JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enzyme-Mediated Solvent Extraction of Carotenoids from Marigold Flower (*Tagetes erecta*)

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Marigold flowers are the most important source of carotenoids for application in the food industry. However, the extraction gives almost 50% losses of the carotenoids depending on conditions for silaging, drying, and solvent extraction. In the past decades, macerating enzymes have been successfully applied to improve the extraction yield of valued compounds from natural products. In this work, an alternative extraction process for carotenoids is proposed, consisting of a simultaneous enzymatic treatment and solvent extraction. The proposed process employs milled fresh flowers directly as raw material, eliminating the inefficient silage and drying operations as well as the generation of hard to deal with aqueous effluents present in traditional processes. The process developed was tested at the 80 L scale, where under optimal conditions a carotenoid recovery yield of 97% was obtained.

KEYWORDS: Marigold flowers; *Tagetes erecta*; carotenoids; lutein; enzymatic reaction; enzymatic extraction

INTRODUCTION

Marigold (*Tagetes erecta*) is an annual plant reported to be native to Mexico (*1*). Its flowers are commercially cultivated, harvested, and processed on an important industrial scale as a source of high value colorants of the carotenoids family. Philip and Berry (*2*) reported that marigold flowers are the most concentrated common source of carotenoids, with lutein, a dihydroxylated compound, accounting for 85% of the total carotenoids present in the flower. Carotenoids in flowers are mostly esterified with lauric, myristic, palmitic, and stearic acids in different proportions, which makes them readily soluble in hexane (*3*, *4*).

Crude flower extract is used mainly as an ingredient of poultry feed to promote the coloration of chicken skin and yolk according to consumer's demand (*5*). Lutein esters are efficiently absorbed into the human blood stream (*6*), resulting in restraint of mamma tumor growth and an enhanced proliferation of lymphocyte (*7*). Also, lutein ingestion has been linked to an attenuation of age-related degeneration of the human eye macula (*8*, *9*), possibly by a mechanism that increases the density of macular pigment. Such properties have inspired the consideration of marigold extracts as "nutraceuticals" with high added value, nowadays being an ingredient in many products commercialized by nutritional companies.

The traditional commercial extraction of marigold carotenoids dates back several decades. After they are harvested, the flowers are silaged, pressed/dried, and milled to form a meal that is pelletized and extracted with hexane. All of these stages of the process result in substantial losses of the carotenoids. Silage undergoes a spontaneous noncontrolled fermentation that occurs when the harvested flowers are stored for 3−4 weeks in open yards with minimum protection. This process results in the partial degradation of tissue cell walls exposing the internal lipid vesicles for a more efficient extraction process. However, substantial amounts of carotenoids are lost by oxidation due to existing uncontrolled conditions. Furthermore, as a consequence of the intensive microbial activity during silage, an important volume of tissue water ends up as an effluent with a high biological oxygen demand (BOD). In addition to silage, drying of the flowers contributes to a severe carotenoid loss, mostly by oxidation, since it is carried out with hot air. In rough numbers, the dry flowers retain only 50% of the carotenoids present in fresh flowers. Clearly, any rational initiative aimed for a reduction in the mentioned losses should favorably impact the extraction process of carotenoids from marigold, as long as it is sound in economic and environmental terms.

Enzyme treatment has been proposed as an alternate stage to solvent extraction processes to improve the yield and quality of several oily products. This has been reported for rapeseed, soybean, canola, mustard, rice bran, cotton, sunflower, and castor

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10.1021/jf025550q CCC: $22.00
Published on Web 06/28/2002

(*10*−*14*). All-aqueous enzymatic processes (i.e., organic solvent-free) have been reported for palm, peanut, corn germ, olive, coconut, avocado, rapeseed, flaxseed, sunflower, soybean, and jojoba seeds (*15*−*20*). Finally, enzyme treatment previous to mechanical extraction has resulted in improved yields in the case of oil from sunflower, colza, and canola (*21*, *22*). In all instances mentioned above, hydrolytic enzymes have been used in different combinations as agents that interact on cell walls, breaking down the structural integrity rendering the intracellular materials more exposed for extraction (solvent or mechanical). Following this approach, enzymes have been explored as a mean to enhance the extraction of carotenoids in marigold flowers. Matoushek (*23*) described a process in which fresh flowers in water (10% w/v) were pretreated with cellulase for 16 h (pH 4−6, 45 °C) in preparation for chloroform or hexane extraction. This author reports a 36% increase in yield as compared to the control with no enzyme. Also, Delgado-Vargas and Paredes-López (*24*) studied the effect of an aqueous enzymatic treatment of marigold flowers meal (not previously subjected to silage) on the extraction yield with a mixture of hexanes−ethanol−acetone−toluene (10:6:7:7 v/v). In this case, a 5% (w/v) meal suspension in water at pH 5 was treated with 0.1% (w/w) Econase-cep (a commercial enzyme from EDC, New York, NY) for a period of 120 h to yield 24.7 g/kg of carotenoids, an important increment when compared to a yield of 11.4 g/kg for the nontreated control. In both cases, the proposed enzymatic treatments had limitations to make them practical, these being the long reaction times employed and the need for the removal, prior to the solvent extraction step, of the substantial amount of water used for the enzymatic reaction.

On the basis of the current knowledge regarding the ability of hydrolytic enzymes to act in organic solvents with minimum amounts of water, the present work reports a novel one pot process for the simultaneous break down of marigold cell wall and the extraction of the carotenoids in a reasonable process time and better yields as compared to current industrial processes. Furthermore, the process can use directly freshly milled flowers as substrate, eliminating the inefficient silage and drying operations and reducing considerably the generation of hard to deal with aqueous effluents.

MATERIALS AND METHODS

Freshly chopped or silaged marigold flowers (*T. erecta*) were obtained from Bioquimex Natural dehydration plants in the state of Querétaro, México. The enzymes Viscozyme, Pectinex, and Neutrase came from Novo-Nordisk (Denmark), Corolase came from Rhöm (Germany), and HT-Proteolytic came from Enmex (México). Viscozyme is a preparation containing various enzymatic activities, mainly pectinase, cellulase, and hemicellulase. Pectinex is a fungal pectinase, and Corolase, HT Proteolytic, and Neutrase are bacterial proteolytic enzymes. The hexane used was of industrial grade and high-performance liquid chromatography (HPLC) grade for analytical purposes (hazard evaluation and safety precautions concerning hexane can be consulted in http://www.dhs.cahwnet.gov/ohb/HESIS/nhexane.htm). Experiments were carried out at various scales from test tubes to pilot plant. Reaction/extraction was conducted in stirred vessels of 1 L (one turbine impeller, Di/Dt = 0.63), 14 L (two turbine impellers, L/D = 2 with Di/Dt = 0.56, and one impeller with Di/Dt = 0.32), and 80 L pilot plant (three turbine impellers, L/D = 2.5, Di/Dt = 0.55 designed specifically for this purpose).

Marigold extracts (oleoresin) were determined by weight after hexane evaporation in a Büchi 185EX evaporator (Laboratorium Technik AG, Switzerland) at 80 °C under vacuum. Carotenoids were quantified spectrophotometrically at 474 nm in a Beckman DU650 spectrophotometer, from a hexane solution of the oleoresin obtained ($E^{1\%}_{1cm}$ of 2300) using a concentration curve constructed with an oleoresin of known composition supplied by Bioquimex Natural. Each batch of flowers used was characterized in terms of total oleoresin and carotenoids. Initial and residual oleoresin was obtained by thorough hexane extraction, until no color was obtained. The extraction yield was calculated by comparison of the residual and extracted carotenoids with the original amount present in the flower on dry basis. All experiments were carried out in triplicate. Results shown never exceeded more than 5% deviation. Initial carotenoid concentration ranged from 6.7 to 7.1 g/kg of dry flowers; kinetic results are reported in terms of grams of carotenoids/kilogram of hexane.

Enzyme Selection. To select the optimal mixture of enzymes, reactions were carried out in small volumes. Eight grams of the chopped flowers was mixed with 0.8 mL of distilled water containing the enzymes dissolved at 1% (v/w or w/w flower, depending on the enzyme presentation). Controls were performed in the same way except for the enzymes. After it was incubated for 1 h at a specified temperature, the resulting mixture was extracted with 32 mL of hexane in a rotary shaker at room temperature (flower:hexane ratio of 1:4). After 24 h, the mixture was centrifuged at 3000 rpm and the carotenoids were quantified directly from the hexane fraction. Oleoresin was also quantified after hexane evaporation.

Extraction Process. Various process conditions were studied in a 1 L reactor. These included the effect of the enzyme concentration, the incubation time previous to extraction, the flower:hexane ratio, the effect of agitation in the extraction process, and the hexane reuse. For these studies, chopped fresh flowers were sprayed with 10 mL of an enzyme solution prepared in such a way as to reach a final enzyme concentration of 0.1, 0.2, 0.3, 0.5, and 1 mL (or gram) per 100 g of flower and incubated for various times at room temperature (24−25 °C) until extraction. The kinetics of carotenoids extraction was then studied at 45 °C in a stirred 1 L vessel with various flower:hexane ratios. Products were analyzed as described previously.

RESULTS AND DISCUSSION

The hypothesis sustaining the proposed research was that a set of hydrolytic enzymes should be able to degrade the components of the cell walls in a predominantly organic media (i.e., hexane) with a low amount of water. Clearly, such water should include the amount needed for hydrolysis in addition to that required to sustain enzyme activity (*25*). Although an excess of water allows enzymatic hydrolysis, it may retard the extraction process due to the formation of an aqueous interface that prevents the solvent from contacting the intracellular lipid vesicles that carry the carotenoids. Therefore, the minimum amount of water required for the process to occur had to be defined.

Preliminary experiments demonstrated that the water present in fresh or partially silaged flowers (75−80% w/w) in addition to that used as vehicle to spray the enzyme onto the flowers (∼10% w/w) was enough to sustain both enzymatic hydrolysis and carotenoids extraction into the solvent. In other words, the addition of a substantial volume of water was unnecessary. Although continuous countercurrent extractors are used in industrial plants, our system was evaluated in stirred tanks to minimize mass transfer limitations in the flower−water−solvent multiphasic system. The temperature was chosen according to that reported by the supplier as optimal for enzyme activity, while the pH was not adjusted.

The effect of different commercial enzymes was studied under the conditions described in Materials and Methods. The results are shown in **Figure 1**. In the absence of enzymes (control with freshly chopped flowers), a carotenoids recovery yield of 44% was obtained, whereas the yield increased substantially when enzymes were mixed with the flower prior to extraction. It was observed that Viscozyme, a commercial cocktail consisting mainly of polysaccharases, raises the yield more than 45%, indicating that it performs most of the necessary modifications

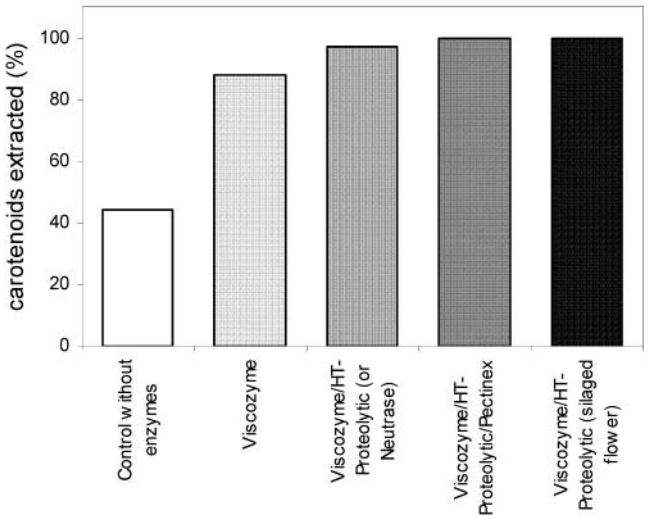

Figure 1. Effect of different commercial enzymes on the yield of carotenoids extracted from fresh marigold flowers. All enzymes were added at 1% (v or w/w flower depending on the enzyme presentation). All extractions were carried out with 32 mL of hexane and a 1:4 flower:solvent ratio for 24 h at 25 °C.

of the cell tissue to allow extraction. Further addition of proteolytic (HT or Neutrase) or pectinolytic (Pectinex) progressively incremented the yield to reach values close to 100%.

Among other factors affecting the extraction of colorant, enzyme concentration and flower:hexane ratio were found to be particularly important. Domínguez et al. (*12*) have also observed that the oil extractability from seeds is affected by enzyme concentration in a simultaneous enzymatic treatment−solvent extraction system. For marigold, preliminary experiments indicated that 1:4 and 1:3 ratios (flower:solvent) were adequate. Larger values underuse solvent and enzymes, whereas lower values did not mix well. The results for these ratios as a function of enzyme concentration in the added solution are presented in **Figure 2**. For the 1:4 ratio, no differences are observed for 1 and 0.1% enzyme. This represents an increase of more than 20% in comparison with nontreated control. However, for the 1:3 ratio, a significant difference of more than 20 and 50% was observed for the 1% enzyme sample as compared to the 0.1% and untreated samples, respectively. It is also found that the extraction yield for nontreated flower drops sharply in the system with a high solids load (i.e., 1:3 ratio as compared to 1:4 ratio in **Figure 2**). This suggests that insufficient mixing in the 1:3 ratio limits the extraction process more than a deficiency in enzyme activity. In contrast, when the flower is thoroughly macerated with 1% enzyme, mixing improves enhancing carotenoid extraction. These results demonstrate that good yields can be obtained with appropriate combinations of flower:solvent ratios and enzyme concentrations.

The system with a 1:4 ratio was used to optimize the rate of carotenoids extraction as a function of enzyme concentration in the range of 0.1−0.5%. The results are presented in **Figure 3**. Above 0.2% enzyme, carotenoid extraction occurred rapidly reaching a high value in just 3 h. For the sample supplemented with 0.1% enzyme, the extraction rate diminished, reaching total carotenoid extraction in 4.5 h. The enhancement in the rate of reaction associated to enzyme action is evident since only 50% of the carotenoids are extracted in nontreated samples after 9 h of contact with the solvent under stirring.

As already pointed out by Rosenthal et al. (*15*), a critical step in the extraction process of oil seeds and other vegetable materials using enzymes is the particle size obtained after grinding. In the case of marigold flowers, chopping facilitates agitation and has a positive effect on enzyme/substrate contact. Maceration during silage or controlled enzyme treatment reduces the particle size even more. This is shown in **Figure 4**. Clearly, the initial rate of carotenoid extraction is practically the same for chopped silaged flowers and for freshly chopped flowers mixed with a 0.5% enzyme solution of Viscozyme/Neutrase, with a dramatic improvement in the rate as compared to only chopped fresh flowers used as control (see curve in **Figure 3**). Because all experiments were conducted at constant flower:solvent ratios, the difference in maximum carotenoid extracted (0.45 vs 0.6 g/kg) was due to a reduction in the initial carotenoid content resulting from color loss during silage, as described before. Clearly, for a target concentration of carotenoid extracted, an appropriate combination of maceration by silage and enzyme treatment may be implemented. The optimum condition represents a compromise between silage time, carotenoids losses, and enzyme concentration. Subject to economic advantages, mainly their price, enzymes can even totally replace the current silage step avoiding loss of carotenoids during pretreatment. Obviously, the rate of handling of harvested flowers at the plant level dictates the timing of the whole process. Nevertheless, agitation also plays an important role due to the highly heterogeneous nature of the system and the need to transfer enzymes to the solid substrate as well as products from the macerated tissue to the solvent bulk. As a consequence of

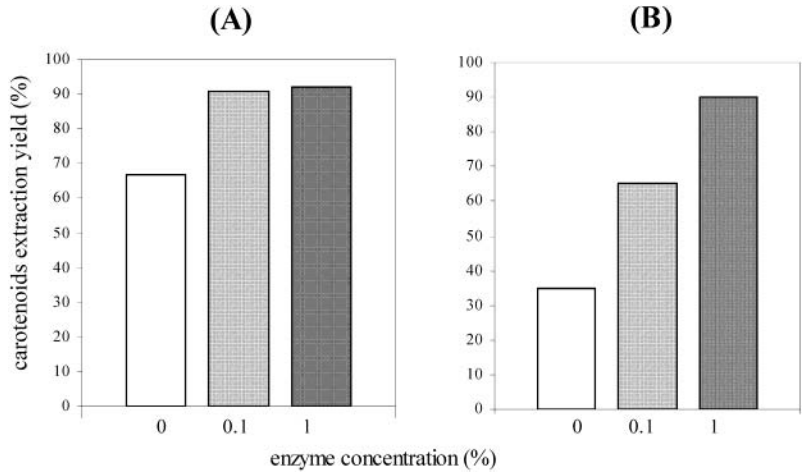

Figure 2. Effect of flower:hexane ratio and enzyme concentration (% v/w flower) in carotenoids extraction yield. The enzyme treatment was with Viscozyme/HT-Proteolytic/Pectinex. Extractions were at 45 °C for 5 h and 700 rpm. Flower:hexane ratio of 1:4 (A) and 1:3 (B).

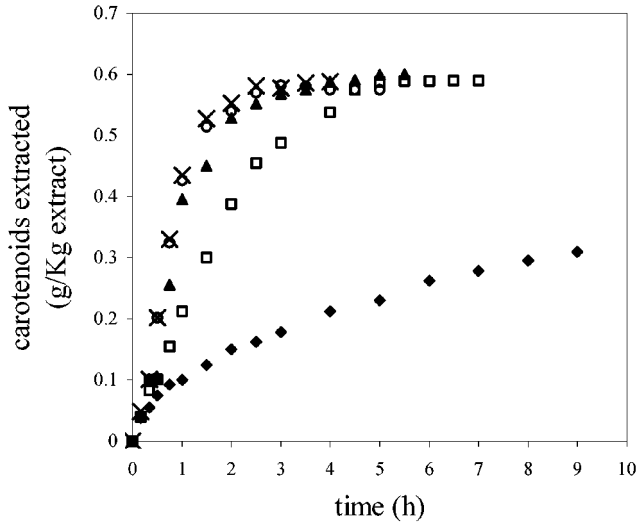

Figure 3. Effect of enzyme concentration on carotenoids extraction rate. The enzyme treatment was with Viscozyme/Neutrase (various concentrations in v/w flower) and a flower:hexane ratio of 1:4 at 45 °C and 700 rpm. Control without enzyme (◆); 0.1% (□); 0.2% (▲); 0.3% (○); and 0.5% v/w flower (×).

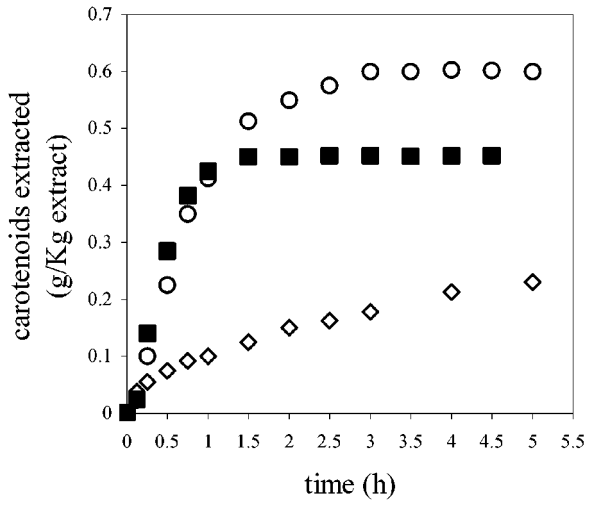

Figure 4. Comparison of enzyme treatment and flower conditioning by silage on carotenoids extraction rate. The enzymatic treatment was with Viscozyme/Neutrase (0.5% v/w flower). Extractions were carried out with a chopped flowers:hexane ratio of 1:4 at 45 °C and 700 rpm. Control without enzyme (◇); silaged (■) and fresh flower (○). Note that the difference in the maximum concentrations is due to differences in initial xanthophylls due to color loss during silage.

enzyme action (or silage), flowers became softer and flowed easier, making mixing less difficult. It was obvious that poor extraction experiments were related to deficient mixing. Even with degraded flowers, the classical design with Di/Dt = 1/3 did not fulfill mixing requirements, so larger impellers were employed.

During industrial processing of marigold, silage might be considered a necessary step to store harvested flowers before entering the production line. Flower incubation with macerating enzymes as an alternative to silage was explored. To that end, samples of freshly chopped flowers were sprayed with enzyme solution and incubated for specific times prior to hexane extraction. The results are shown in **Figure 5**. As expected, the longer the incubation time, the faster the extraction rate. Clearly, when the dosed flowers are preincubated for 1 h, all carotenoids are extracted after 1.5 h of contact with hexane. This behavior

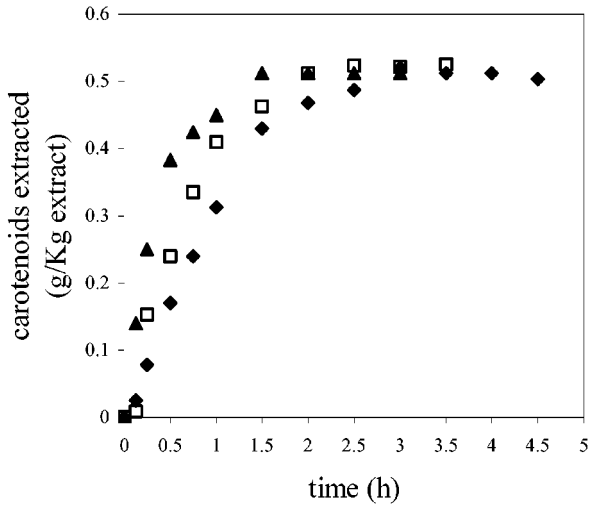

Figure 5. Effect of incubation time previous to extraction on the rate of carotenoids extraction, for fresh flowers sprayed with enzymes. Viscozyme/Neutrase was added at 0.3% (v/w flower). Extractions were with enzymatically treated flower:hexane ratio of 1:4 at 45 °C and 500 rpm. No enzyme added (◆); 30 min (□); and 1 h (▲).

is similar to the one observed for silaged samples suggesting that silage and enzymatic preincubation performed the same function, with the advantage that the enzymatic process can be easily controlled and does not result in carotenoid loss.

As an alternative to improve the process, and considering that at the end of the reaction/extraction treatment the flower suffers a considerable degradation, the possibility of double processing capacity via flower recharge was explored. This was studied in a 1 L reactor with a 1:4 flower:hexane ratio. For this experiment, 100 g of freshly chopped flowers were sprayed with a 0.3% (v/w flower) solution of Viscozyme/Neutrase and loaded into the vessel containing hexane. When the maximum concentration of extracted carotenoids was reached, a second load of 100 g of flower/enzyme was loaded into the vessel, bringing the flower:hexane ratio to 1:2. Although some difficulties were observed at the beginning to maintain proper agitation, after a couple of hours, the system eventually flowed more steadily to reach a 97% extraction yield. The recharge process was then scaled up to 14 and 80 L using the same reaction conditions. These results are shown in **Figure 6**. For the 14 L reactor, almost 100% recovery of carotenoids was obtained, despite the initial stirring difficulties. For the 80 L reactor, agitation was fixed at 200 rpm and the second load was reduced to 60% of the initial load to maintain adequate mixing. Again, the final recovery yield was almost complete (97%), demonstrating the feasibility to conduct the process through stepwise additions of flower, with the limitation being the capacity of the vessel to maintain an adequate level of mixing.

For systems not limited in the rate of agitation, the system can be improved even more by maximizing solvent usage by recycling it. To test this hypothesis, the 14 L reactor was loaded with a 1:2 flower:solvent ratio and stirred for 3 h and the solvent was recovered by centrifugation. After the volume was restored to its initial value with fresh hexane, the colored solvent was added to the vessel containing the same amount (1:2 ratio) of flower (with enzyme). The results are presented in **Figure 7**. A high global yield (88%) was obtained, demonstrating that hexane reuse is a feasible option to maximize throughput.

In summary, a novel process for the solvent extraction of marigold colorants from fresh flowers has been developed. It relies on the enzymatic maceration of flower tissue simulta-

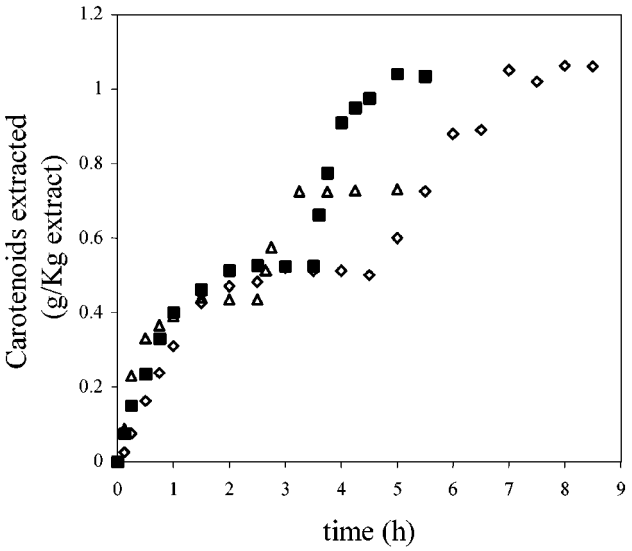

Figure 6. Effect of scale-up and hexane reuse by the stepwise addition of flower. Viscozyme/Neutrase treatment was with 0.3% (v/w flower). Extractions were carried out with a flower:hexane ratio of 1:4 at 45 °C and 500 rpm. Recharge was with the same amount of treated flower (60%) for 80 L reactor as described in the text. For the 80 L reactor, flowers were incubated for 1 h after enzyme spraying and before hexane extraction. 1 (◇); 14 (■); and 80 L reactor (△).

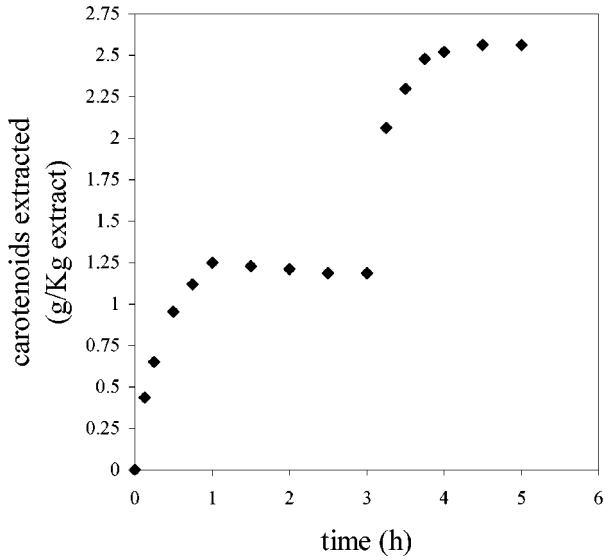

Figure 7. Hexane reuse by solvent recycling. Viscozyme/Neutrase treatment was with 0.3% (v/w flower). The first and second extraction were with enzyme-treated flower:hexane ratio 1:2 at 45 °C and 500 rpm. The hexane recovered from the first extraction was used in the second extraction, with initial volume adjusted for losses.

neously to carotenoid extraction with solvent in one single stirred vessel. Its main advantage is that it obviates the drying and silage operations normally used that result in a substantial degradation of carotenoids. In addition, it prevents the generation of aqueous effluents with a high BOD. The results presented show that high yields (>85%) of carotenoids recovered can be obtained with different arrays and scales in simple stirred vessels. The capacity of the system is limited by the rate of agitation to obtain adequate mixing of dispersed solids. The high added value of the product, the increase in reaction yields, and the absence of adverse environmental consequences makes this process attractive as an alternative to the traditional one.

ACKNOWLEDGMENT

We thank Elena Arriaga, Mario Trejo, Sandra Perez Munguía, Fernando Gonzalez, and Mario Caro for technical support.

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Received for review March 21, 2002. Revised manuscript received May 27, 2002. Accepted June 3, 2002. This project was partially financed by Bioquimex Natural.

JF025550Q