Efficient two-frequency ultrasound extraction of β-carotene from the fungus *Blakeslea trispora*

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Abstract
Carotenoids are known for their lower environmental pollution, lower costs, shorter extraction time compared to regular extraction methods and higher extraction efficiency. Ultrasound has significant advantages in the extraction of a great number of biologically active compounds including carotenoids. Benefits of using ultrasound for extraction of various compounds are numerous and include effective mixing and micro-mixing, more efficient energy transfer, reduced thermal and concentration gradients, reduced temperature. The aim of this work was to investigate the influence of two-frequency ultrasound main parameters such as ultrasonic intensity, sonication time, and temperature. Application of two-frequency ultrasound (simultaneous influence of ultrasound with frequency of 20–46 kHz, intensity of 1.5–2.5 W/cm^2 and frequency of 1.0–2.0 MHz, intensity of 2.0–3.0 W/cm^2) raises the percentage of β-carotene extraction from biomass of the fungus *Blakeslea trispora* from 90–92% up to 98–99% in comparison with one frequency ultrasound. The recommended oil temperature for the extraction process is 35 °C, because lower temperature leads to reduction of percentage of β-carotene extraction, and higher temperature promotes unjustified increase of expenditure of energy.

Keywords: ultrasound, β-carotene, *Blakeslea trispora*, two-frequency ultrasound.

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β-Carotene is an organic compound and chemically is classified as a hydrocarbon and specifically as a terpenoid (isoprenoid), reflecting its derivation from isoprene units. It has special properties that make it important to food quality and human health. As natural pigment, it gives pleasing yellow, orange and red colors of many foods [1,2]. It is a precursor of vitamin A, the deficiency of which persists as a serious public health problem in developing countries. Both provitamin A and vitamin A-inactive carotenoids can reduce the risk of the development of degenerative diseases such as cancer, cardiovascular diseases, cataract and macular degeneration [3–5]. A large number of precursors and methods for obtaining this compound are described in previous works. It was shown that β-carotene can be produced from many precursors including sucrose [6], yeasts, such as *Rhodotorula mucilaginosa* [7], cellobiose [8], grape must [9], citrus byproducts [1], lipids [10], algae [11], whey [12], glycerol [13] and fungi *Blakeslea trispora* [8,14,15].

The maximum yield has been obtained by *Blakeslea trispora*, a heterothallic Zygomycota with two mating types (termed plus and minus) producing hormone precursors (prohormones) from β-carotene. Carotenes extraction/production from *Blakeslea trispora* using different methods was conducted using stimulation of the biosynthesis [16] with oxygen-vectors [17], by enhanced aeration [18], from synthetic medium [19], effects of amines [20], metabolic stimulators and inhibitors [21], and media optimization [22].

Wider implementations of these technologies on industrial scale are limited by many factors, such as: a number of processing parameters is difficult to control, investing in such new technologies is expensive and risky, no regulatory implementations exist, whereas consumers' reluctance cannot be neglected. Also, the application of ultrasound has a significant reduction on many parameters necessary to create biologically active compounds including carotenoids. The effects of ultrasound in extraction must be considered especially from the point of effective macro and micro-mixing, energy saving, reduced processing temperature, etc. [23,24]. Analysis of β-carotene extraction techniques

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and the stability of β-carotene extraction under ultrasound treatment are described in previous works [25,26]. There are several studies about ultrasound extraction of β-carotene from *Spirulina platensis* [27], grape pomace [28], citrus peels [29], tomato waste [30] and peach palm fruit [31]. Also, the effect of high power ultrasound on extraction of β-carotene was presented, and the results were satisfactory [32]. The highest yields have been synthesized from the mixture of + and – strains of *Blakeslea trispora* [33].

In recent years, large number of studies showed the use of the simultaneous influence of low (18–47 kHz) and high frequency (1.0–2.5 MHz) ultrasound for the intensification of some technological processes: the destruction of complexes of metals with organic compounds in table salt and brines, the intensification of a mineralization of products of an animal and phytogenesis, acid extraction of fats and oils. In our previous work, it was explained that simultaneous influence of low- and high-frequency ultrasound is more effective in comparison with one frequency ultrasound [34]. The cavitation force of ultrasound can successively accelerate the heat and mass transfer rate so as to disrupt plants cell walls and facilitate the release of important extractable compounds. More specifically, there is almost no information available in the literature about the application of simultaneous influence of low- and high-frequency ultrasound in extraction of β-carotene from *Blakeslea trispora*.

The use of one frequency ultrasound requires the higher temperature in the range 150–170 °C. In the present work, the possibility of use of two-frequency ultrasound in extraction of β-carotene from biomass of the fungus *Blakeslea trispora* was investigated. After 4320 min (72 h) of culture growth the β-carotene content in medium can reach 1.0–1.6 g/L [35]. β-Carotene is extracted from the produced mycelium *Blakeslea trispora* after drying and powdering by heating with oil. Powdering of a dry fungus mycelium is necessary operation because the protein-vitamin bonds do not allow extracting β-carotene [36]. The essential limitation of this method is a low percentage of extraction 45–50% [37].

EXPERIMENTAL

The comminution of biomass of the fungus *Blakeslea trispora* and the extraction of β-carotene by oil was carried out in the chemical reactor, which allowed treating the brines by low (18–48 kHz) and high (0.5–3.0 MHz) frequency ultrasound simultaneously [35]. As sources of ultrasound magnetostriction and piezoelectric radiators with work frequencies from 18 kHz to 3.0 MHz, the lamp generator 24-UZGI-K-1,2 (Russia) and ultrasonic modernized dispergator, UZDN-1 M (Ukraine) were used [34]. For comminution of the biomass of the fungus *Blakeslea trispora* a laboratory disintegrator 2 BC-1-6M (Australia) was also utilized.

Four samples of biomass of the fungus *Blakeslea trispora* with the various content of β-carotene (Table 1) were selected and each sample of a biomass was divided into two parts to obtain desired mass of 20.0 g. A solution of β-carotene (0.50 g) was added in every second part of sample, mixed for 30 min and then left for 180 min. During this time the formation of protein-vitamin bonds was completed [34]. From every sample we selected three laboratory samples (each sample was 5.00 g). The extraction was carried out in the 50 mL chemical reactor. 15 mL of sunflower oil (Olejna) was added to the selected samples and reactor was treated simultaneously by low and high frequency ultrasound. The intensity of low-frequency ultrasound was 1.0–3.5 W/cm^2, and the intensity of high-frequency ultrasound was 1.0–4.0 W/cm^2. Duration of ultrasound was 1.5–5.0 min. β-Carotene was separated from the solution by siphoning and centrifugation. Extraction was repeated, and extracts were mixed.

Simultaneously, the extraction of β-carotene from the same samples of biomass of the fungus *Blakeslea trispora* was performed according to literature method [38] following these conditions: ultrasound frequency 22 kHz, intensity 3 W/cm^2 and the treatment time 8 min. Extraction was carried out by oil Olejna at 150 °C. Standard statistical methods and software package MS-Excel were used for analysis and the graphical presentation of the data has been done in Tables 1–3 and Figures 1–4 [16,17].

Total yield of β-carotene was determined and presented as percentage of the extraction of the added part of β-carotene. Each experiment was repeated three times and main values were presented. Also, total yield of β-carotene was controlled with use of biomass of the fungus *Blakeslea trispora* which was freed from β-carotene by triple extraction with hot oil and the subsequent double extraction with acetone in accordance with the previous work [34]. Afterwards, the known quantity of β-carotene as a solution in acetone was added into the biomass [34]. For the solution preparation, 98% crystalline β-carotene, fabricated by Bioscience Ptu. Ltd. (Australia), was used. The experiments were carried out with variation of oil temperature from 18 to 70 °C. Sunflower oil Olejna (Ukraine), corn oil (Ukraine) and olive oil Renieris (Greece) were used as extraction medium. The content of β-carotene was determined by spectrophotometer SF-46 (Russia) according to the technique from previous work [39].

The content of β-carotene in oil extract was carried out in next mode [34]. 200.0 mg of an oil extract was putted into graduated flask of 100 mL. The flask was filled with chloroform up to one third, mixed well, and filled up to the mark with chloroform and shaken. 10

mL of solution were taken into graduated flask of 100 mL. The flask was filled up to mark with chloroform and solution was mixed well.

Determination of β-carotene was carried out in a spectrophotometer at a wavelength of 465 nm [39]. A cell with 10 mm layer thickness was used. The chloroform was used as a reference solution.

The mass concentration of β-carotene was calculated by the formula:

$$X = \frac{100Dn}{2396m} \quad (1)$$

where D is optical density of the tested solution (instrument readings), n is number of dilutions, m is sample weight in g, 2936 is specific absorption coefficient (at a wavelength of 465 nm for 100% solution of β-carotene in chloroform).

The average of the three replicates was taken as final result. The acceptable deviation in replicates wasn't more than 2% relative to average.

RESULTS AND DISCUSSION

The simultaneous influence of low- and high-frequency ultrasound for extraction of β-carotene from a biomass of the fungus *Blakeslea trispora* rises the extraction percentage up to 98–99% in comparison with the use of one frequency ultrasound (90–92%) and disintegrator (45–50%, Table 1). The validity of the obtained results is proved by good accordance with results of the spectrophotometric analysis. The yield of β-carotene is the percentage of the extraction of added part of β-carotene (Table 1).

It is necessary to notice that the percentage of β-carotene extraction was 90–91% when the oil temperature was 18 °C, and when the temperature was above 35 °C, the percentage was 98–99% (Figure 1). This fact shows that the use of two-frequency ultrasound allows isolation of a product with higher biochemical and physiological activity [34].

Optimal time of ultrasound treatment was found to be between 2–4 min (Figure 2). When the exposure to ultrasound was less than 2 min, the extraction is not completed, but longer treatment (more than 4 min) possibly leads to partial destruction of β-carotene by ultrasound and decrease of percentage of β-carotene extraction could be noticed (Figure 2) [34,40]. Time--dependent extraction of different biomass of β-carotene extraction in % has moderate increasing trend to approximately 30 °C, then steeply increase up to approximately 35 °C reaching a constant value (Table 1). Dependence of the values of β-carotene extraction in %*versus* time in min for different biomass showed high slope to approximately 2 min, and then have approximately constant values up to 4 min, after when they start to decrease (Figure 2).

Higher efficiency of simultaneous influence of low- and high-frequency ultrasound is caused by features of formation and collapse of cavitation bubbles, because in this case the percentage of small spherical cavitation bubbles is extremely high (more than 90%). When the small spherical cavitation bubbles collapse, the protein–vitamin bonds in a biomass of the fungus *Blakeslea trispora* are broken and β-carotene becomes accessible to be extracted [34, 40]. Also, the collapse of small cavitation bubbles promoes the bigger efficiency in mass exchanged processes in comparison with big cavitation bubbles [40]. Frequency variation of low-frequency ultrasound from 20 to 46 kHz does not influence on the percentage of β-carotene extraction (Figure 3). The intensity of low-frequency ultrasound should be constant.

Comparison of the results obtained with use of high-frequency ultrasound (0.5–3.0 MHz) has shown that the best results have been received for ultrasound frequency of 1.0–2.5 MHz (Table 2). This value of intensity of 1.5–2.5 W/cm^2, in the case when high-frequency

Table 1. Comparison of methods of β-carotene extraction as an oil concentrate

Sample	Mass of added β-carotene, g	Use of disintegrator			Use of one frequency ultrasound (22 kHz)			Use of two-frequency ultrasound (20 kHz + 1 MHz)		
		β-Carotene found, g	S_r[a]	Total yield[b] %	β-Carotene found, g	S_r	Total yield %	β-Carotene found, g	S_r	Total yield %
1	0	0.201	0.112	45	0.403	0.087	90	0.438	0.081	99
	0.5	0.454	0.107		0.851	0.078		0.929	0.072	
2	0	0.339	0.111	50	0.652	0.085	91	0.710	0.080	98
	0.5	0.559	0.112		1.048	0.076		1.176	0.069	
3	0	0.287	0.121	48	0.514	0.083	91	0.560	0.079	98
	0.5	0.490	0.103		0.926	0.075		1.038	0.068	
4	0	0.275	0.122	49	0.515	0.084	92	0.569	0.080	98
	0.5	0.492	0.102		0.933	0.075		1.041	0.065	

[a]The relative standard deviation, S_r, was found at the 95% confidence level and n = 3; [b]the yield of β-carotene is the percentage of extraction of the added part of β-carotene

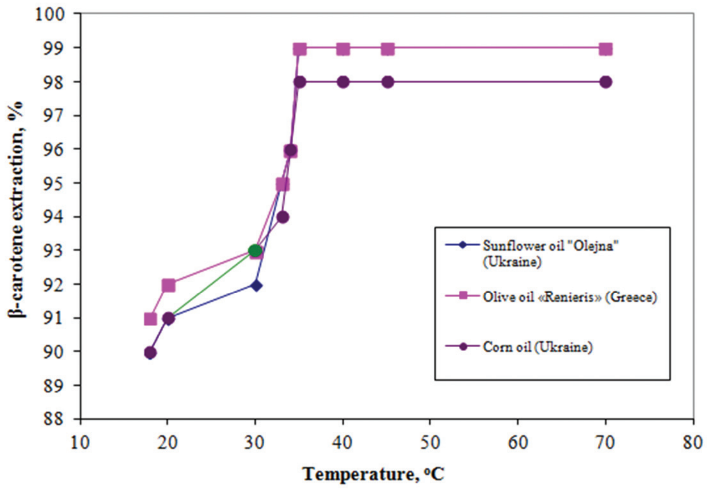

Figure 1. Percentage of β-carotene extraction from different biomass influenced by temperature. Note: The frequency of high-frequency ultrasound was 1.0 MHz, ultrasound intensity was 2 W/cm^2. Intensity of low-frequency ultrasound was 2 W/cm^2. The time of ultrasound treatment was 3 min.

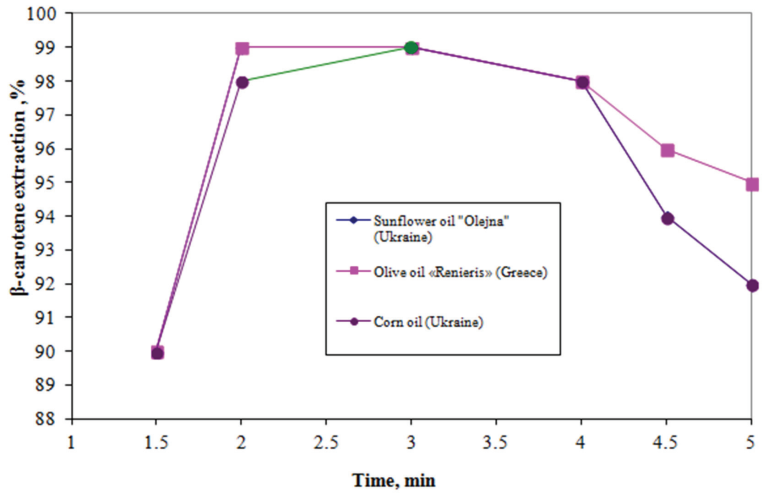

Figure 2. Percentage of β-carotene extraction from different biomass influenced by time. Note: The sample of the fungus 3 was used. The frequency of low-frequency ultrasound was 20.0 kHz. The frequency of high-frequency ultrasound was 1.0 MHz. The intensity of low- and high-frequency ultrasound was 2 W/cm^2.

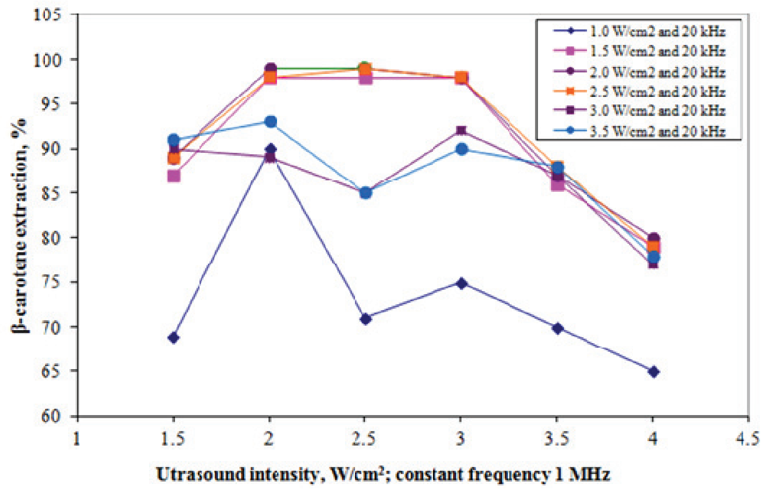

Figure 3. Percentage of β-carotene extraction from different biomass influenced by low-frequency. Note: The frequency of high-frequency ultrasound was 1.0 MHz, the intensity was 2 W/cm^2. The intensity of low-frequency ultrasound was 2 W/cm^2. Time of ultrasound treatment was 3 min.

Table 2. The influence of frequency of high-frequency ultrasound on percentage of β-carotene extraction from a biomass of the fungus B. trispora; the frequency of low-frequency ultrasound was 20.0 kHz, the intensity was 2 W/cm^2. The intensity of high-frequency ultrasound was 2 W/cm^2. Time of ultrasound treatment was 3 min

Sample	Frequency, MHz					
	0.5	1.0	1.5	2.0	2.5	3.0
1	90	99	98	99	93	78
2	89	98	99	98	94	77
3	89	99	98	99	93	78
4	90	98	98	98	93	76
Mean	89.50	98.50	98.25	98.50	93.25	77.25

ultrasound is applied increases to 2.5–4.0 W/cm^2 (Table 3 and Figure 4). Thus, combination of frequency and intensity of low-frequency and high-frequency ultrasound provides the optimal cavitation activity for this process, which promotes the greatest possible percentage of β-carotene extraction [34].

Also, it is established that primary extraction allows obtaining more than 90% of β-carotene in the sample, and repeated extraction is generally necessary of residual oil in a biomass of the fungus *B. trispora*. The recommended oil temperature is 35 °C, because lower temperature leads to reduction of percentage of β-carotene extraction, whereas higher temperature leads to unjustified increase of expenditure of energy (Figure 1). The received results practically do not depend on oil type; the experiments were carried out with use of sunflower, olive and corn oils (Figure 1 and Table 3).

Table 3. The influence of ultrasound intensity on percentage of β-carotene extraction from a biomass of the fungus B. trispora; the sample of the fungus 3 was used. The frequency of low-frequency ultrasound is 20.0 kHz. The frequency of high-frequency ultrasound is 1.0 MHz. Time of ultrasound treatment is 3 min

The intensity of low-frequency ultrasound, W/cm^2	Intensity of high-frequency ultrasound, W/cm^2					
	1.5	2.0	2.5	3.0	3.5	4.0
1.0	69	90	71	75	70	65
1.5	87	98	98	98	86	79
2.0	89	99	99	98	87	80
2.5	89	98	99	98	88	79
3.0	90	89	85	92	87	77
3.5	91	93	85	90	88	78

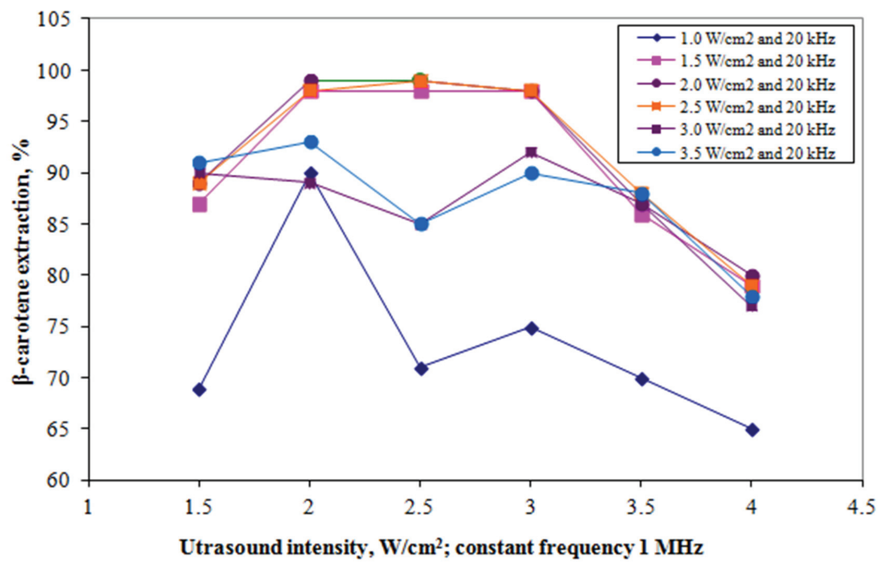

Figure 4. Dependence of percentage of β-carotene extraction from ultrasound intensity of two frequency for sample 3. Note: The frequency of low-frequency ultrasound was 20.0 kHz. The frequency of high-frequency ultrasound was 1.0 MHz. Time of ultrasound treatment was 3 min.

CONCLUSION

In this paper, based on research results in laboratory conditions, the influence of application of simultaneously low- and high-frequency ultrasound on the extraction efficiency of β-carotene from the biomass of the fungus *Blakeslea trispora* was defined. By applying the defined optimal conditions for extraction, in comparison with the use of single-frequency ultrasound, the yield was increased to 98–99%. In addition, this method can be applied at temperature of 35 °C, which allows to receive more biochemical and physiological activity of the obtained product and to reduce the power expenses. The use of one frequency ultrasound requires the higher temperature 150–170 °C. Based on this investigation, in laboratory conditions, it is developed and experimentally tested the technique of β--carotene extraction from a biomass of the fungus *B. trispora* with product yield 98–99%.

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IZVOD

EFIKASNA ULTRAZVUČNA EKSTRAKCIJA β-KAROTENA IZ GLJIVE *Blakeslea trispora* UZ POMOĆ ISTOVREMENOG TRETIRANJA NISKOM I VISOKOM FREKVENCIJOM

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Karotenoidi su vrlo dobro poznati po svom uticaju na životnu sredinu u smislu niske toksičnosti, jeftini su, potrebno je kraće vreme za njihovu ekstrakciju u poređenju sa standardnim metodama ekstakcije određenih komponenti i što je vrlo bitno efikasnost ekstrakcije je visoka. Ultrazvuk ima značajne prednosti kada se radi o ektrakciji velikog broja biološki aktivnih komponenti uključujući i karotenoide. Prednosti ultrazvučne ekstrakcije određenih komponenti su mnogobrojne i uključuju efikasno mešanje, efikasniju razmenu energije, redukovani termički i koncentracioni gradijent i niža temperatura ekstrakcije. Cilj ovog rada je ispitivanje uticaja glavnih parametara ultrazvučne ekstrakcije izvršene istovremenim tretiranjem niskom i visokom frekvencijom kao što su ultrazvučni intenzitet, vreme trajanja tretiranja i temperatura. Primena ultrazvuka sa niskom i visokom frekvencijom (simultano tretiranje ultrazvukom frekvencije od 20–46 kHz, intenziteta od 1,5–2,5 W/cm^2 i frekvencije od 1,0–2,0 MHz, intenziteta od 2,0–3,0 W/cm^2) podiže procenat ekstakcije β-karotena iz biomase gljive *Blakeslea trispora* sa 90–92 na 98–99% u poređenju sa tretiranjem ultrazvukom sa jednom frekvencijom. Preporučena temperatura ulja u procesu ekstrakcije je 35 °C, zbog toga što niža temperature dovodi do redukcije procenta ekstrakcije β-karotena a visa temperatura vodi ka neopravdanom povećanju troškova energije.

Ključne reči: ultrazvuk • β-Karoten • *Blakeslea trispora* • Dve frevencije ultrazvuka