y]}{\partial X_2} = 0.012 - 0.26X_1 - 0.98X_2 = 0$$

The system solution based on the Newton−Raphson method is the following: $X_1 = 0.105$ and $X_2 = -0.04$. Using the transformation (X_i = optimum factor value [(factor upper boundary − factor lower boundary)/2] + mean factor value), the decoded factor values can be obtained. This procedure indicates that the enzymatic treatment should take place at 33 °C and 196 rpm.

Effects of Enzymatic Treatment on Carotenoid and Capsaicinoid Content in Chili Powder. All chili samples treated with the *R. nigricans* enzymatic extract at a protein concentration of 113.039 μg/mL showed an increase in the yield of both carotenoids and capsaicinoids compared with the control (nontreated chili powder), which was clearly correlated with enzymatic activity. In particular, in the *bydagi* chili fruits, yield increases of 110% (average) for carotenoids [7.1 (±0.1) g of total carotenoids/kg of powder (dry weight)] and 11% (average) for capsaicinoids [5.2 (±0.1) g of capsaicinoids/kg of powder

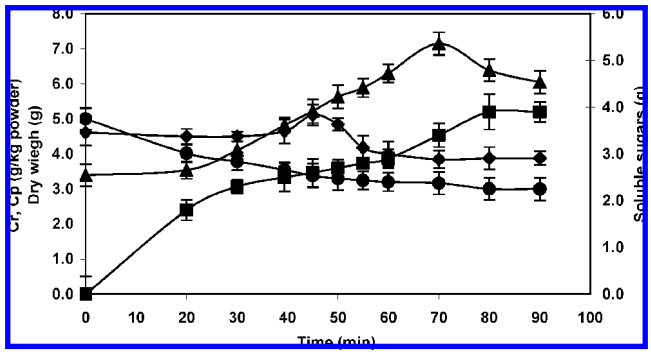

Figure 1. Concentration profiles during enzymatic treatment on carotenoids [Cr (▲)], capsaicinoids [Cp (◆)], and soluble sugars (■); weight lost (●) in the chili samples var. *bydagi*.

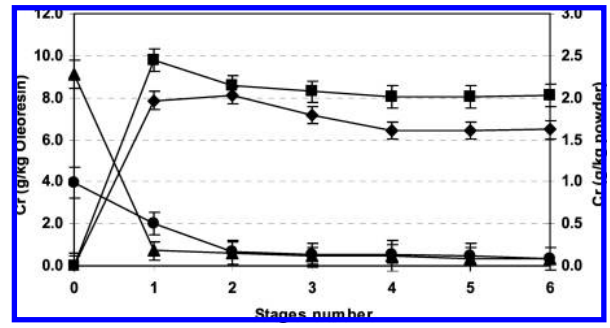

Figure 4. Kinetics of the carotenoid extraction during lixiviation of chili powder (var. *cayenne*) [treated sample, light phase (■, right axis) and solid phase (▲, left axis)] and untreated sample [light phase (♦, right axis) and solid phase (●, left axis)].

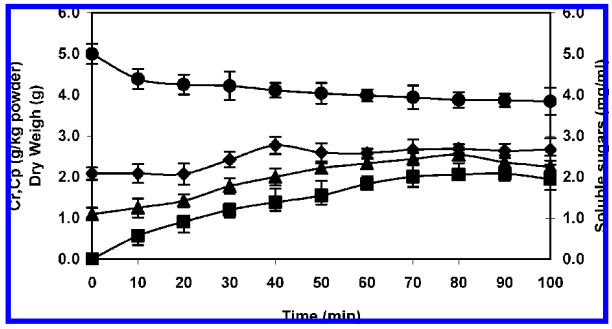

Figure 2. Concentration profiles during enzymatic treatment on carotenoids [Cr (▲)], capsaicinoids [Cp (◆)], and soluble sugars (■); weight lost (●) in the chili samples var. *cayenne*.

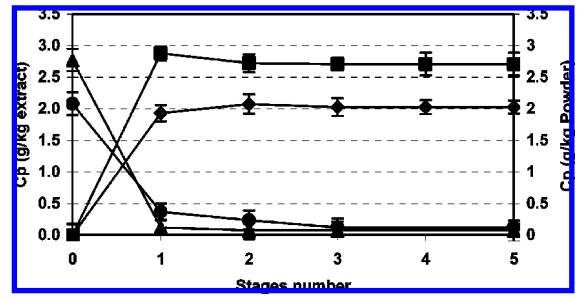

Figure 5. Kinetics of the capsaicinoid extraction during lixiviation operation for chili powder (var. *cayenne*) treated with enzymatic extract [light phase (■, right axis) and solid phase (▲, left axis)] and untreated chili powders [light phase (♦, right axis) and solid phase (●, left axis)].

(dry weight)] were observed after 70 and 45 min of processing, respectively (**Figure 1**). For the *cayenne* chili fruits, the yield increases reached 130% (average) for carotenoids [2.6 (±0.1) g of total carotenoids/kg of powder (dry weight)] and up to 33% (average) for capsaicinoids [2.8 (±0.1) g of capsaicinoids/kg of powder (dry weight)] after 80 and 40 min of treatment, respectively (**Figure 2**) These results compare favorably with those reported by Santamaría et al. (*7*) and by Desikacharya et al. (*8*). Both studies reported yield increases over the control batch with enzymatic treatments using commercial enzymes. The significant differences observed between the carotenoid and capsaicinoid yields that are reported here appear to be linked to the type of cellulase activity and the cellulase concentration. Moreover, visual inspection of the samples treated revealed the structural disintegration of the chili fruit (mesocarp and placental structure), which directly correlated with the treatment time. Structural disintegration of the chili fruit by the enzyme complex plays a key role, and it leads to the transfer of some hydrosoluble components from the solid to the liquid phase, increasing the concentration of the other components in the solid phase. These phenomena have been verified by the weight lost in the solid phase and the increase of soluble sugars in the liquid phase (**Figures 1** and **2**) in accordance with similar observations by Santamaría et al. (*7*). In addition, it was noted that, with longer processing times, beyond the reported time for the maximum recovery of carotenoids and capsaicinoids, the content of these valuable constituents in the powder was decreased (**Figures 1** and **2**). This behavior is also due to the transfer of some capsaicinoids and carotenoids from the solid to the liquid phase after structural disintegration of the placenta and mesocarp of the chili fruit, which increase cell wall permeability and facilitate diffusive mechanisms of mass exchange between nearly immiscible phases during enzymatic treatment. However, in both cases, the concentration loss is not significant because of the

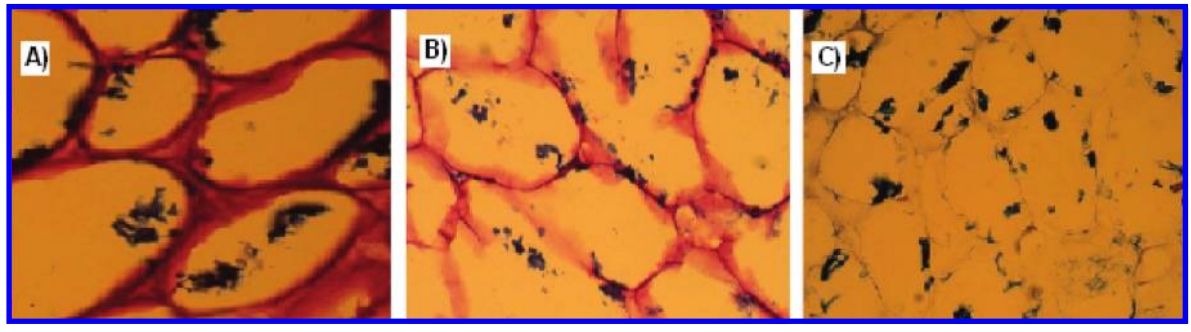

Figure 3. Micrographs of mesocarp tissue from chili cells stained with PAS-Amido Black (proteins stain blue, cellulose stains red): (**A**) untreated sample; (**B**) sample treated for 20 min; (**C**) sample treated for 45 min.

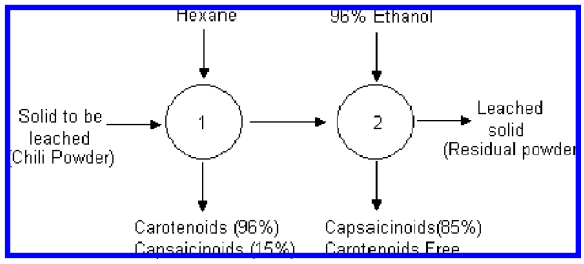

Figure 6. Sequential and selective cross-current extraction system for the recovery of carotenoids and capsaicinoids in separate fractions from chili powder.

low solubility of capsaicinoids and carotenoids in water. Moreover, if the enzymatic treatment is interrupted at the time when the content of carotenoids and capsaicinoids in the solid phase is at a maximum, the loss of these valuable constituents can be avoided.

Effect of Enzymatic Treatment upon Structural Cell Walls. In a previous study, the ability of the hydrolytic enzymes secreted by *R. nigricans* to degrade cellulose was demonstrated (*10*). Histological studies of the chili fruits confirmed these observations. The studies included analysis of the mesocarp structures, which contain larger quantities of carotenoids, and the placental structures, which contain larger quantities of capsaicinoids. Independent of the chili variety, the cellulose structure disappeared, and it was clearly correlated with the time of enzymatic treatment. The same effect was observed in the mesocarp and placental cells (**Figure 3**). These structural changes were correlated with the increase in cell permeability, facilitating mass exchange during carotenoid and capsaicinoid extraction. Santamaría et al. (*7*) noted partial cell wall degradation by enzymatic treatments using commercial enzyme solutions and were, therefore, able to achieve only modest yield increases. The differences observed in the chili cell micrographs appear to be related to the cellulase activity and concentration in the enzymatic extract used for the treatments.

Carotenoid and Capsaicinoid Extraction in a Continuous Multistage Extraction System. Chili powder obtained from the enzymatic treatments at the time that the carotenoids content was at a maximum and untreated chili powders were lixiviated in a cross-current extraction system using hexane for carotenoid recovery. The results showed an extraction efficiency of 96% in one stage for the treated chili powders, whereas, for the nontreated powders, three stages were required to achieve similar results (**Figure 4**). For the capsaicinoids, a 93% extraction efficiency was observed in one stage for the treated powders, and, again, three stages were required to achieve a similar efficiency with the nontreated samples (**Figure 5**). The improved efficiency for the treated samples is the result of the increase in chili cell permeability, leading to larger transfer rates from the solid to the liquid phase. Note that, from **Figures 4** and **5**, additional stages (beyond three) in the extraction train do not lead to improved carotenoid and capsaicinoid recovery. Due to the nonselective character of the extraction, it appears that additional stages led to the recovery of secondary components that increased the total mass recovery, but they did not preferentially lead to further extraction of carotenoids and capsaicinoids.

Sequential and Selective Extraction Process. On the basis of the multistage extraction results, and to obtain separate fractions of the carotenoids and capsaicinoids from the treated chili fruits, a sequential and selective extraction process in a cross-current extraction system was implemented. The process begins with carotenoid extraction using hexane in the first stage, followed by capsaicinoid extraction using ethanol in a second stage. Under this configuration, yield recoveries up to 96% of the total carotenoids and up to 85% of the total capsaicinoids were achieved in separate fractions (**Figure 6**). The relatively low yield obtained for capsaicinoid recovery is due to the fact that a significant portion of the capsaicinoids (15%) was extracted along with the carotenoids in the first stage of extraction.

HPLC Analysis. The chili powder obtained from the enzymatic treatment was analyzed by HPLC to determine the profile of the major components responsible for color and pungency. The chromatograms shown in **Figures 7** and **8** indicate that the profiles of the major carotenoids and capsaicinoids were preserved after the enzymatic treatment. In addition, the resultant signals for the chromatograms were

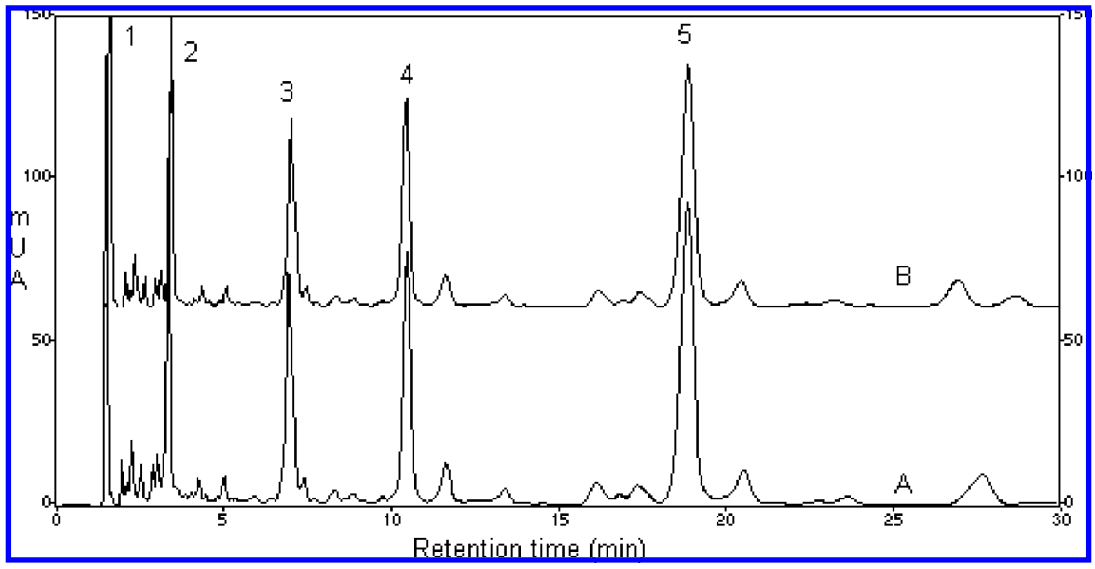

Figure 7. HPLC chromatogram for carotenoids in the control sample (**A**) and the treated sample (**B**) for chili var. *cayenne*. The possible components of the signals are (1) β-carotene, (2) β-cryptoxanthin, (3) undefined, (4) zeaxanthin, and (5) capsanthin, based on a chromatogram standard of the paprika used in the quality control area of the Alcosa industry (Mexican industry that processes natural colorants).

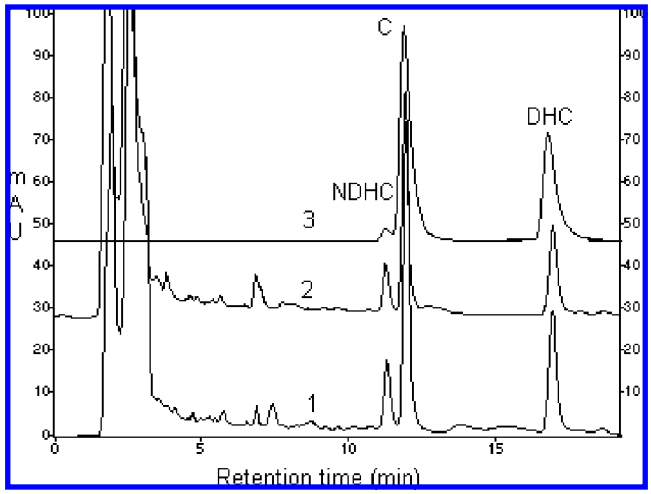

Figure 8. HPLC chromatogram for capsaicinoids in the chili powder control sample (1), treated chili powder (2), and a mixture of capsaicinoid standards (3): NDHC, nordihydrocapsaicin; C, capsaicin; DHC, dihydrocapsaicin.

associated with a particular component by retention time comparison based on a chromatogram standard of the paprika used in the quality control area of the Alcosa industry (Mexican industry that processes natural colorants) and by a calibration curve constructed using external standards of capsaicin (98% and 2% of nordihydrocapsaicin; Sigma-Aldrich Chemie GmbH) and dihydrocapsaicin (90%; Sigma-Aldrich Chemie GmbH). These chromatograms can be favorably compared with those reported by Kozukue et al. (*2*) for capsaicinoid analysis and by Deli and Molnár (*20*) for carotenoid analysis on ripe red paprika in reverse phase.

Conclusions. The results presented in this work confirm that carotenoid and capsaicinoid extraction from chili powders can be improved by enzymatic treatment. Higher yields for capsaicinoids (110 and 11%, respectively) and carotenoids (130 and 33%, respectively) were obtained from chili powder made from the *bydagi* and *cayenne* chili varieties using an enzymatic extract obtained as a result of *R. nigricans* metabolism. The values reported in this study compare favorably with those previously reported by Santamaría et al. (*7*) and Desikacharya et al. (*8*), who used similar processes that relied upon commercial enzyme preparations. Additional studies into the extraction process showed that, when the powders were leached in a sequential and selective two-stage extraction process using hexane in the first stage and ethanol in the second stage, up to 96% of carotenoids and up to 85% of capsaicinoids could be recovered separately. Likewise, the enzymatic treatment process described here requires significantly less processing time than enzymatic treatments using commercially available enzymes. The reasons for these differences are unclear. However, the type and activity of the cellulase associated with the enzymatic extract obtained from *R. nigricans* are probably the main candidates for the observed differences. An additional explanation may lie in the composition of the enzymatic extract synthesized by *R. nigricans*, which may contain other enzymes in addition to cellulases (for example, pectinases) that, together with cellulase, help to soften the cell wall and facilitate the degradation of the chili cells, as described by Lakshmesha et al. (*21*). HPLC analysis of the powder obtained from these treatments showed that there were no alterations in the total carotenoid and total capsaicinoid profile. These results, namely, a high concentration of carotenoids and capsaicinoids in the treated chili powder, a high recovery of these components using a simple extraction process, on-site enzyme production, and a reduction in processing time, have a significant impact on the cost efficiency of the overall process, establishing this process as a viable alternative to improve the efficiency of carotenoid and capsaicinoid production from chili (*C. annuum*) fruits.

ACKNOWLEDGMENT

We gratefully acknowledge the technical assistance provided by Miriam del Rocío Medina Herrera and Carmina Martínez Hernández.

LITERATURE CITED

(1) Surh, Y. J.; Lee, R. C. J.; Park, K. K.; Mayne, S. T.; Liem, A.; Miller, J. A. Chemoprotective effects of capsaicin and diallyl sulfide against mutagenesis or tumorigenesis by vinyl carbamate and *N*-nitrosodimethylamine. *Carcinogenesis* **1995**, (16), 2467–2471.

(2) Kozukue, N.; Han, J. S.; Kozukue, E.; Lee, S. J.; Kim, J. A. E.; Lee, K. E.; Levin, C. E.; Friedman, M. Analysis of eight capsaicinoids in peppers and pepper-containing foods by high-performance liquid chromatography and liquid chromatography−mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 9172–9181.

(3) Todd, G. N., inventor; Kalamazoo Holdings, Inc. (Kalamazoo, MI), assignee. High temperature countercurrent solvent extraction of *Capsicum* solids. U.S. Patent 5,773,075, 1998.

(4) Dull, B. J., inventor; Pillsbury Co. (Minneapolis, MN), assignee. Treated peppers products having capsaicinoids removed. U.S. Patent 6,123,970, 2000.

(5) Torres, L., inventor; New Mexico Tech Research Foundation (Socorro, NM), assignee. Method of providing an essential oil extract of capsicum and the extract. U.S. Patent 6,919,095, 2005,

(6) Treybal, R. E. *Mass-Transfer Operations*, 3rd ed.; McGraw-Hill: New Yor, 1987; pp 717−744.

(7) Santamaría, R. I.; Reyes-Duarte, M. D.; Bárzana, E.; Fernando, V.; Gama, F. M.; Mota, M.; López-Munguía, A. Selective enzyme-mediated extraction of capsaicinoids and carotenoids from chili guajillo puya (*Capsicum annuum* L.) using ethanol as solvent. *J. Agric. Food Chem.* **2000**, *48*, 3063–3067.

(8) Desikacharya, S. S. R., Naidu, M. M., Sowbhagya, H. B., Naik, J. P., Krishnamurthy, N., inventors; Morgan & Finnegan, L.L.P., assignee. Process of extracting chili (*Capsicum*) oleoresin. U.S. Patent Application 20,040,191,364, 2004.

(9) Navarrete-Bolaños, J. L.; Jiménez-Islas, H.; Botello-Alvarez, E.; Rico-Martínez, R. Mixed culture optimization for marigold flower ensilage via experimental design and response surface methodology. *J. Agric. Food Chem.* **2003**, *51*, 2206–2211.

(10) Navarrete-Bolaños, J. L.; Jiménez-Islas, H.; Botello-Alvarez, E.; Rico-Martinez, R. Improving xanthophylls extraction from marigold flower using cellulolytic enzymes. *J. Agric. Food Chem.* **2004**, *52*, 3394–3398.

(11) Navarrete-Bolaños, J. L.; Rangel-Cruz, C. L.; Jimenez-Islas, H.; Botello-Alvarez, E.; Rico-Martinez, R. Pre-treatment effects on the extraction efficiency of xanthophylls from marigold flower (*Tagetes erecta*) using hexane. *Food Res. Int.* **2005**, *38* (2), 159–165.

(12) Botello-Álvarez, E.; Pérez-Castrejón, S.; Jiménez-Islas, H.; Rico-Martínez, R.; Navarrete-Bolaños, J. L. Hydrodynamic analysis and inoculum effect on *Rhizopus nigricans* growth for cellulase production in a bubbling column bioreactor. In *Modern Multidisciplinary Applied Microbiology*; Mendez-Vilas, A., Ed.; Wiley-Verlag: Weinheim, Germany, 2006; pp 221−226.

(13) Kruger, N. J. The Bradford method for protein quantification. In *The Protein Protocols Handbook*, 2nd ed.; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1992; pp 15−22.

(14) Montgomery, D. C. Response surface methods and designs. In *Design and Analysis of Experiments*, 6thed.; Montgomery, D. C., Ed.; Wiley: New York, 2005; pp405−463.

(15) AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Arlington, VA, 1984.
(16) ISO. *Chillies and Chilli Oleoresins. Determination of Total Capsaicinoid Content. Part 1: Spectrometric Method*; International Organization for Standardization: Geneva, Switzerland, 1994; ISO 7543-1.
(17) Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **1959**, *31*, 426–428.
(18) Clark, G. *Staining Procedures*, 4th ed.; Williams and Wilkins: Baltimore, MD, 1981; pp 317−327,
(19) Hoffman, P. G.; Lego, M. C.; Galetto, W. G. Separation and quantification of red pepper major heat principles by reverse-high-pressure liquid chromatography. *J. Agric. Food Chem.* **1983**, *31*, 1326–1330.
(20) Deli, J.; Molnár, P. Paprika carotenoids: analysis, isolation, structure elucidation. *Curr. Org. Chem.* **2002**, *6* (13), 1197–1219.
(21) Lakshmesha, K. K.; Lakshmidevi, N.; Mallikarjuna, A. Inhibition of cell wall degrading cellulase enzyme—the incidence of anthracnose disease caused by *Colletotrichum capsici* on *Capsicum* fruit. *Arch. Phytopathol. Plant Prot.* **2005**, *38* (4), 295–302.

Received for review February 26, 2008. Revised manuscript received September 9, 2008. Accepted September 10, 2008. We gratefully acknowledge the financial support of Concyteg-Conacyt.

JF801823M