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A Study on the Different Methods of Preparation of Lutein from Supercritical Fluid Processed Lutein Esters

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Abstract

Lutein is a dietary oxycarotenoid which is found to reduce the risks of Age-related Macular Degeneration (AMD). Supercritical fluid extraction of lutein esters from marigold petals was carried out and was found to be much effective than conventional solvent extraction. The saponification of pre-concentrated lutein esters to produce free lutein was studied which showed a composition of about 88% total carotenoids (UV-VIS spectrophotometry) and 90.7% lutein (HPLC). The lipase catalyzed hydrolysis of lutein esters in conventional medium was investigated. The optimal temperature, pH, enzyme concentration and water activity were found to be 50°C, 7, 15% and 0.33 respectively and the activity loss of lipase was about 25% after 8 times re-use at 50°C for 12 days. However, the lipase catalyzed hydrolysis of lutein esters in conventional media resulted in poor conversions (38.7%).

Keywords: Lutein; Preconcentration; Saponification; Lipase

Introduction

Since the beginning of the food industry, natural or synthetic pigments were used to give an attractive presentation, perception of freshness, taste, and quality of food. Today, natural colorants are emerging globally due to the perception of their safer, eco-friendly nature and pharmacological applications.

Lutein is an oxycarotenoid/xanthophyll containing two cyclic end groups and the basic C_{40} isoprenoid structure [1]. It is a non-vitamin A carotenoid that cannot be synthesized by humans and lutein and zeaxanthin are the only dietary carotenoids present in the macular region of the retina and the lens [2]. Epidemiological studies have shown that the risks of age-related macular degeneration and cataracts are inversely correlated with dietary intake and the concentrations of these xanthophylls in the serum and macula [3,4]. Lutein has proposed protective functions in the eye as an antioxidant and blue light filter. The chemical formula of lutein is $C_{40}H_{56}O_2$ and the molecular weight is 568.88 (lutein). Lutein is recently gaining importance as a nutraceutical compound [5]. Marigold flower petals are excellent sources of lutein as they contain high levels of lutein (of the order 4500 mg/lb) and no significant levels of other carotenoids [6]. Extracts of lutein are normally diesterified with lauric, myristic and palmitic acids with two fatty acid groups occupying the sites of the hydroxyl groups [7]. Lutein esters must be de-esterified before they are absorbed by the body since the *in vivo* hydrolysis of lutein esters into lutein occurs with an efficacy of less than 5% [4,8]. Also a high amount of fat content in the diet exceeding the level recommended by the American Heart Association is required for the absorption of lutein [9]. Age may play a role in the human body's ability to absorb lutein since enzyme activity normally decreases with the aging process [10].

The traditional process of lutein production consists of solvent extraction of lutein esters and saponification which gives free lutein [11]. Khachik F [12] showed a method of isolating, purifying and recrystallizing lutein from saponified marigold oleoresin. However, this method involved a multiple solvents for extraction and purification. Another study described a saponification process of crude oleoresin from marigold extract using ethanol, water and 45% alkali for about 3-5 hours at a temperature of about 45-80°C [13]. Even though the yield was higher in this method, the process was uneconomical because of the high amount of alkali used and the lower lutein ester content in the substrate.

Although organic solvents have been used quite extensively in the processing of biomaterials, concerns over their use in the food industry and environmental issues are also growing. This in turn has lead to the growing attention to the use of supercritical or near-critical fluids [14,15]. A fluid heated to above the critical temperature and compressed to above the critical pressure is known as a supercritical fluid [16]. For this reason, during the last two decades there has been growing attention to the use of compressed gases, in particular supercritical or near-critical fluids. An attractive feature of supercritical fluids is that the properties lie between that of gases and liquids. Thus, a supercritical fluid can diffuse faster in a solid matrix than a liquid, yet possess a solvent strength to extract the solute from the solid matrix [17]. Supercritical fluid extraction of lutein esters is much effective than conventional solvent extraction, since it has improved mass transfer properties. Supercritical carbon dioxide is the most commonly utilized SCF, due to its low critical temperature and pressure (31°C and 7.38 MPa, respectively), chemical inertness and relatively low cost. There have been a lot of studies regarding the supercritical fluid extraction of lutein esters from marigold flowers and other sources [18-20]. Most of these processes invariably used co-solvents with $SCCO_2$ for their extraction. Even though the solubilities of lutein esters further increase with a co-solvent, the direct extraction of food colors using $SCCO_2$ is considered to be economically viable. During the saponification process, generally high temperatures and concentrated alkali solutions are applied to obtain complete lutein ester hydrolysis. In most cases, the starting material used for saponification is derived from solvent extraction which involves the use of a huge amount of organic solvents of environmental concern to produce an oleoresin containing lesser

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amount of lutein ester than that extracted using supercritical fluids. Also the yield and recovery of lutein obtained after saponification of the lutein ester substrate is very low as a result of the lower lutein ester content in the substrate. This leads to the application of more amounts of alkali and solvents for the production of lutein. Moreover, this demands a tedious purification procedure, which also results in the inevitable loss of valuable lutein at each step due to purification. The production of lutein by chemical saponification thus becomes an uneconomical and less attractive process. Thus there arises a need for pre-concentrating the lutein esters and then subjecting it for saponification thereby resulting in a high yield of lutein. Another study described a process for using ketonic solvents for producing a lutein esters concentrate containing lutein and zeaxanthin esters containing 90-95% of trans-lutein esters and 3.5-6% of zeaxanthin esters [21].

Even using supercritical extraction, it is not practically feasible to increase the concentration of lutein esters in the substrate beyond 45%. Thus there has been a long standing desire to find an alternative process, which reduces the amount of alkali and organic solvents, overcoming the difficulty in pre-concentration and at the same time resulting in a maximum yield and recovery of lutein. The present study attempts to develop a simple, effective and a high yielding process involving supercritical extraction of lutein esters followed by a pre-concentration step using a solvent and then saponifying the pre-concentrated resin.

On the other hand environmentally benign enzymatic hydrolysis of lutein esters using lipases was also investigated since chemical saponification causes product impairment [22] and lipase enzymes are highly specific catalysts which catalyze the hydrolysis of fatty acid ester bonds. The finding that enzymes especially lipases are stable and active in organic solvents and Supercritical fluids especially $SCCO_2$, has broadened immensely the scope of their applications as highly enantioselective catalysts in organic synthesis. Water-insoluble substrates can thus be transformed by enzymes in non-aqueous media [23-25]. With this background, the present study has been conducted to evaluate the efficiency of lipase catalyzed hydrolysis of lutein esters using the two different methods.

Materials and Methods

Marigold meal and chemicals

Marigold meal (fermented, dried and pelleted material) was a gift from Novo Agritech, Pvt Ltd, Hyderabad, India. All the chemicals and solvents used for extraction were of analytical grade and the solvents used for HPLC were of HPLC grade purchased from Qualigens Fine chemicals, Mumbai, India.

Enzyme

Immobilized enzymes of Candida antarctica Lipase B (CALB) with lipase activity of about 5988 U/g were purchased from Advanced Enzyme Technologies Ltd, Mumbai, India.

Standards

Lutein standard (70%; Catalog No # X6250-5MG) was purchased from Sigma Aldrich, USA.

Supercritical fluid extraction of lutein esters

A 5 litre pilot scale supercritical fluid extractor (Model A2630-IND2-NX-001) from NATEX process technologies, Ternitz, Austria of maximum working pressures of 100 MPa was used for the supercritical extraction of lutein esters. The marigold meal pellets were ground to a particle size of 0.5 mm and dried to a moisture content of 5% in a rotary drier. The marigold powder thus prepared was refluxed with hexane at a temperature of 60-65°C for a period of 2-3 hours. The hexane extract was thus subjected to UV-VIS Spectrophotometer at 446 nm to estimate lutein ester content in the marigold meal powder. The lutein ester content was found to be 2.46%. The supercritical fluid used in the extractor was CO_2 without any co-solvent/entrainer. 3 kg of the powdered marigold meal was loaded into a cylindrical basket and both the ends were secured with fine steel meshes and clamped tightly. The basket was then placed inside the extractor and closed. The pressure used in the extractor was 45 MPa and the temperature was 70°C. The pressure developed in the first separator S_1 was 12 MPa and the temperature was 55°C. The pressure and temperature in the second separator S_2 were 4.5 MPa and 20°C respectively. The first fraction (350 g) collected in the first separator is called the total extract and it contained lutein ester content of about 26% as shown by UV-VIS spectrophotometry. It also contains undesirable odour and substances. The residue obtained as waste was about 2.65 kg. In order to improve the concentration of the lutein esters and to remove the undesirable odour, this total extract fraction was subjected to liquid-liquid fractionation using $SCCO_2$. The pressure used in the extractor for liquid-liquid extraction was 27.5 MPa and the temperature was 60°C. The pressure developed in the first pressure and temperature in the second separator S_2 were 4.5 MPa and 20°C respectively. Depending upon the extraction time and the requirement the first fraction can be concentrated with lutein ester between 23-40%.

Pre-concentration of lutein ester

The lutein ester total extract obtained from supercritical extraction was further concentrated by organic solvent since the supercritical fluid extraction technique is not feasible for concentrating more than a lutein ester concentration of 45%. 200 g of the total extract with 26% lutein ester content was admixed with isopropanol in the ratio ranging from 1:3 to 1:6 (Total extract:isopropanol). The addition of isopropanol helps in preferentially dissolution of fatty acids and other non-ester compounds. This admixture was kept under continuous agitation at a temperature of 10-25°C for a period of 3-6 hours. For every one hour a sample was drawn and the lutein ester concentration was estimated using UV-VIS spectrophotometer at 446 nm. It was then filtered and the resultant filtrate was then dried under vacuum at room temperature and dried to obtain a solid resinous concentrate. The lutein ester content of the final concentrate was estimated and found to be ranging from 60-68%. The yield of the concentrated lutein ester was about 50 g.

Calibration for HPLC analysis

Calibration curve of the lutein standard samples were determined five times each, 8 different solutions of known concentrations of lutein standard included between 5 and 40 ppm to determine the linearity between lutein peak areas against injection mass concentrations. The curve equation $y = bx + m$ calculated with linear regression method to determine samples concentration was utilized.

Saponification of pre-concentrated lutein esters

For saponification 50 g of the pre-concentrated lutein ester (60%) resin was mixed with about 100-150 ml of isopropanol. The mixture was continuously agitated and kept at a temperature of about 60-75°C until the solution became homogenous. Then 20-25 ml of an aqueous

Treatments in Conventional Aqueous media	Treatment in Non-aqueous (Hexane) media	pH	Temperature (°C)	Enzyme concentration (% weight of the substrate)
CT_1	OT_1	5	40	5
CT_2	OT_2	5	50	5
CT_3	OT_3	5	60	5
CT_4	OT_4	5	40	15
CT_5	OT_5	5	50	15
CT_6	OT_6	5	60	15
CT_7	OT_7	5	40	25
CT_8	OT_8	5	50	25
CT_9	OT_9	5	60	25
CT_{10}	OT_{10}	6	40	5
CT_{11}	OT_{11}	6	50	5
CT_{12}	OT_{12}	6	60	5
CT_{13}	OT_{13}	6	40	15
CT_{14}	OT_{14}	6	50	15
CT_{15}	OT_{15}	6	60	15
CT_{16}	OT_{16}	6	40	25
CT_{17}	OT_{17}	6	50	25
CT_{18}	OT_{18}	6	60	25
CT_{19}	OT_{19}	7	40	5
CT_{20}	OT_{20}	7	50	5
CT_{21}	OT_{21}	7	60	5
CT_{22}	OT_{22}	7	40	15
CT_{23}	OT_{23}	7	50	15
CT_{24}	OT_{24}	7	60	15
CT_{25}	OT_{25}	7	40	25
CT_{26}	OT_{26}	7	50	25
CT_{27}	OT_{27}	7	60	25
CT_{28}	OT_{28}	8	40	5
CT_{29}	OT_{29}	8	50	5
CT_{30}	OT_{30}	8	60	5
CT_{31}	OT_{31}	8	40	15
CT_{32}	OT_{32}	8	50	15
CT_{33}	OT_{33}	8	60	15
CT_{34}	OT_{34}	8	40	25
CT_{35}	OT_{35}	8	50	25
CT_{36}	OT_{36}	8	60	25

Table 1: Treatment combinations for the lipase catalyzed hydrolysis in non-aqueous medium (Hexane).

solution equivalent to 30% potassium hydroxide was added slowly to the reaction mixture over a period of 30-60 min. The reaction is carried over for a period of 4-5 hours to ensure complete saponification. An aliquot of 1 ml was drawn from the reaction mixture every 1 hour and the sample was analyzed by HPLC to determine the completion of saponification which is indicated by the complete disappearance of the lutein ester peaks. After saponification, the reaction mixture is cooled to about 50-60°C and neutralized with a 10-30% aqueous acetic acid. Then about 100-200 ml of distilled water was added to the reaction mixture and the temperature was increased to about 60-70°C and this mixture is stirred continuously for a period of 15-30 min. The resultant mixture is then centrifuged for a period of 20-30 min in a tubular centrifuge and the centrifugation continued for 2-3 cycles with continuous replenishment of distilled water until the supernatant becomes clear. The precipitate was collected and washed with warm distilled water 2-3 times to remove the impurities and dried under vacuum for 3 hours to produce a fine crystalline powder. The experiment was conducted in duplicate.

Lipase catalyzed hydrolysis of lutein esters in conventional medium

20 g of the lutein ester samples of concentrations (20% after liquid-liquid extraction) were taken in 250 ml conical flasks. To this substrate immobilized lipase enzyme of Candida Antarctica Lipase B (CALB) was added in the range from 5-25 % concentration to the weight of the substrate. The immobilized enzymes and the substrate were pre-equilibrated to water activities (aw) ranging from 0.11-0.95 using a series of saturated solutions providing different water activities [26]. The reactions were carried out in different pH's ranging from 5-8 using different buffers. About 5 ml of lecithin solution was added to the reaction mixtures for improved emulsification. The reaction mixture was incubated at different temperatures ranging from 40-60°C.

The reaction mixture was continuously agitated using a Teflon-coated magnetic stirrer at 150-180 rpm. The reactions were carried for a period of 72 hours and a representative sample was taken periodically from the reaction mixture between 8-72 hours and was analyzed for the lutein content. Five replications were done for each parameter. Control experiments were also conducted simultaneously without the enzymes. After the reaction was over the immobilized enzymes were filtered through a muslin cloth and collected. The collected material was washed twice with chloroform to ensure the recovery of any possible adsorbed lutein-based products from the immobilized biocatalyst particles. The representative samples of reaction mixture were extracted with a required amount of chloroform. The chloroform extract was collected and dried completely under vacuum. A known quantity of the dried material was again re-dissolved in methanol and made up to 50 ml with methanol in a 50 ml volumetric flask and subjected for UV-VIS spectrophotometric and HPLC analysis. Table 1 shows the different treatment combinations for the lipase catalyzed hydrolysis of lutein esters in aqueous and non aqueous media.

Lipase catalyzed hydrolysis of lutein esters in non-conventional medium (hexane)

Lipase catalyzed hydrolysis in organic solvents was carried out after pre-concentration of lutein esters. This provides maximum availability of the lutein esters for lipase enzyme action thus making the reaction more specific. After concentration of lutein esters to a required degree, the lutein ester concentrate was re-dissolved again in dried hexane to known required concentrations. The solvent hexane is selected because of its high log *P* value (3.5-3.98). The log *P* value is proposed as a quantitative measure of solvent polarity and the lipase enzyme catalyzed reactions generally increase with the increase in log *P* value of the solvent. 20 ml of the hexane concentrate (12% lutein ester) was taken in 250 ml conical flasks. To this solution immobilized lipase enzyme of Candida Antarctica Lipase B (CALB) with lipase activity of about 5,988 FIP U/g was added to the reaction mixture in the range from 5-25% concentration to the weight of the substrate. The immobilized enzymes and the substrate were pre-equilibrated to water activities (aw) ranging from 0.11-0.95 using a series of saturated solutions providing different water activities [26]. The reactions were carried out in different pH's ranging from 5-8 using different buffers. About 5 ml of lecithin solution was added to the reaction mixtures for improved emulsification. The reaction mixture was incubated at different temperatures ranging from 40-60°C. The reaction mixture was continuously agitated using a Teflon-coated magnetic stirrer at 150-180 rpm. The reactions were carried for a period of 72 hours and a representative sample was taken periodically from the reaction mixture between 8-72 hours and was analyzed for the lutein content. Five replications were done for each parameter. Control

experiments were also conducted simultaneously without the enzymes. After the reaction was over the immobilized enzymes were filtered through a muslin cloth and collected. The collected material was washed twice with chloroform to ensure the recovery of any possible adsorbed lutein-based products from the immobilized biocatalyst particles. The representative samples of reaction mixture were extracted with a required amount of chloroform. The chloroform extract was collected and dried completely under vacuum. A known quantity of the dried material was again re-dissolved in methanol and made up to 50 ml with methanol in a 50 ml volumetric flask and subjected for UV-VIS spectrophotometric and HPLC analysis. Table 1 shows the different treatment combinations for the lipase catalyzed hydrolysis of lutein esters.

Lipase catalyzed hydrolysis of lutein esters in non-conventional medium ($SCCO_2$)

Lipase catalyzed hydrolysis of lutein esters in supercritical carbon-di-oxide was investigated in the pilot scale supercritical fluid extraction plant. The enzyme concentrations used were 5, 25 and 50%. The different pressures tried ranged from 200 and 275 bar. The different CO_2 flow rates used were 20 and 25 CO_2/hr. The immobilized lipases (CALB) were placed inside a rectangular 30 µ nylon porous bag of dimensions 7. 5 × 6 cm. Four cylindrical stainless steel rings of 45 g weight each with dimensions (Inner diameter 3.7 cm, Outer diameter-4 cm; Height-3.3 cm) were tied to the strings which were attached to the centers of the four sides of the bag. This arrangement was made to keep the bag buoyant and to expose the largest surface area for the enzyme reaction. 1kg of the substrate with lutein ester content of 21.6% was taken and filled inside the extractor. 50 ml of water was added to keep the water content of the reaction mixture at 5%. This was done to compensate for the loss of minimum water for the enzyme hydrolysis by the stripping action of $SCCO_2$ which dissolves water and has a saturation level of 0. 3%. Therefore a calculated amount of water was added prior to the reaction since there is no provision for adding water continuously in required amount during the reaction. The bag was then placed within the extractor (5000 ml). With this arrangement, the contact of substrate and catalyst was brought through the continuous supercritical phase. To initiate the reaction, the vessel was closed and filled with CO_2 up to an initial pressure of 120 bar and then slowly raised to 200-275 bar and maintained at 55-60°C. This slow increase in pressure was to acclimatize the enzyme to the high pressure environment. The pressure in the first separator S_1 was 120 bar and temperature was 50°C, whereas the pressure and temperature in the second separator were 45 bar and 20°C respectively. The reaction and extraction was continued for a period of about 6 hours in the case of lower enzyme concentration (5%) and 2 hours in the case of higher enzyme concentration (50%). The depressurization in the extractor was carried very slowly over a period of 90 minutes as against 30 minutes in normal liquid-liquid extraction to avoid denaturation of the enzymes. The bag was removed after the reaction was over and leached with chloroform to get a thick concentrate. The resulting chloroform concentrate was dried under vacuum to remove the chloroform and a known weight of the dried material was dissolved in methanol for HPLC and UV-VIS spectrophotometric analysis. The materials from the extractor and the separators S_1 and S_2 were collected separately and analyzed for the lutein content by HPLC and UV-VIS spectrophotometry. In all cases, five replications of the experiments were conducted.

Estimation of total carotenoids and lutein esters by UV-VIS Spectrophotometer

The lutein esters content of the samples were measured by UV-VIS spectrophotometer (GBC Model 916, Melbourne, Australia) at 446 nm in hexane with an extinction coefficient of 2671 for a 1% solution. The total carotenoids of the samples were estimated at 450 nm in chloroform with an extinction coefficient of 2550 for a 1% solution.

HPLC analysis

HPLC analysis were carried out using a Waters HPLC equipment equipped with a 515 HPLC pump (Model 2487, Dual wavelength UV detector, USA) and a Supelco C_{18} siloxane column with specifications of 250 × 4.6 mm, 5 microns (516 DB; Supelco Analytical; Sigma Aldrich; USA). The elution was isocratic using a mobile phase of Methanol: Water: Methyl tert-Butyl alcohol with a composition of (800:50:150 v/v). The sample solution and the mobile phase were filtered through a 0.45 µm PTFE filter membrane (Millipore, USA). The flow rate was maintained at 1ml/min for a run time of about 60 min. The injection volume of the sample was 25 µl and the wavelength was 446 nm. From the chromatograms the sample peak area units were compared with the lutein reference standard peak area units and the percentage of free lutein against the lutein reference standard solution was estimated.

Determination of lipase activity

Hydrolysis activity of the CALB lipase was assayed titrimetrically on olive oil emulsion [23]. The immobilized lipase was added to 10 ml of 10% v/v olive oil emulsion and was preincubated at 37°C for 20 min. The emulsion was then stirred magnetically for about 20 min. Then the reaction was terminated by adding 10 ml of 1:1 acetone: ethanol (v/v). The liberated free fatty acid was titrated with 0.1 M NaOH solution. One unit of lipolytic activity was defined as the amount of lipase that released 1 µmol of fatty acid liberated per minute at 37°C. A control was also performed using the same procedure without the addition of enzyme to the emulsion.

Karl Fischer Titration

Water activities of the enzymes were determined by Karl Fischer titration using Mettler–Toledo DL18 Karl Fischer titrator, USA.

Results and Discussion

Determination of lutein standard curves

Calibration curve of the lutein standard samples were determined five times each, 8 different solutions of known concentrations of lutein standard included between 5 and 40 ppm to determine the linearity between lutein peak areas against injection mass concentrations. The curve equation $y = bx + m$ calculated with linear regression method to determine samples concentration was utilized. The equation of the curve Y = -229454.96 + 422030.51*X and the R^2 value (0.99) showed good linearity of the lute in peak areas and the injection mass concentration of the samples.

Saponification of pre-concentrated lutein esters

The UV-VIS spectrophotometric analysis of the final purified product showed a total carotenoid/xanthophyll content of about 88% by purity. The HPLC analysis of the final purified product showed that the percentage of free lutein in the final product was 90.7% by comparing it with the peak area of the lutein standard chomatogram (Figure 1). The results showed comparatively better results and higher

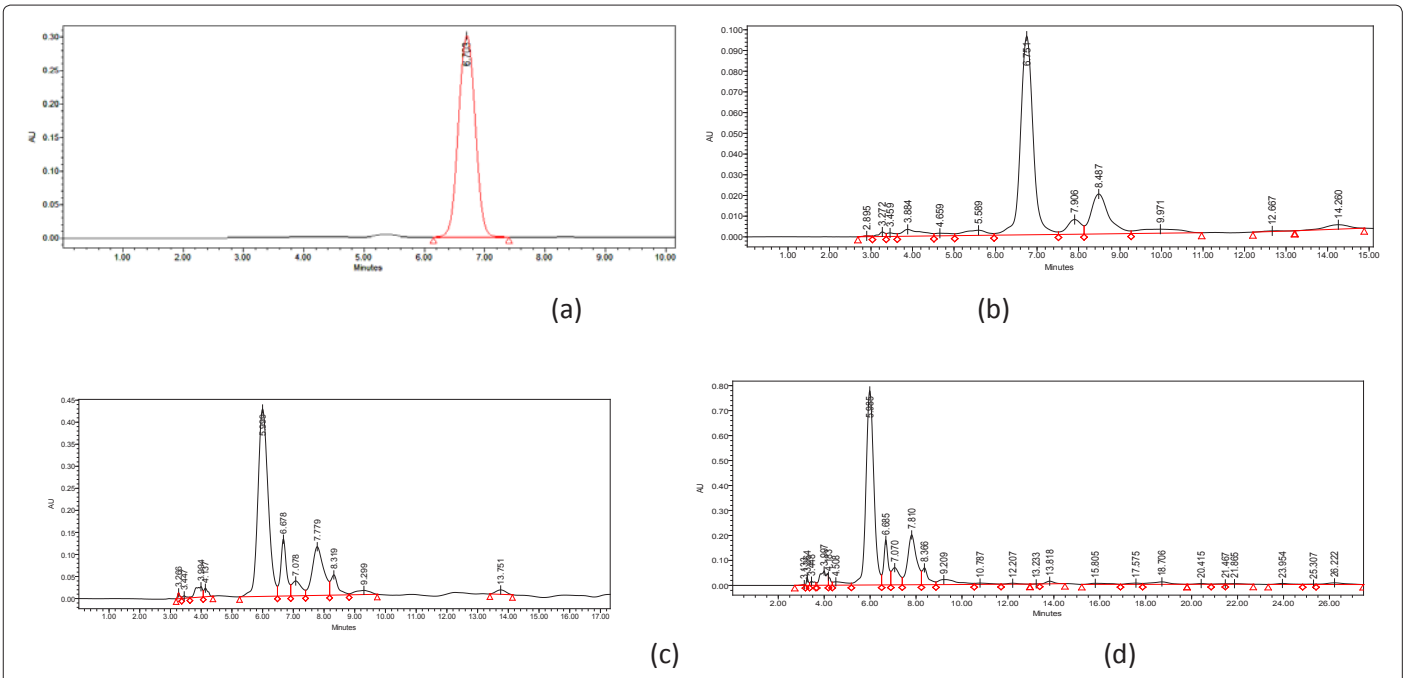

Figure 1: (a-d). a. HPLC chromatogram of lutein standard showing lutein peak eluting at approximately 6.7 min. b. HPLC chromatogram of the CALB enzyme hydrolyzed lutein ester sample in aqueous media. c. HPLC chromatogram of the CALB enzyme hydrolyzed lutein ester sample in non-aqueous media. d. HPLC chromatogram of the CALB enzyme hydrolyzed lutein ester sample in $SCCO_2$ media.

Treatments	MeanLutein concentration (%)	Treatments	Mean Lutein concentration (%)	Treatments	Mean Lutein Concentration (%)
CT_1	1.056	CT_{13}	1.108	CT_{25}	1.584
CT_2	0.33	CT_{14}	1.252	CT_{26}	1.66
CT_3	1.048	CT_{15}	0.792	CT_{27}	0.962
CT_4	1.156	CT_{16}	0.518	CT_{28}	1.096
CT_5	0.388	CT_{17}	0.994	CT_{29}	1.504
CT_6	0.652	CT_{18}	0.674	CT_{30}	0.772
CT_7	0.788	CT_{19}	1.404	CT_{31}	2.0254
CT_8	0.34	CT_{20}	1.73	CT_{32}	1.156
CT_9	1.266	CT_{21}	0.132	CT_{33}	0.17
CT_{10}	1.458	CT_{22}	2.18	CT_{34}	1.198
CT_{11}	0.474	CT_{23}	2.722	CT_{35}	1.29
CT_{12}	1.056	CT_{24}	0.856	CT_{36}	0.34
Critical difference (CD):0.268227; SE(d):0.135703					

Table 2: Mean values of lutein concentration of the treatments of lipase catalyzed hydrolysis in conventional medium.

yield compared to the study [12] which described a method for the purification of saponified marigold extract though the use of a series of filtrations and washes with water/alcohol and to obtain crude lutein crystals. It was also found from the study that the preconcentrated lutein esters resulted in higher conversions and yield of lutein. Also it can be observed from the study that the amount of alkali and solvent used is comparatively less than that used for obtaining the same yield without preconcentrating the lutein esters. By preconcentrating the lutein esters, the purification and the recovery of lutein after saponification becomes much easier. This results in the production much less effluent and the associated problem in the treatment of the same. The impurities present in the final product are other minor carotenoids which are of dietary origin. The time used for saponification and the amount of alkali used can be considerably reduced if the lutein esters are preconcentrated. The reaction becomes more specific when the alkali for saponification is more particularly used for hydrolyzing the lutein esters.

Lipase catalyzed hydrolysis of lutein esters

It is the first investigation towards the enzymatic hydrolysis of lutein esters at a very high substrate concentration and volume under different ranges of temperatures, pH, time, enzyme concentrations and water activities. The present study attempted to conduct lipase catalyzed hydrolysis of lutein esters without the use of bile salts as activators for enzymes since it affects the final product quality and increases the cost of production significantly. Table 2 shows the relation between the different treatments and their corresponding mean concentration (%) as determined by UV-VIS spectrophotometric analysis.

Effect of temperature on reaction rate

The reaction rates were monitored by UV-VIS spectrophotometric analysis and HPLC analysis of the methanol extract of the sample. The lowest temperature studied was 25°C. Lower temperatures resulted in slower initial reaction rates, since the reaction temperature had a great

Treatments	Mean Lutein concentration (%)	Treatments	Mean Lutein concentration (%)	Treatments	Mean Lutein Concentration (%)
OT_1	1.086	OT_{13}	2.894	OT_{25}	4.800
OT_2	1.338	OT_{14}	3.760	OT_{26}	4.848
OT_3	1.336	OT_{15}	3.548	OT_{27}	3.678
OT_4	1.616	OT_{16}	2.852	OT_{28}	4.364
OT_5	1.958	OT_{17}	3.406	OT_{29}	4.852
OT_6	1.714	OT_{18}	3.222	OT_{30}	3.250
OT_7	0.834	OT_{19}	4.364	OT_{31}	4.818
OT_8	1.272	OT_{20}	4.864	OT_{32}	5.288
OT_9	1.112	OT_{21}	4.826	OT_{33}	4.340
OT_{10}	2.186	OT_{22}	4.750	OT_{34}	4.120
OT_{11}	2.544	OT_{23}	5.720	OT_{35}	4.260
OT_{12}	2.394	OT_{24}	4.322	OT_{36}	4.220
Critical difference (CD):0.268227; SE(d):0.135703					

Table 3: Mean values of lutein concentration of the treatments of lipase catalyzed hydrolysis in hexane.

influence on the rate constant for lutein ester hydrolysis. The reactions at 25°C did not show any observable change or conversion as compared to the control in both conventional and non-conventional hexane media. At this temperature in conventional medium, the substrate was very viscous and thick in which the immobilized enzymes got trapped and the agitation with magnetic stirrer was not possible. At this temperature the substrate conversion was very low in which the immobilized enzymes showed almost no activity. The absence of enzyme reaction may be due to the mass transfer limitations between the biocatalyst particles and the substrate.

However, the reactions conducted at 40°C showed initiation of enzyme reaction (increase in absorbance) compared to reactions at 25°C. The melting point of lutein ester substrate (50-53°C at atmospheric pressure) also plays an important role. At 50°C the reaction rate was comparatively higher and resulted in good conversions. The reactions conducted at 50-60°C showed the highest conversion and correspondingly higher reaction rates. Even though the reactions above 50°C especially at and above 60°C showed increased acceleration of reaction, after 12 hours the reaction rate decreased considerably compared to the reactions conducted in the range of 50°C. The conversion also was decreased considerably. The lipase activity was found to decrease by an order of 30% in the case of lipase enzymes subjected to reactions at temperatures 60°C whereas the lipase activity of the enzymes used in the reaction at temperatures ≤ 50°C did not change significantly [27]. Higher temperatures accelerate reactions, but the influence of high temperature on the enzyme efficiency is an important factor to be considered. Enzyme denaturation can occur at elevated temperatures because of both the partial unfolding of the enzyme molecule and covalent alterations in the primary structure of the molecule [28]. This factor is essential because it determines the reusability of the immobilized enzymes. As expected, conversion was found to increase with increasing temperatures. Thus it was found from the study that the optimum temperature range for *Candida antarctica* Lipase B for lutein ester hydrolysis in both conventional and non-conventional hexane media was 50-60°C and the optimum temperature at which maximum efficiency was observed was 50°C.

Effect of temperature on enzyme stability

It was found that the activity loss of lipase immobilized CALB after six times re-use in conventional and hexane medium was about 25% and 40% respectively at 50°C for 12 days.

Effect of initial water activities

The initial water activities of the substrates and enzyme were adjusted by pre-equilibrating with the desired saturated salt solution, catalyst and reactants in a sealed container *via* the vapor phase before use. The effects of the initial water activities of the reactants and enzyme on the conversion were studied. It was found that the highest yield was achieved at an initial water activity of 0.33 in conventional medium, whereas in the case of hexane medium it was 0.22. The increased rate of hydrolysis of a highly hydrophobic substrate like lutein esters at low water activities were consistent with the results of the study which established the fact that in non-aqueous media the hydrolysis of lutein esters is favored at lower water activities [11]. Therefore, high levels of biocatalyst hydration result in a hydrophilic microenvironment around the biocatalyst particles that suppresses the hydrolysis reaction. This establishes that even in aqueous media the hydrolysis of a highly hydrophobic substrate like lutein esters is favored at lower water activities as a result of a facilitated access of the substrate to the immobilized enzyme.

Effect of reaction time

The lipase enzyme hydrolysis reaction was studied over for a period of 72 hours. As indicated in previous sections, long reaction times normally favor the hydrolysis of lutein esters and also give high conversions. It was found that the initial hydrolysis reaction rate was very fast, but then the reaction rate decreased progressively after a certain period of reaction time. The reaction rate was faster for a period of 8 hours after which the rate decreased gradually. The maximum conversion was attained in a period of 18 hours after which the reaction became stable and there was no significant conversion for a period of 48 hours. An interesting phenomenon was observed after 48 hours in the case of samples treated at temperatures of 60°C. The free lutein content as observed by UV-VIS spectrophotometric analysis (increase in absorbance) started decreasing (decrease in absorbance) and after 72 hours there was virtually no free lutein. This phenomenon was due to the re-esterification of free lutein formed with the free fatty acids in the system. CALB is a versatile enzyme which catalyzes the reversible reaction after a certain reaction period is over at low water activity. This observed phenomenon was consistent with the results of the earlier studies conducted on the hydrolysis of lutein esters and other ester compounds [27].

Effect of enzyme concentration

Lipase catalyzed hydrolysis of lutein esters with different enzyme concentrations in the range of 5-30% of the weight of the substrate was investigated. It is very important to provide effective mixing of reactants and enzyme is important for good transport and contact of the reaction

partners. It was found that the yield increased with increasing enzyme concentration until a maximum value was reached at 15% (w/w) enzyme load. It is not economical to increase the enzyme concentration more than 15% due to the highly expensive nature of immobilized enzymes. The effective mixing of the reactants and enzymes was also found to be hindered due to increased enzyme concentration. The enzymes availability becomes futile for the hydrolysis of the lutein esters substrate and finally affects the reaction rate due to the mass transfer limitations as a result of improper mixing status. Therefore the optimum enzyme concentration at which maximum hydrolysis is obtained was found to be 15%.

Effect of pH

It is important to determine the optimal pH because different enzymes usually have different pH optima depending on substrate concentration and temperature [24]. The catalytic activity of the lipase changes with pH in a bell-shaped fashion, thus yielding a maximum rate in the stability range. In the present study the lipase catalyzed hydrolysis of lutein esters was investigated at different pH ranging from 5-8.

The inherent pH of the substrate was found to be ranging from 6.7-7. The pH of the reaction mixture was controlled using different pH buffers with pH ranging from 5-8. The reactions conducted at pH 5 did not yield good conversions and the reaction rates were found to be slower, whereas the reactions conducted at pH 6 yielded comparatively higher conversions and the reaction rate was faster. The maximum conversion was observed at the pH of 7. At pH 8 the reaction rate was faster initially after which it started decreasing. From this study the optimum pH of *Candida antarctica* lipase B for the hydrolysis of lutein esters was found to be 7.

Overall the lipase catalyzed hydrolysis of lutein esters in conventional media did not yield good results and resulted in poor conversions. Figure 1(b) shows the HPLC chromatogram of the lipase hydrolyzed lutein ester sample. HPLC and UV-VIS spectrophotometric analysis showed that the maximum conversion was observed in Treatment 23 (CT_{23}- 2.722%) which was significantly more than the other treatments (P>0.05). The percentage of conversion of lutein ester was 16.4% as indicated by HPLC for CT_{23}. The mean difference between CT_{31} and CT_{20} was more than the critical difference at 5% level of significance and hence the CT_{31} yielded significantly more conversion than CT_{20}.

However, the hydrolysis of lutein esters in hexane yielded moderate conversions. Figure 1(c) shows the HPLC chromatogram of the lipase hydrolyzed lutein ester sample. HPLC and UV-VIS spectrophotometric analysis showed that the maximum conversion was observed in Treatment 23 (OT_{23}- 5.720%) which was significantly more than the other treatments (P>0.05). The percentage of conversion of lutein ester was 38.7% as indicated by HPLC for OT_{23}. The mean difference between OT_{31} and OT_{20} was more than the critical difference at 5% level of significance and hence the OT_{31} yielded significantly more conversion than OT_{20}.

The results corresponded well to the previous studies on the enzymatic treatment of natural esterified red and green pepper carotenoids using *C. rugosa* lipase which was only partially successful [8]. Similarly an inability to demonstrate efficient, quantitative enzymatic hydrolysis of natural carotenoid esters was reported using *Pseudomonas fluorescens* cholesterol esterase [29]. The concentrations of the lutein esters used in these previous studies were very less. In this study, the enzymatic hydrolysis was tried at higher lutein ester concentration which resulted in mass transfer limitations and due to the highly hydrophobic nature of the substrate. The conduction of enzyme hydrolysis at lower lutein ester concentrations is often not commercially viable and unattractive. In one study the lipase catalyzed hydrolysis of carotenoid esters such as lutein esters from marigold flowers and capsanthin esters from paprika were studied by enzymatic assays [25]. It was reported that the maximum hydrolysis using a commercial lipase was observed in the case of *Candida antarctica* lipase which yielded 44% release in the case of lutein and 69% release in the case of capsanthin from their respective esters. All these commercial lipases required bile salts for their activation. Bile salts proved to be essential auxiliaries for all commercial enzymes; omitting them at least halved the hydrolysis rates for both substrates. Moreover, the concentration of lutein ester oleoresin taken for the hydrolysis was very low and no study was conducted on the enzymatic hydrolysis under different ranges of temperatures, pH, time and enzyme concentrations. Similarly a recombinant enzyme of Human Pancreatic Lipase (rHPL) and porcine co-lipase was used in a study to demonstrate the hydrolysis of xanthophylls esters, which found that the activity of rHPL was extremely low with all substrates. The present study, however, did not use any bile salts in order to protect the quality of the end product and also to reduce the cost of the hydrolysis process and the enzyme reactions were conducted in very high substrate concentrations and volume as compared to any study for commercial applicability. The enzymatic hydrolysis reactions were conducted under different ranges of temperatures, pH, time and enzyme concentrations.

Effect of Different Parameters on the Lipase Catalyzed Hydrolysis of Lutein Esters in $SCCO_2$

Effect of temperature on reaction rate

All the treatment reactions were carried out at a constant temperature of 55°C which is the required temperature for the dissolution of the lutein esters in the $SCCO_2$ to maintain the required pressure and $SCCO_2$ flow rate and were also found to be optimum for the activity of CALB. Higher temperatures also affect the stability of lutein formed from the reaction since free lutein is not heat labile. Though conversion will increase with increasing temperatures in atmospheric pressures, the same will adversely affect the stability of lutein under high pressures especially under supercritical conditions.

Effect of $SCCO_2$ on enzyme stability

The enzyme stability under supercritical conditions depends on the reaction conditions such as pressure, temperature, depressurization regime and the reaction time. CALB is a very versatile lipase which is catalytically active even in supercritical fluids. The immobilized CALB is even more thermo stable. It was found that the activity loss of lipase immobilized CALB was about 70% after 3 times re-use in $SCCO_2$ at a temperature of 55°C, and a pressure of 200 bars for a reaction period of 6 hours. In comparison with the reactions in conventional medium and hexane, the loss of activity of CALB appears slightly higher in supercritical fluids and the number of times the enzymes could be reused was also found to be less.

Effect of initial water activities

The effect of the initial water content of the reaction mixture was on the conversion were studied. Water is crucial for enzymes and affects the enzyme action by influencing enzyme structure via non-covalent binding and disruption of hydrogen bonds, by facilitating reagent diffusion and by influencing the reaction equilibrium. However, even a small amount, perhaps even a monolayer on the enzyme molecules

is sufficient. As per the previous established studies it was found that lipase hydrolysis reactions are favored at lower initial water activities in $SCCO_2$ achieved at initial water content of 5% to the weight of the substrate. The use of an enzyme in pure $SCCO_2$ leads to the removal of water (pure $SCCO_2$ dissolves more than 0.3% water), which is included or bonded to the enzyme. The quantity of the removed water is temperature and pressure depended. The water content of the enzymes was determined by Karl-Fischer titration after the reaction was over. It was found that the water content of the enzymes were 0.26 (w/w) on an average. This indicated that there was sufficient water for the enzymes for hydrolysis as already established [11].

Effect of reaction time

As established by previous studies, longer reaction times normally favor increased conversion in $SCCO_2$. Mora-Pale et al. [11] showed that 70% of lutein ester conversion to lutein over a period of 24 hours. However, it is not economically feasible to conduct the reactions for more than 6 hours and also the unreacted lutein esters become more concentrated due to the extracting effect of $SCCO_2$ which removes the fatty acids and other unwanted components from the raw material. Due to this phenomenon the processing and removal of the material from the extractor and the separators becomes difficult. The reaction and extraction was continued for a period of about 6 hours in the case of lower enzyme concentration (5%) and 2 hours in the case of higher enzyme concentration (50%). The reduced reaction time in higher enzyme concentration was mainly because of the comparatively lower amount of substrate used which got exhausted easily and more concentrated after a period of about 2 hours. The initial reaction rate was observed to be faster and then progressively the rate got decreased after 5 hours.

Effect of enzyme concentration

Lipase catalyzed hydrolysis of lutein esters in $SCCO_2$ was conducted under the enzyme concentrations such as 5, 25 and 50% concentration to the weight of the substrate. The lowest enzyme concentration showed very less conversions as indicated by UV-VIS spectrophotometric and HPLC analysis. However, the reaction with an enzyme concentration was 25% was observed to show higher conversion (ST_2-26.36%) significantly more than that of 5% enzyme concentration (ST_1-7.101%). The maximum conversion (ST_3-42.87%) was observed at a very high enzyme concentration of 50% [11] similarly used a very high enzyme concentration of about 500 mg of enzyme for a lutein ester substrate of 40 mg. But, such high level of enzyme usage is not economically attractive. The effect of enzyme concentration on the lutein ester conversion is presented in Figure 1(d).

Effect of pressure

Pressure is likely to affect the reaction rate by changing either the reactants solubility or the rate constant directly. Indeed, an increase in pressure leads to enhanced fluid density and, therefore, improved solvating power of the fluid. On the other hand, the solubility of substances increases with higher pressures because of a higher fluid density and this is essential to bring the initial products in the reactor and remove the end products from the reactor. Therefore a pressure increase is, in most cases, positive for enzymatic reactions. The reactions were conducted at two different pressures 200 and 275 bars to study the effect of pressure over conversion. The reactions conducted at lower pressure of 200 bars showed significantly more conversions (ST_1,ST_2,ST_3) than that of high pressure of 275 bars (ST_4, ST_5, ST_6). An increase in pressure of the SF normally enhances the conversion rate due to increased analyte solubility, however, at some point; the enzyme activity starts to decrease with increasing pressure. This has been attributed to the lower mass transfer rates of reactants with an increase in $SCCO_2$ density. Rantakyla and Aaltonen described a higher enzyme activity at near-critical conditions compared to supercritical conditions in CO_2 at higher pressure. Such effects can be explained by the lower solubility of water in CO_2 (leaving more water available for the enzyme), faster diffusion rates, and enhanced electron-accepting power of the $SCCO_2$.

Effect of CO_2 flow rate

The $SCCO_2$ flow rate has the role of transporting the analytes from the sample matrix, through the enzyme-bed and finally to the collection device. The flow rate also can accelerate mixing, which even at low flow rates removes the rate-limiting effect of external diffusion (i.e. the diffusion from the bulk to the surface of the enzyme), due to the high solute diffusivities in $SCCO_2$. A higher flow rate leads to a shorter mean residence time of the substrates in the enzyme bed. Therefore in dynamic systems, the lowest flow rate possible is commonly applied in order to maximize the reaction of analytes as they travel though the enzyme bed. The yield of lutein was significantly affected by CO_2 flow rate. Similar to the effect of pressure, the yield of lutein increased initially with the rise of CO_2 flow rate. However, under certain conditions, further increases in flow rate resulted in a decline in the yield. Therefore the reactions were conducted at two different $SCCO_2$ flow rates such as 20 and 25 kg CO_2/h. The reactions at lower $SCCO_2$ flow rate of 20 kg CO_2/h showed higher conversions than that exhibited by higher flow rates of 25 kg CO_2/h. Dumont et al. has demonstrated that a higher $SCCO_2$ flow rate increased the conversion rate of myristic acid and ethanol to ethyl myristate, although the mean residence time of analytes in the enzyme bed decreased. A similar observation was also reported by Sun and Temelli for the extraction of carotenoids from carrots using canola oil as a co-solvent.

Among the 6 different treatments, the treatment 3 (ST_3) gave the highest conversion which was significantly higher than other treatments as shown by HPLC and UV-VIS spectrophotometry analysis. Treatment 2 (ST_2) showed significantly higher conversions than Treatment 1 (ST_1) at 5% level of significance, whereas the treatment 6 (ST_6) showed significantly higher conversions than treatments ST_4 and ST_5. From the HPLC chromatograms of the samples from the extractor, it was observed that at lower enzyme concentrations the reaction rate was very slow and a large peak eluted at the 9th minute with a larger area which was the lutein monoester. No other significantly large lutein diester peaks eluted after the 9th minute. It was very obvious that these were converted from the lutein diesters as a result of lipase activity over the lutein diesters. The free lutein peak was small and indicated that the lutein monoesters have not converted to lutein. This thereby shows that the enzyme concentration is insufficient to act on a highly hydrophobic substrate like lutein ester. Conversely, in the case of reactions at higher enzyme concentrations especially at 50% concentration, the lutein monoester peak which eluted at the 9th minute was very small, and the lutein peak represented the largest peak. This clearly indicates that the lipase has acted on the lutein diesters and monoesters and converted them completely into lutein. Thus from the study on the lipase catalyzed de-esterification of lutein esters in supercritical CO_2, it is clearly shown that the higher conversions are realized at higher enzyme concentrations, lower pressures and lower flow rates. The optimum pressure and $SCCO_2$ flow rate for lipase hydrolysis was thus found to be 200 bars and 20 kg CO_2/h respectively.

Conclusion

The saponification of lutein esters after preconcentration gives a much higher yield of lutein compared to the lipase catalyzed hydrolysis. There is a definite possibility for the reduced use of alkali and less saponification time for the preconcentrated lutein esters. On the contrary, in conventional saponification of lutein esters, there is a need for higher alkali usage and longer reaction times for complete saponification. This makes the hydrolyzed lutein more susceptible for degradation due to the high temperatures and concentrated alkali. However, with the improvised and modified saponification method adopted in the present study, these existing problems with chemical saponification can be easily overcome. The lipase catalyzed hydrolysis of lutein esters is a more environment friendly biocatalytic process which involves mild reaction conditions. However, the conversion and recovery of lutein is often moderate, presumably because of the high hydrophobicity of the substrate. Among the three methods of lipase catalyzed hydrolysis of lutein esters, simultaneous extraction and hydrolysis/de-esterification in $SCCO_2$ is found to be the most efficient and resulted in a maximum conversion in a comparatively lesser reaction period. The improved mass transfer properties of $SCCO_2$ enhance the reaction rate and the separation of reactants and products becomes easier. But, in the present study, even though the conversion was greater in the case of $SCCO_2$ reactions, the enzyme concentrations used were very high (50%) as compared to that of the reactions conducted in conventional aqueous media and organic solvents (15%). This is often not economically viable as the enzymes are very expensive and thereby increases the cost of production despite the fact that the enzymes can be reused. In the present study, in contrary to the previous established studies which describe the improved stability of immobilized enzymes in $SCCO_2$, the observed stability after a number of uses was relatively low to that of the reactions conducted in other media. Also in the present study, it was observed that the reactants and products were not amenable for separation after a certain reaction time, the extension of which would have certainly resulted in more conversions. These observations encountered were contradictory to the previous established results and can be attributed to the fact that the present study was conducted in a pilot scale reactor as opposed to the previous studies which were all conducted in a lab scale module. The results from these studies cannot be extrapolated to commercial scale as there are a multitude of factors which influence the efficient hydrolysis. These factors which often have negligible effects in a lab scale reaction might become magnified in a commercially scaled up process.

However, these limitations can be overcome by undertaking a lot of studies with a multi-pronged approach. The multiplicity of parameters involved and its effect on the hydrolysis reaction has to be thoroughly understood and the optimal and minimal concentration of enzymes where maximum conversion results has to be fine tuned. Enzyme biocatalysis in supercritical fluids is an innovative and effective way which would certainly replace the existing less efficient conventional biocatalysis methods.

Thus the lipase catalyzed hydrolysis of lutein esters provides a good alternative for the production of free lutein which is a potent nutraceutical that can be incorporated into different foods after proper encapsulation to improve its stability in foods. The lipase catalyzed hydrolysis in supercritical fluids offers a new possibility for the simultaneous extraction and de-esterification of lutein esters to produce free lutein. This reduces the multiple steps involved in the extraction and the subsequent hydrolysis, which are highly expensive and energy demanding processes.

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