JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Improving Xanthophyll Extraction from Marigold Flower Using Cellulolytic Enzymes

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In this work is studied the effect of a noncommercial enzyme preparation on xanthophyll extraction from marigold flower (*Tagetes erecta*). The enzymatic extract was synthesized by endogenous microorganisms previously isolated and identified as *Flavobacterium IIb*, *Acinetobacter anitratus*, and *Rhizopus nigricans*. The results show that the extraction yield depends directly on the extent of the enzymatic hydrolysis of cell walls in the flower petals and that it is possible to reach yields in excess of those previously reported for treatments with commercially available enzymes (29.3 g/kg of dry weight). HPLC analysis of the product indicates that the original xanthophyll profile is not altered. The enhanced extraction system appears to be very competitive when compared to the traditional process and current alternatives.

KEYWORDS: Xanthophyll extraction; marigold flower; cellulolytic enzymes

INTRODUCTION

Marigold flower (*Tagetes erecta*) extract (oleoresin) is a product with increasing demand in international markets. The saponified extract is used as an additive to poultry feed (*1*−*3*) due to its pigmenting properties. Additional studies have shown that the marigold extracts may be used as a human nutritional supplement, based on important biological functions such as cancer prevention agent, ligament repair in muscular tissue, and prevention agent for age-related macular degeneration (*4*, *5*). These applications have fostered the interest in improving xanthophyll production processes and developing new alternatives. These efforts had included chemical synthesis and fermentation technology, as well as the extraction from marigold flower (*6*−*8*). The commercial oleoresin production from marigold flower involves the following stages: ensilage, pressing, drying, hexane extraction, distillation, and saponification (*9*). Previous studies have shown that an enzymatic treatment of the marigold flower, based on cellulases, can advantageously improve xanthophyll extraction (*10*−*12*). Matoushek (*10*) described a process in which fresh flowers in water (10% w/v) were pretreated with cellulase, followed by the extraction using a solvent (chloroform or hexane). The author reports a 36% increase of yield, as compared to the control without enzyme treatment. Delgado-Vargas and Paredes-López (*11*) determined the effects of solids content and the elimination of water-soluble substances on the carotenoid profiles of marigold samples treated with enzymes. Their results show that enzymatic treatment produced a flour with the highest *all-trans*-lutein content (25.1 g/kg of dry weight), an important increment when compared to a reference yield of 11.4 g/kg for the untreated control. In both cases, the proposed enzymatic treatments had practical limitations, namely, long reaction times and the removal of a substantial amount of water used in the enzymatic reaction. Bárzana et al. (*12*) proposed a simultaneous enzymatic treatment and solvent extraction. They used a set of hydrolytic enzymes that can degrade the cell wall components in a predominantly organic medium (i.e., hexane) with low humidity. Their results show that, in the absence of enzymes, 44% of the carotenoids can be recovered, whereas recoveries in excess of 85% can be obtained when the enzymes are mixed with the flower prior to extraction. Perhaps the main drawback in these efforts is the use of expensive commercial enzymes. In previous work (*13*), we studied marigold flower ensilage seeking to improve the xanthophyll extraction. There, we evaluated the use of a mixed culture, associated as normal microbiota of the marigold flower, as starter inoculum, reaching yields of 24.9 (±0.04) g of xanthophylls/kg of flour (dry weight), similar to those previously reported for enzymatic treatments (*11*). In addition, we demonstrated that the endogenous microorganisms *Flavobacterium IIb*, *Acinetobacter anitratus*, and *Rhizopus nigricans* are the most significant in marigold flower ensilage and exhibit high cellulase activity. On the basis of the current knowledge regarding the

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10.1021/jf0351366 CCC: $27.50
Published on Web 05/05/2004

ability of hydrolytic enzymes to act on structural cell wall degradation, the present study seeks to characterize the relationship between the polymer structural changes and the cellulase activity, which facilitate xanthophyll extraction. Here, we also report a novel pretreatment that allows, in a reasonable processing time, improved xanthophyll extraction yields.

MATERIALS AND METHODS

Fresh Material. Fresh marigold flowers (*T. erecta*) were supplied by Industrias Alcosa, S.A. de C.V. Guanajuato, Mexico. A single batch was used for all experiments. The flowers were separated from the receptacles, and the petals were mixed homogeneously. The petals obtained were divided in two sets; one was processed fresh, and the other portion was dehydrated at environmental conditions to 10% (±1%) moisture content.

Enzymatic Extract. For enzymatic activity studies, *F. IIb*, *A. anitratus*, and *R. nigricans* were propagated, according to the method of Navarrete et al. (*13*), to obtain a raw cellulase extract. The effects of cellulase, present in these extracts, on petals' structural wall breakdown, as well as the residual enzymatic activity via essays on a cellulose carboxymethyl (CCM, high viscosity; Sigma Chemical Co., St. Louis, MO) solution, were evaluated.

Commercial Cellulase. Commercial enzyme with endo-1,4-β-D-glucanase (EC 3.2.1.4 from *Aspergillus niger*, Sigma Chemical Co.) activity was used for enzymatic treatment. To compare against the enzyme extract produced by endogenous microorganisms, enzymatic solutions at 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001% (w/v) were prepared and a calibration curve was constructed. The pH of all enzymatic solutions was adjusted to 5.0 (recommended by the manufacturer) with diluted hydrochloric acid (Beckman Instruments, Inc., Irvine, CA).

Enzymatic Activity for the Raw Enzymatic Extract and Commercial Cellulase Solutions. For both the commercial enzyme solutions and the raw enzymatic extracts, enzymatic activity assays involved the addition of 10 mL to 150 mL of a cellulose carboxymethyl solution of 5.25 g/L that presented a viscosity (η) of 2400 cP (Brookfield digital rheometer model DV-III+). The solutions obtained were kept on a rotary shaker at 28 °C and 175 rpm (Forma Scientific, model 4520) for 24 h. The enzymatic activity of these samples was measured indirectly as a function of viscosity reduction.

Structural Cell Wall Breakdown. To evaluate the effect of commercial cellulase and raw cellulase extract on the marigold petals, one set of Erlenmeyer flasks containing 10 g of fresh petals was blended with 120 mL of raw enzymatic extract or commercial cellulase solution. The samples were divided in two sets: The first set was kept on a rotary shaker at 28 °C and 175 rpm for 24 h, whereas the second set was kept at the same conditions but without agitation. Additional samples were prepared in the same manner using dry petals. Portions from all sets were taken at regular time intervals. These portions were filtered, and their solid and liquid phases were analyzed separately. The solid phases were dehydrated in a vacuum oven (Shel Laboratory, model 1430) to 10% (±1%) moisture content and milled (0.5-mm sieve) using a Brinkmann mill (Brinkmann, Westbury, NY). The flour obtained was analyzed according to AOAC method 970.64 (*14*) to determine the total xanthophylls concentration. In addition, fiber acid detergent (*15*) for quantifying lignin and cellulose, fiber neutral detergent (*16*) for quantifying wall cell components and cell content, and lignin neutral detergent (*15*) for quantifying lignin were also measured. The liquid phases obtained were used to evaluate the residual enzymatic activity, as described below.

Residual Enzymatic Activity. Aliquots of 10 mL from the liquid phase of the processed marigold flower samples were added to 150 mL of a cellulose carboxymethyl solution of 5.25 g/L (2400 cP). The solutions obtained were kept on a rotary shaker at 28 °C and 175 rpm for 24 h. The residual enzymatic activity of these samples was measured, once again, indirectly as a function of viscosity reduction.

Sample Preparation for High-Performance Liquid Chromatography (HPLC) Analysis. Treated marigold flower samples were dried in a vacuum oven (Shel Laboratory, model 1430) to 10% (±1%) moisture content. The dried samples were extracted with a mixture of hexane, ethanol, acetone, and toluene (HEAT) of analytical grade (J. T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ) under mild conditions (56 °C during 45 min). When the extraction was finished, the liquid phase was separated by centrifugation at 6000 rpm (Hermle Z383 K), mixed with a solution of potassium hydroxide (Sigma Chemical Co., St. Louis, MO) in methanol (40% w/v), and kept during 20 min at 56 °C for saponification. The resulting solution was dissolved in hexane and sodium sulfite (Sigma Chemical Co.) solution (10% w/v). After settling, the immiscible phases were separated. The light phase (that contains the free xanthophylls) was used in the HPLC analysis.

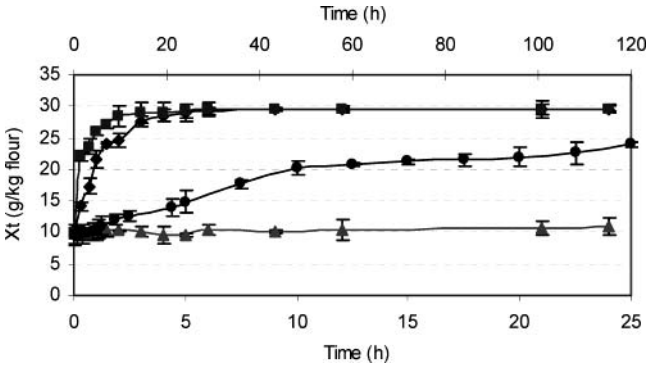

Figure 1. Effect of enzymatic treatment on xanthophyll concentration in agitated systems: treatment with raw enzymatic extract on dry petals (■) and fresh petals (◆); treatment with commercial enzyme on dry petals (●) and fresh petals (▲).

HPLC. The equipment used for the HPLC essays was a Varian chromatograph model 9050 equipped with a quaternary pump solvent delivery and degassing system. Samples consisting of 20 μL of saponified pigment were injected. The analysis of carotenoids was performed using a silica OH column (SGE BPX5 from SGE International). The solvent elution was operated at 1.0 mL/min of a mixture containing 66.5% hexane, 1.5% 2-propanol, and 32.0% ethyl acetate. The separation was performed at room temperature. The pigments were monitored using a 447 nm detector and 1 s and 2 nm as time and wavelength resolutions, respectively. Each 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 HPLC chromatogram area.

The experimental results described below were performed in duplicate, and the reported values represent the mean values of these duplicated experiments.

RESULTS AND DISCUSSION

Cellulase Activity of Extract from Endogenous Microorganisms. The results of viscosity reduction on CCM solutions showed that the quantity of enzyme synthesized by the endogenous microorganisms corresponds to the commercial enzyme solutions of 0.05 (±0.01)% (w/v).

Enzymatic Treatment Effect on Xanthophyll Yield. The xanthophyll concentration in the flours obtained via raw enzyme extract treatment shows an increase clearly correlated to the enzymatic activity. For the samples incubated with agitation, the increase appears to be independent from the condition of the petals (**Figure 1**). Flours with 29.0 (±0.1) g of total xanthophylls/kg of flour (dry weight) can be obtained after 5 h of treatment on fresh petals. Additional processing did not affect the xanthophyll concentration in the flours. Similar behavior was observed on the samples formed by dry petal samples (**Figure 1**). Flours with 29.3 (±0.1) g of total xanthophylls/kg of flour (dry weight) can be obtained in the same treatment time. Nevertheless, **Figure 1** also shows that the enzymatic extract acts more rapidly on the dry petals than on the fresh petals. Similar studies using a commercial enzyme solution of 0.05% (w/v) were realized, and the results showed that it is possible

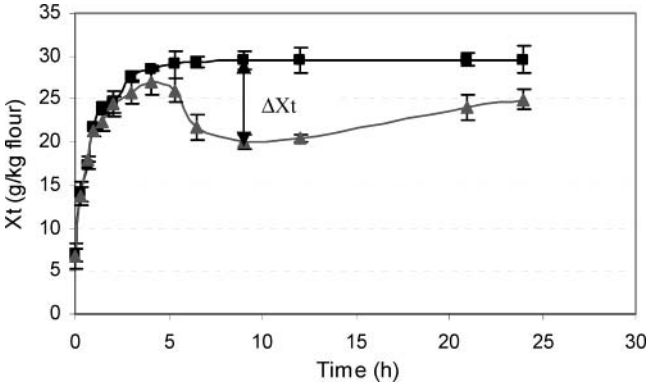

Figure 2. Effect of enzymatic treatment in agitated systems using the raw enzymatic extract on fresh petals: treatment with efficient filtration system to prevent xanthophyll losses (■); treatment coupled with a loose filtration system (▲).

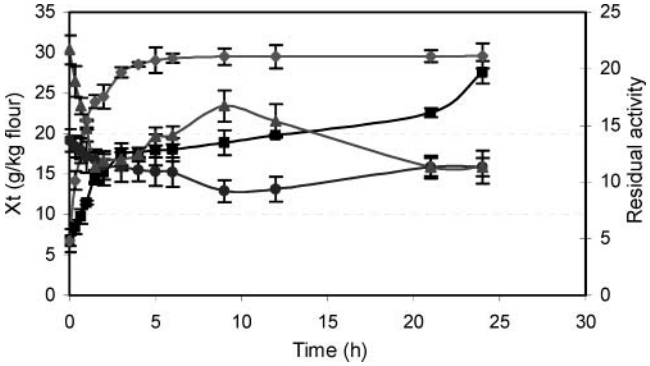

Figure 3. Effect of enzymatic treatment on xanthophyll concentration on fresh petals for nonagitated samples (left axis, ■) and agitated samples (left axis, ◆) and changes in the residual enzymatic activity as measured via the reduction of CCM solutions for nonagitated samples (right axis, ●) and agitated samples (right axis, ▲).

to obtain a marigold flower flour with 23.9 (±0.1) g of total xanthophylls/kg of flour (dry weight) in 120 h of treatment on marigold flower dry petals (**Figure 1**). These results compare favorably with those reported by Delgado-Vargas and Paredes-López (*11*); the differences observed are not significant and can be attributed to uncontrolled variables such as the variety and degree of maturity of flower and cellulase activity type. We note that the enzymatic treatment, using the raw enzymatic extract, reduces the processing time by ∼95% (±1%) compared to the enzymatic treatment using commercial enzyme. In addition, the use of commercial enzyme did not affect the xanthophyll yield from fresh marigold flower petals with respect to that of the untreated samples, also described by Delgado-Vargas and Paredes-López (*11*). At first, this behavior was attributed to cell wall modification during the drying process of the petals; however, the treatment using raw enzymatic extract from the proposed endogenous culture allowed us to obtain marigold flower flour with high xanthophyll concentration also using fresh petals. Currently, we do not have a logical explanation for the failure of the commercial enzyme preparation to affect the yield from fresh flowers. We believe that the enzymatic extract synthesized by the endogenous microorganisms contains, in addition to cellulase, other enzymes that facilitate cellulase activity on cell wall petals.

Enzymatic Treatment Effect on Marigold Flower Petals. Visual inspection of the samples treated, after 1.5 h, reveals the presence of fine petal particles suspended in the supernatant, resulting from the hydrolytic enzymes activity. After this time, we observed a gradual increase in the suspended solid material directly correlated to the treatment time. At ∼5 h of treatment, the petals' structure has been totally destroyed and the maximum xanthophyll concentration has been reached (**Figure 2**). However, if the product obtained after 5 h of treatment is not adequately separated, up to 30% of the xanthophylls may be lost (ΔXt, **Figure 2**). Such observation is consistent with previous findings (*12*), where inefficiencies of the traditional commercial extraction (ensilage-based) were declared. Nevertheless, the losses in the enzymatic treatment system can be partially reduced. Such recovery involves a loose filtration stage in which the xanthophyll-rich liquid phase is separated from the leached solid. As the solid particles are accumulated on the filter, forming a cake, one observes better xanthophyll recovery due to the presence of the cake, which acts as a secondary barrier for filtration. However, full recovery was never reached (**Figure 2**). Additional pressure was needed to prevent the xanthophyll from being retained in the cake.

To evaluate the residual enzyme activity during the essays, treated samples were filtered to separate the phases; supernatant aliquots were added to CCM solutions to evaluate the residual enzymatic activity. The results showed that the viscosity of the CCM solution diminished steadily during the first 1.5 h, correlated with the increase in the yield values (**Figure 3**). Apparently, viscosity reduction in the CCM solutions is associated with enzyme adsorption into the petals, leading to the yield increase, as the result of the enzymatic activity. During the following 7.5 h of the treatment, the residual enzymatic activity gradually recovers, reaching 80% (±2%) of its original value at zero time (**Figure 3**). Apparently, the enzyme activity increases due to enzyme desorption, characterized by changes in the solid structure and an increase of suspended particles in the supernatant. However, we do not observe further recovery of the enzymatic activity beyond 9 h of treatment. Seemingly, this behavior may be related to the enzyme being trapped in the solid phase when the samples were filtered, similar to the phenomena observed for the loose filtration mentioned above.

Likewise, the nonagitated samples showed lower yields for the same treatment times (**Figure 3**). Yields similar (27.5 g of total xanthophylls/kg of flour in dry weight) to those obtained on agitated systems are observed only after ∼24 h of treatment (**Figure 3**). **Figure 3** shows trends related to adsorption and activity similar to those observed for agitated samples. However, in nonagitated samples one never observes the total degradation of petals characterized by the presence of particles in the supernatant; furthermore, the residual enzymatic activity did not exhibit any significant recovery.

From these results, we can conclude that agitation plays an important role in the enzymatic treatment system. It favors the adsorption, activity, and desorption of the enzyme, maximizing the efficiency and extraction yields. Agitation appears to facilitate enzyme diffusion from the supernatant (liquid phase) to the marigold flower petals (solid phase). The fast enzyme adsorption accelerates the cell wall lysis, leading to an increase in the yield extraction. In addition, the agitation favors the pulverization of solid material. Pulverization leads to an increase in the interfacial area available for enzyme desorption. A speedy enzyme desorption is a desirable characteristic for the separation, recovery, and reutilization steps.

Enzyme Treatment Effect on Cell Wall Structural Components. Agitated samples from treated fresh petals were analyzed at different process times to estimate profile changes in the structural components in petal cell walls. The neutral detergent fiber (NDF) fraction was quantified as described by

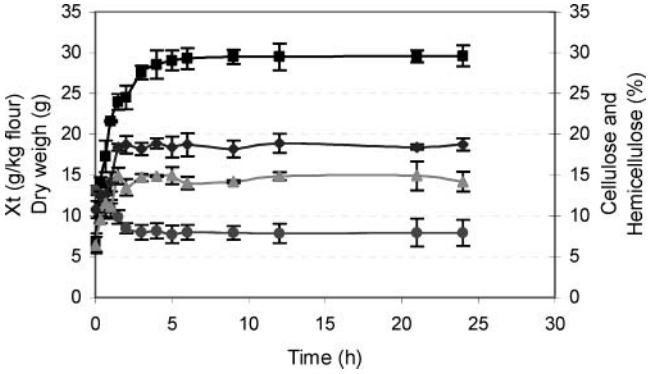

Figure 4. Effect of raw enzymatic extract on fresh petal composition [cellulose (◆) and hemicellulose (▲) right axis], weight loss in the solid phase [(●), left axis], and xanthophyll concentration [(■), left axis).

Van Soest and Wine (*15*), whereas cellular content fraction was determined by the difference (1 − NDF). The acid detergent fiber (ADF) fraction, in turn, was quantified as described by Van Soest (*16*). The ADF residue was treated with sulfuric acid (72%) to obtain the lignin detergent (LD) fraction (*16*). The difference (NDF − ADF) represents the hemicellulose content, and the ADF − LD fraction was identified as cellulose content.

The results (**Figure 4**) showed that, for the agitated samples, cellulose and hemicellulose curves are very similar to the xanthophyll yield curve. This characteristic trend supports the simultaneous occurrence of enzyme adsorption and activity. The change in the cellulose and hemicellulose content appears to be correlated with the permeability change in the cell walls during the treatment, easing mass transfer between the solid phase (petals) and the liquid phase (enzymatic extract). During the process, the solid-phase mass diminishes steadily. As a result, the insoluble components (i.e., xanthophylls) increase their concentration in the solid phase. Such phenomena are further confirmed by the weight change in the samples (**Figure 4**).

The overall analysis of these results appears to suggest that the adsorption−reaction stages are the limiting ones, and they are enhanced via agitation. As a consequence, higher extraction yields and substantial reduction in processing time are achieved for the agitated samples. In this study, the overall data analysis shows that the treatment time should be ∼5 h. At this time the maximum xanthophyll extraction yield is obtained, the xanthophyll loss to the liquid phase can be minimized, the substrate for hydrolytic activity of the enzyme has been depleted, the interfacial area has been maximized, and the enzyme has desorbed from the solid. Although we did not explore this issue, enzyme recovery could be started at such time for reuse.

HPLC Analysis. Flours obtained from the enzymatic treatment in the agitated system were analyzed by HPLC to determine the concentration and profile of the main components. The chromatogram showed (**Figure 5**) that the main components are lutein (84.7%) and zeaxanthin (4.5%). This analysis, by comparison with previously reported profiles (*17*), indicates that the concentrations are preserved.

Conclusion. The results presented here confirm that xanthophyll extraction from marigold flower can be improved using cellulolytic enzymes. Flours with high contents of xanthophylls (29.3 g of total xanthophylls/kg, dry weight) can be obtained after only 5 h of treatment, using the raw enzymatic extract synthesized from *F. IIb*, *A. anitratus*, and *R. nigricans*, in an agitated system. Clearly, the process described in this study has significant advantages when compared to the traditional commercial processes and similar previous studies: (1) the treatment with hydrolytic enzyme synthesized by microorganisms associated as normal flora of the marigold flower is more cost-efficient due in part to the straightforward enzyme synthesis, and (2) a substantial reduction in processing time can be achieved while attaining high xanthophyll content. The on-site enzyme production and process time reduction are decisive characteristics when the economic viability of the process is evaluated. Furthermore, HPLC analysis of the flour obtained from this treatment shows no alteration in the total xanthophyll profile and composition. The identified processing time (5 h) and proportion of substrate to enzymatic extract volume (10% w/v) reported here are the first steps toward the design of more efficient continuous or semicontinuous processes that are currently being investigated.

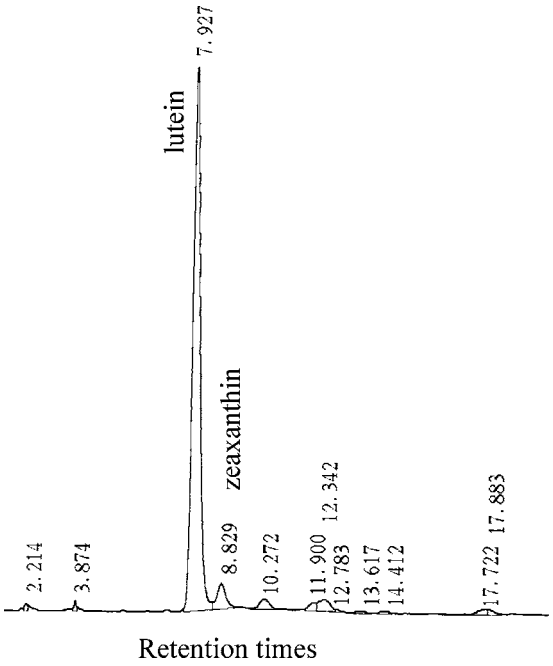

Figure 5. HPLC chromatogram of the saponified oleoresin obtained as final product from treated marigold flower flour.

ACKNOWLEDGMENT

We gratefully acknowledge technical assistance provided by Rosa Martha Figueroa-Vizcaíno, Janet Zavala-Nigoa, and Claudia L. Rangel-Cruz.

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Received for review October 4, 2003. Revised manuscript received March 11, 2004. Accepted April 1, 2004. We gratefully acknowledge financial support by FOMIX (CONACYT/CONCYTEG; 02-09-B-005) and COSNET (896.03-P).

JF0351366