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Supercritical Fluid Extraction of Lutein Esters from Marigold Flowers and their Hydrolysis by Improved Saponification and Enzyme Biocatalysis

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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 in at 50°C for 12 days. However, the lipase catalyzed hydrolysis of lutein esters in conventional media resulted in poor conversions (16.4%).

***Keywords*—**lutein, preconcentration, saponification, lipase

I. 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 [22]. 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 [17]. 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], [8]. 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 is recently gaining importance as a nutraceutical compound [14]. 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 [27].

Extracts of lutein are normally diesterified with lauric, myristic and palmitic acids with two fatty acid groups occupying the sites of the hydroxyl groups [26]. 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% [1], [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 [23]. Age may play a role in the human body's ability to absorb lutein since enzyme activity normally decreases with the aging process [4].

The traditional process of lutein production consists of solvent extraction of lutein esters and saponification which gives free lutein [20]. Ref [15] 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 [28]. 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 [11], [18]. A fluid heated to above the critical temperature and compressed to above the critical pressure is known as a supercritical fluid [9]. 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

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extract the solute from the solid matrix [7]. 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 [13], [21] and [19].

Most of these processes invariably used co-solvents with SC CO_2 for their extraction. Even though the solubilities of lutein esters further increase with a co-solvent, the direct extraction of food colors using SC CO_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 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 [16].

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 [29] and lipase enzymes are highly specific catalysts which catalyze the hydrolysis of fatty acid ester bonds [10], [24] and [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.

II. MATERIALS AND METHODS

A. .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.

B. 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.

C. Standards

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

D. 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 SC CO_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%.

E. 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.

F. 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 was used to determine the samples concentration.

G.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 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.

H.Lipase catalyzed hydrolysis of lutein esters

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 (a_w) ranging from 0.11-0.95 using a series of saturated solutions providing different water activities [6]. 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. I shows the different treatment combinations for the lipase catalyzed hydrolysis of lutein esters.

İ. 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.

J. 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 × 4.6mm, 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 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.

K. Determination of lipase activity

Hydrolysis activity of the CALB lipase was assayed titrimetrically on olive oil emulsion (Huang et al. 1997). 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.

L. Karl Fischer Titration

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

III. Results and Discussion

A. .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 ranged from 5 to 40 ppm to determine the linearity between lutein peak areas against injection mass concentrations. The equation of the curve Y = -229454.96 + 422030.51*X and the R^2 value (0.99) showed good linearity of the lutein peak areas and the injection mass concentration of the samples.

B. 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 chromatogram (Fig.1). The results showed comparatively higher yield than any other chemical saponification method [15]. 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 of 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.

C. Lipase catalyzed hydrolysis of lutein esters

When investigating the lipase catalyzed hydrolysis reactions of lutein esters it was found that any concentration of lutein esters higher than 20% in the substrate was not suitable to conduct enzyme hydrolysis reactions. At high concentrations, the substrate becomes very hard which needs a temperature more than 85°C to become viscous. These high temperatures are not suitable for conducting enzyme reactions as the lipase enzymes are not stable at such high temperatures and are denatured. It was 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. II shows the relation between the different treatments and their corresponding mean concentration (%) as determined by UV-VIS spectrophotometric analysis.

D.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.

E. 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 influence on the rate constant of lutein ester hydrolysis. The reactions at 25°C did not show any observable change or conversion as compared to the control. At this temperature the substrate was very viscous and thick in which the immobilized enzymes got trapped and the agitation with magnetic stirrer was not possible. 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 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 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 [2].

TABLE I TREATMENT COMBINATIONS FOR THE LIPASE CATALYZED HYDROLYSIS IN CONVENTIONAL MEDIA

Treatments	*pH*	*Temperature (°C)*	*Enzyme concentration (% weight of the substrate)*
T_1	5	40	5
T_2	5	50	5
T_3	5	60	5
T_4	5	40	15
T_5	5	50	15
T_6	5	60	15
T_7	5	40	25
T_8	5	50	25
T_9	5	60	25
T_{10}	6	40	5
T_{11}	6	50	5
T_{12}	6	60	5
T_{13}	6	40	15
T_{14}	6	50	15
T_{15}	6	60	15
T_{16}	6	40	25
T_{17}	6	50	25
T_{18}	6	60	25
T_{19}	7	40	5
T_{20}	7	50	5
T_{21}	7	60	5
T_{22}	7	40	15
T_{23}	7	50	15
T_{24}	7	60	15
T_{25}	7	40	25
T_{26}	7	50	25
T_{27}	7	60	25
T_{28}	8	40	5
T_{29}	8	50	5
T_{30}	8	60	5
T_{31}	8	40	15
T_{32}	8	50	15
T_{33}	8	60	15
T_{34}	8	40	25
T_{35}	8	50	25
T_{36}	8	60	25

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 [5]. 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 is 50-60°C and the maximum efficiency was observed at 50°C.

F. Effect of temperature on enzyme stability

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

G.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. 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[20]. 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.

H.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 give also 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 [2].

İ. 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%.

J. 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 (Salter and Kell 1992). 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. Fig.2 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 (T_{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 T_{23}. The treatment T_{31} was significant than the other treatments (at 5% level of significance) and yielded the maximum conversion. 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 [1].

TABLE II MEAN VALUES OF LUTEIN CONCENTRATION OF THE DIFFERENT TREATMENTS OF LIPASE CATALYZED HYDROLYSIS

Treatments	***Mean Lutein concentration*** *(%)*	***Treatments***	***Mean Lutein concentration*** *(%)*	***Treatments***	***Mean Lutein Concentration*** *(%)*
T_1	0.826	T_{13}	1.108	T_{25}	1.584
T_2	1.056	T_{14}	1.252	T_{26}	1.660
T_3	0.330	T_{15}	0.792	T_{27}	0.962
T_4	1.048	T_{16}	0.518	T_{28}	1.096
T_5	1.156	T_{17}	0.994	T_{29}	1.504
T_6	0.388	T_{18}	0.674	T_{30}	0.772
T_7	0.652	T_{19}	1.404	T_{31}	2.025
T_8	0.788	T_{20}	1.730	T_{32}	1.156
T_9	0.340	T_{21}	0.132	T_{33}	0.170
T_{10}	1.266	T_{22}	2.180	T_{34}	1.198
T_{11}	1.458	T_{23}	2.722	T_{35}	1.290
T_{12}	0.474	T_{24}	0.856	T_{36}	0.340

Critical difference (CD):0.186064; SE(d):0.094315

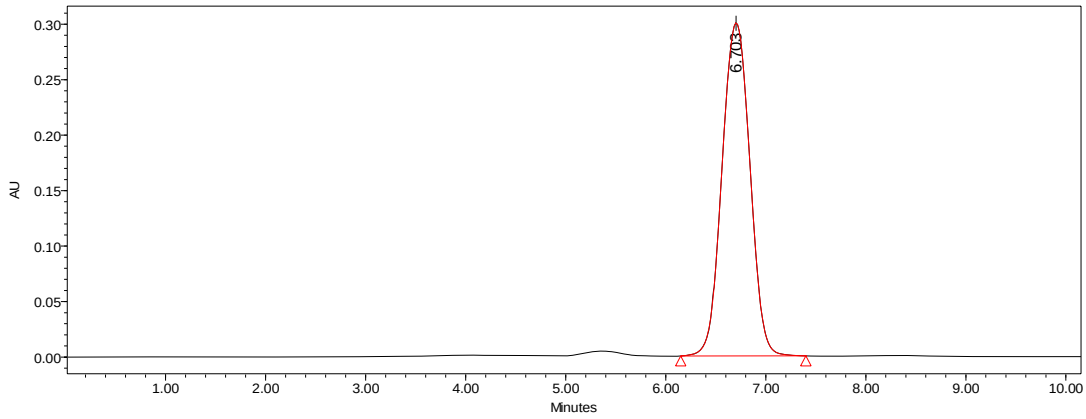

Fig.1 HPLC chromatogram of the saponified preconcentrated lutein ester sample showing the lutein peak eluted at 6.703 min

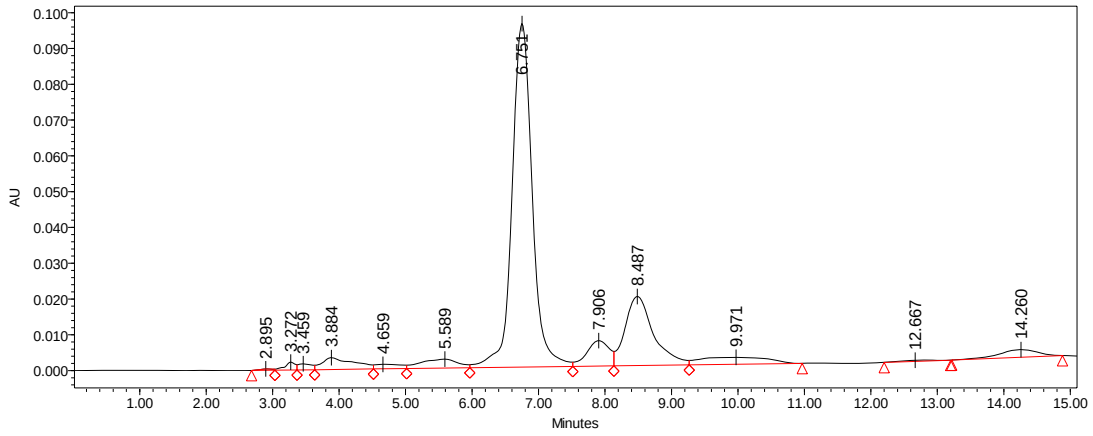

Fig.2 HPLC chromatogram of the CALB enzyme hydrolyzed lutein ester sample showing the lutein peak eluted at 6.751 min, cryptoxanthin peak-7.906 min, zeaxanthin peak-8.487 min and the subsequent peaks of lutein esters

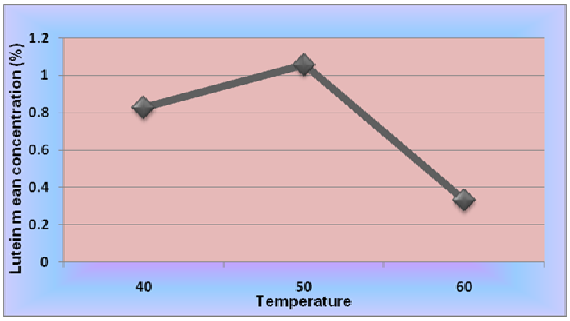

Fig. 3(a): Effect of temperature

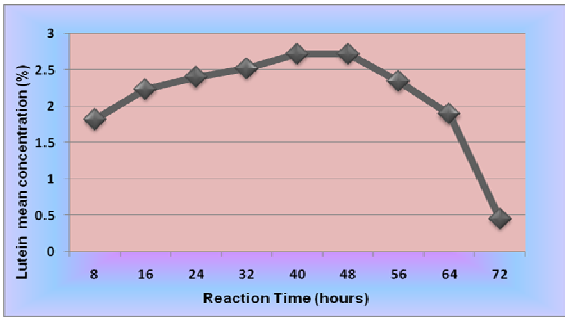

Fig. 3(b): Effect of reaction time

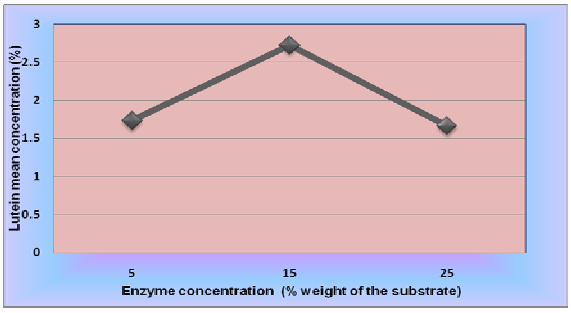

Fig.3(c): Effect of Enzyme concentration

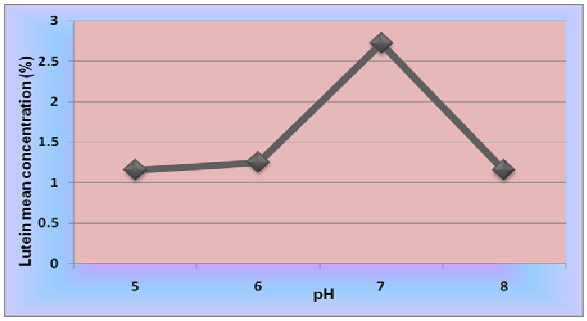

Fig.3 (d): Effect of pH

Fig.3: Effect of various factors on the lipase catalyzed hydrolysis of lutein esters in conventional media

Similarly an inability to demonstrate efficient, quantitative enzymatic hydrolysis of natural carotenoid esters was reported using *Pseudomonas fluorescens* cholesterol esterase [12]. 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.

IV. 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 very less, presumably because of the high hydrophobicity of the substrate. The cost of production also becomes very high due to the highly expensive nature of the enzymes. Also, the separation of the lutein becomes difficult. Thus, the modified saponification method of the pre-concentrated lutein esters serves to be an efficient and economical process for the production of lutein. The lutein thus produced is a potent nutraceutical and a natural colourant that can be incorporated into different foods after proper encapsulation to improve its stability in foods.

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