Lutein extraction from microalgae *Chlorella vulgaris* IG-R96: effect of solvent and processing time

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Abstract
Lutein is a carotenoid that has multiple roles in human and plant life. Recent research shows that this compound can play an important role in preventing and treating many diseases, such as cancer, cardiovascular disease and cataract, in human. Currently, the petals of marigold are mostly used for lutein extraction; but this flower has many problems as the main source. Since species of microalgae are a rich source of carotenoids, these microalgae can be seen as a competitive source to Marigold flowers in lutein production. In this study, solvent extraction of lutein from *Chlorella vulgaris IG-R96* is investigated. The results show that methanol is the best solvent for lutein extraction compared to alternative solvents such as acetone, chloroform, hexane, and ethanol. Lutein solvent extraction is better to be performed in multi-stage mode. The results show that a 3-stage process with incubation time of 30 min between each stage recovers the most lutein from algal biomass and consumes the least volume of solvent. In these conditions the most economic potential of algal feedstock is demonstrated.

Keywords: microalgae, carotenoids, extraction, lutein, solvent

Introduction
Carotenoids are a group of pigments that are essential to absorbing light in plants. There are many different structures of these fat-soluble pigments, based on their structure, carotenes and xanthophylls are the two main subclasses of carotenoids. Lutein, and its isomer zeaxanthin, are members of the xanthophyll family of carotenoids. There are two hydroxyl functional groups in the structure. [1] Lutein is beneficial to treat eye diseases such as age-related macular degeneration (AMD) and cataracts. It also serves to protect skin from UV-induced damage and may help reduce the risk of cardiovascular disease, cancer and degenerative human diseases.[2] Lutein is a carotenoid that is not synthesized by the human body and must be obtained through the diet such as fruits and vegetables with green leaves including spinach, kale and carrots and is frequently used as a food additive.[2, 3] Currently, marigold flower petals are used for commercial production of lutein. But this source is only cultivated in certain seasons and requires land, and ultimately despite of the high cost, it has low lutein content. In recent years, microalgae have been considered as a suitable source to lutein extraction.[4, 5] So, several microalgae with higher lutein contents, such as *Muriellopsis* sp. and *Chlorella* sp. have been used as lutein sources. *Chlorella vulgaris IG-R96* is a microalgae

that contains carotenoids.[6] In general, the extraction process of lutein from microalgae consists of 3 steps: cell disruption, saponification and extraction. In the first step, dried biomass is powdered, the cell is disrupted, and then lutein is extracted by modified methods. A number of techniques such as bead-beater, ultra-sonication, microwave, pestle and mortar and solvent soaking methods for cell disruption and organic solvent extraction and super critical fluid treatments for extraction, were commonly used. Among all of the disruption and extraction methods, bead-beater and organic solvent extraction are the most efficient methods.[7]

This study focuses on determining suitable solvent and optimizing the total extraction time and number of stages of lutein extraction from microalgae. Methanol, ethanol, acetone, hexan, choloroform and a mixture of aceton and methanol were examined and the number of processing stages in a specified time span was optimized with the objective of maximizing lutein extraction.

Experimental

Microalgae strain *Chlorella vulgaris IG-R96* was purchased from Iranian Biological Resource Center (ibrc.ir) with code number IBRC-M 50184.

To prepare algal culture, 1 liter of BG11 growth medium was autoclaved and cooled under sterile conditions. The composition of BG11 medium is (per 1 L): $NaNO_3$ (1.5 g); $MgSO_4.7H_2O$ (0.075 g); $K_2HPO_4.3H_2O$ (0.04 g); $CaCl_2.2H_2O$ (0.036 g); Na_2CO_3 (0.02 g); $FeCl_3$ (0.004 g); Citric acid (0.006 g); EDTA (0.001 g) and trace element (1 mL) containing $ZnSO_4.7H_2O$ (222 mg); $Na_2MoO_4.2H_2O$ (39 mg); $CuSO_4.5H_2O$ (79 mg); $MnCl_2.4H_2O$ (1810 mg); H_3BO_3 (2860 mg) and $Co(NO_3)_2.6H_2O$ (4.9 mg).

After cooling, the medium was inoculated with microalgae from prepared solid culture so that the initial optical density of the medium under 680 nm was about 0.07 to 0.1. The light intensity in the culture was 12,000 lux and the light/dark period was 16/8. After 4 days, 200 mL of the cell suspension was transferred to the 15 mL falcon tubes and centrifuged (Eppendorf, Centrifuge 5702) at 4400 rpm for 10 min. The supernatant was discarded and the wet biomass was transferred to a pre-weighed container and left in the oven (WiseOven) for 24 hours at 45 ˚C to dry to a constant weight. The dried biomass was powdered in a ball mill and stored in the fridge for further extraction.

For lutein extraction, glass bead vortexing (GBV) was applied. In a 50 mL falcon tube, 0.006 g of oven-dried biomass, 0.25 mL of distilled water, 3 mL of solvent, and two 0.5-mm diameter glass beads were mixed. Vortexing was conducted in a laboratory vortex (Wised, WiseMix, VM-10, South Korea) close to its maximum speed for 1 minute. To complete the extraction process, the sample was incubated in the dark for 10 min and then centrifuged (4400 rpm, 10 min) to seprate biomass from extract. The supernatant (extract) was removed for lutein content analysis.

In multi-stage process, the biomass was subject to 0.25 mL of solvent in every stage with dark incubation times of 5 min, 15 min, 30 min, 60 min, and 1440 min (24 h), and number of stages of 3, 4, and 6. The supernatants of all stages were mixed and analyzed for lutein content.

To determine lutein content of the extract, the absorption of supernatant was measured at the wavelength appropriate to the solvent using a spectrophotometer (UNICO Corporation, UV-2100), and then the content of lutein was calculated using the following equation:

$$C=(A/\varepsilon)\times(1/b)\times 568.88\times(V/M)\times 1000 \qquad \text{Eq. 1}$$

Where C is lutein content of the sample (mg lutein/g biomass), A is absorption of sample in lutein maximum wavelength in the solvent, ε is molar absorptivity (L/mol.cm), b is length of optical path which is 1 cm, V is solvent volume (L), M is mass of algal biomass (g), and 568.88 shows lutein molar weight in g/mol.[9]

Results and discussion

This study was conducted in two parts. At the first part, different solvents including methanol, ethanol, acetone, hexane, chloroform and a mixture of acetone and methanol (vol ratio 3:7) were examined at the fixed volume of 3 mL and dark incubation time of 10 min to find the most appropriate one for lutein extraction. Then, at the second part, multi-stage extraction processes were investigated with the objective of maximizing lutein extraction. To determine the optimum number of stages, and total extraction time, the process was performed at different stages, and then incubation times of 5, 15, 30, 60 min, and 24 hours were applied between each stage to obtain optimal extraction time. In this part, to reduce the rate of solvent use, the total volume of solvent for each sample did not exceed 3 mL.

Table 1 shows the lutein content of the algal sample using different extracting solvents. The results indicate that methanol could extract more lutein from biomass than rival solvents. Even, the mixture of methanol with acetone performed lower than each individual solvent. The least performance was observed using hexane that the extracted content was less than 20% of that of methanol. Thus, methanol was selected for further investigation in the next part of the study.

Table 1: Lutein content of algal sample in different extracting solvents. Solvent volume: 0.003 L, mass of algal biomass: 0.006 g.

Solvent	λ (nm)	A	ε (L/mol cm)	C (mg/g)
Aceton	446	1.203	144500	1.47
Chloroform	454	1.686	134800	2.20
Hexane	444	0.576	147300	0.69
Ethanol	444	1.892	145100	1.84
Methanol	442	3.1	149600	3.35
Methanol+Acetone	444	1.087	146030	2.12

The extraction of lutein from algal biomass could hypothetically increase using a multi-stage process. Aliquots of solvent were used for extraction in different stages so that the total volume of the solvent does not exceed 3 mL. Each stage was accompanied with a specified incubation time to complete extraction and the final number of stages determined the total extraction time. Table 2 indicates the extracted lutein content of algal sample under multiple stages and different incubation times. As seen in this table, a 3-stage process with incubation time of 30 min (Run 3) extracts more lutein from algal biomass (4.33 mg/g) than single-stage (3.35 mg/g). Increasing the number stages with shorter incubation times (Run 2 and Run 1) do not extract more lutein. Surprisingly, increasing the number of stages with longer incubation times (Run 4) and very long incubation time of 1440 min (Run 5) are not beneficial to maximum extraction. The results show that there is an optimum number of stages for lutein extraction and in each stage sufficient time need to be given to the extraction process to approach to the equilibrium state. In cases where incubation time is short, extracted lutein content is not good enough even though more stages are applied; since the extraction is

intrinsically a slow process and contact time is a key parameter. Nevertheless, too long incubation time (Run 5), tends to degradation of lutein and reduction of its content. The lower the number of stages, the lower the volume of the solvent required. This means that at the optimum state, either the processing time or the required volume of the solvent are optimum.
Extraction of lutein at the optimum state better demonstrates the ultimate economic potential of algal biomass as the feedstock for production of commodity products such as lutein. The reduction of processing costs including extraction time and solvent volume significantly reduce the total cost and enlights the prospect of bioproducts recovery from algal resources.

Table 2: Lutein content of algal sample in multi-stage experiments. Solvent volume for each stage: 0.25 mL, mass of algal biomass: 0.006 g, Solvent: Methanol.

Run	Incubation time (min)	No. of stages	Total solvent volume (mL)	C (mg/g)
1	5	6	1.5	3.3
2	15	4	1	2.23
3	30	3	0.75	4.33
4	60	4	1	2.99
5	1440	3	0.75	2.94

Conclusions

Lutein is a commodity product with numerous human health applications that can be extracted from algal biomass. Methanol is found to be the best solvent for lutein extraction. Multi-stage extraction process better demonstrates the economic potential of algal biomass. In a 3-stage process with 30 min incubation time between each stage and 0.75 mL solvent, the maximum lutein extraction was obtained. The achieve maximum extraction, the contact time in a multi-stage mode and solvent volume need to be optimized.

References

[1] Alves-Rodrigues, A. and A. Shao, "The science behind lutein". Toxicology Letters, 2004. **150**(1), p:57-83
[2] Calvo, M.M., "Lutein: a valuable ingredient of fruit and vegetables". Crit Rev Food Sci Nutr, 2005. **45**(7-8), p:671-96.
[3] Fuad, N.I.N., et al., "Lutein: A comprehensive review on its chemical, biological activities and therapeutic potentials". Pharmacogn J. 2020. **12**(6)Suppl:1769-1778.
[4] Guedes, A.C., H.M. Amaro, and F.X. Malcata, "Microalgae as sources of carotenoids. Marine Drugs", 2011. **9**(4): p. 625-644.
[5] Fábryová, T., et al., "Purification of lutein from the green microalgae *Chlorella vulgaris* by integrated use of a new extraction protocol and a multi-injection high performance counter-current chromatography (HPCCC) ". Algal Research, 2019. **41**: p. 101574.
[6] Deenu, A., S. Naruenartwongsakul, and S.M. Kim, "Optimization and economic evaluation of ultrasound extraction of lutein from *Chlorella vulgaris*". Biotechnology and Bioprocess Engineering, 2013. **18**: p. 1151-1162.

[7] Gong, M., X. Li, and A. Bassi, "Investigation of simultaneous lutein and lipid extraction from wet microalgae using Nile Red as solvatochromic shift probe". Journal of Applied Phycology, 2018. **30**: p. 1617-1627.

[8] Chen, C.-Y., et al., "Production, extraction and stabilization of lutein from microalga *Chlorella sorokiniana* MB-1". Bioresource Technology, 2016. **200**: p. 500-505.

[9] Jalali Jivan. M, Abbasi. A, "Lutein extraction using nanotechnology (microemulsion): effect of surfactant, co-surfactant and ultrasound pretreatment". Journal of Food Science and Technology (Iran), 2018. **15**(80): p. 413-420.

[10] Chen, C.-Y. and C.-C. Liu, "Optimization of lutein production with a two-stage mixotrophic cultivation system with *Chlorella sorokiniana* MB-1". Bioresource Technology, 2018. **262**: p. 74-79.