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Heat stability and antioxidant potential of beta-carotene isolated from a fungal isolate

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Abstract

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The present study has been conducted for optimizations of disruption, extraction, partial purification and properties of beta-carotene produced from a strain of Mucor (*M. azygosporus*).The major findings of the study conducted highlight maximum cell disruption while macerating cells with DMSO, diatomaceous earth or glass beads in mortar and pestle. The maximum extraction yield was seen in a method that involve extraction using hexane and ethyl acetate (1:1 v/v) after disruption. This method gave the best result due to the highest extraction efficiency within short extraction time. The antioxidant activity was found to increase with concentrating of extract whereas the pigment stability was seen to decrease abruptly with temperature.

Keywords: beta-carotene; extraction; *Mucor azyosporus*; antioxidant activity; heat stability

Introduction

β-Carotene is the carotenoid compound which is present in abundance in human diet (Bogacz-Radomska and Harasym, 2018). The high bioactivity offers its use in medicines (Berman et al., 2014; Harasym and Oledzki, 2014; Zhang et al., 2016). Carotenoids are highly unsaturated isoprene derived, fat soluble class of 600 pigments existing in nature, being synthesized from higher plants to array of microorganisms. These are divided into two important classes – carotene (hydrocarbons) and xanthophylls (oxygenated forms). The most important carotenoids (Yokoyama et al., 1994) are not only effective in neutralizing a highly reactive singlet oxygen but also breaks up chain reactions involved in lipid per oxidation range from alpha-carotene, beta-carotene, lutein, lycopene, cryptoxanthin and zeaxanthin. Carotenoids form complexes with proteins are known as carotenoproteins. These complexes are common among marine animals (Hannibal et al., 2000). The carotenoprotein complexes are responsible for the various colors (red, purple, blue, green, etc.) to these marine invertebrates (Mangels et al., 1993) for mating rituals and camouflage. The most familiar carotenoids are carotene (an orange pigment found in carrots), lutein (a yellow pigment found in fruits and vegetables), and lycopene (the red pigment responsible for the color of tomatoes).The term carotene (carotin, from Latin word carota or carrot) is used for several related hydrocarbons having formulae C40 hydrocarbons (Cooney et al., 1966). It is an orange photosynthetic pigment important for photosynthesis by transmitting light energy they absorb from chlorophyll. Beta carotene is a vitamin A (Egyházi et al., 2004) precursor that is produced by many different species of plants. It is a hydrocarbon of 40C atoms, lipid soluble, yellow to orange colored pigment. It is highly unsaturated pigment containing 11 congugated (c = c) bonds. Most beta-carotene in supplements is synthetic, consisting of only one molecule called all Trans beta-carotene. Natural beta-carotene, found in food, is made of two molecules – all Trans beta-carotene and 9-cis beta-carotene (Demmig-Adams and Adams, 2002). Many factors like temperature, pH, nitrogen and carbon sources, type of fermentations and minerals affect their function. Industrially, carotenes are used in pharmaceuti-

cals, neutraceuticals, animal feed additives as well as colorants in cosmetics and food. Scientific interest in dietary carotenes has increased in recent years due to their wide ubiquitous applications as food colorants (Klaui and Bauernfeind, 1981), antioxidants, anticancers (Demmig-Adams and Adams, 2002), preventing vitamin A deficiency (Krinsky, 1979), strengthing the immune system, promoting proper cell to cell communication (Kumaresan et al., 2008) and antiaging (Mojaat, 2008). Due to all, these wide applications of β-carotene lead to development of many chemical synthetic technologies for synthesis of many carotenoids. In fact, more than 85% of the commercially available β-carotene is produced by chemical synthesis (US Patent 2010/0145116). The chemical industry is increasingly recognizing the urgent need to diminish its dependence on petroleum based raw materials and fuels to minimize its environmental impact while enhancing its competitiveness and increasing public confidence. Moreover, chemical synthesis produces mixtures of stereoisomers which may not be desired (Kim et al., 2007) by the consumers or may have undesired side effects. Alternative natural sources of carotenoids are micro algae and other microorganisms. Micro algae are cultured in large-scale outdoor ponds, thus being influenced by environmental constraints such as rainfall, sunlight and availability of salt water. Although microbial production is at its highest peak of demand due to its natural character, safety nutrient like vitamin, production is being independent of seasonal and geographical conditions giving predictable yields. The use of fungal source proved a promising and profitable choice for production, extraction and purification of beta carotene highlighting the fact that b-carotene is a high added value product that may be obtained under specific operating conditions to have its properties preserved. Keeping in view the beneficial applications of β-carotene on humans and cost effective fermentative production of β-carotene from microorganisms present study is focused on the antioxidant activity and heat stability analysis of beta carotene extracted from *Mucor azygosporus*.

Materials and Methods

Different studies and experiments focusing on cell disruption, extraction, purification and properties (antioxidant activity and heat stability) were carried out using a fungal strain of *Mucor azygosporus*.

All the chemicals used were of analytical grade. All media components used were from Hi-media chemicals.

Pure culture of the strain was procured from Department of Biotechnology, Himachal Pradesh University, Shimla, India. The culture was maintained by streaking on media 1 agar slants containing (g/L) yeast extract 4 g, starch 10 g, K_2HPO_4 10 g, $MgSo_4.7H_2O$ 1 g, agar 20 g and stored at 4°C. Repeated sub-culturing was carried out after every 30 days.

Determination of biomass and estimation of β-carotene

The biomass of *M. azygosporus* MTCC 414 was determined by weighing the dried cell mass and expressed in terms of mg dcw. The total carotene concentration was determined spectrophotometrically by extraction of pigments from the cells (25 mg dcw) with hexane (4 mL). The obtained extracts were passed through a dessicant anhydrous sodium sulphate (3%, w/v). The upper colored layer was separated by centrifugation at 10,000 rpm for 5 min and the intensity of color was measured at 452 nm with UV/Vis spectrophotometer. The concentration of carotene present in the sample was determined by comparison with a standard calibration curve prepared from pure all-trans β-carotene (Sigma, USA).

Optimization of various cell disruption and extraction conditions

A scheme of ten methods was followed according to Valduga's method designed for disruption and extraction (Valduga et al., 2009). The methods are summarized in Table 1.

Table 1
Methods of disruption and extraction (Valduga, 2009)

Method	Cell disruption	Extraction solvents
Method I	Diatomaceous earth	Acetone and petroleum ether
Method II	Glass beads (0.1 mm)	Acetone
Method III	Glass beads (0.1 mm) + DMSO (2:1) v/v	Acetone and methanol (1:1) v/v
Method IV	Glass beads (0.1 mm) + DMSO (2:1) v/v	Acetone and methanol (7:3) v/v
Method V	DMSO (2:1) v/v and glass beads (0.1 mm)	Petroleum ether and methanol(7:3) v/v
Method VI	DMSO (2:1) v/v and glass beads (0.1 mm)	Petroleum ether and methanol (7:3) v/v
Method VII	DMSO (2:1) v/v and glass beads (0.1 mm)	Chloroform and methanol (1:1) v/v
Method VIII	Glass beads (0.1 mm)	Tris HCl (20 mH, 8 pH) buffer, acetone and methanol
Method IX	Glass beads (0.1 mm) + DMSO (2:1) v/v	Hexane and ethyl acetate (1:1) v/v
Method X	Glass beads (0.1 mm) + DMSO (2:1) v/v	Hexane and ethyl acetate (1:1) v/v and sodium phosphate buffer (.1M, 8Ph)

Optimization of disruption and extraction with sonicator

Optimization of pulse rate (1 min on, 1 min off, 12 cycles)

1 gm cells and 60 ml hexane were subjected to sonication for about 12 cycles (1 min on, 1 min off). The samples were centrifuged at 10,000 rpm, 10 min and finally absorbance measured at 452 nm.

Optimization of volume

1 gm cells each with different volume of hexane (50 ml, 60 ml, 70 ml) was subjected to eight cycles (1 min on, 1 min off) of sonication at 39% amplitude. Samples after every 1min was centrifuged for 10 min at 10,000 rpm and absorbance measured at 452 nm.

Optimization of amplitude

1 gm cells each with different volume of hexane (50 ml, 60 ml, 70 ml) was subjected to eight cycles (1 min on, 1min off) of sonication at three different amplitudes (30%, 35%, 39%). Samples after every 1min was centrifuged for 10 min at 10,000 rpm and absorbance measured at 452 nm.

Determination of antioxidant and heat stability properties

Antioxidant activity was measured by β-carotene linoleic acid test. Following procedures was carried out:

Approximately 10 mg of β-carotene was dissolved in chloroform (10 ml), the carotene-chloroform solution (0.2 ml) was pipette out into a boiling flask containing linoleic acid (20 mg and 200 mg tween 40). Chloroform was removed using a vacuum evaporator at for 5 min. 50 ml of distilled water was added slowly with vigorous shaking to form an emulsion. A portion of emulsion (5 ml) was added to a tube containing the sample solution (0.2 ml) and absorbance was measured at 470 nm, with blank consisting of an emulsion without β-carotene. The tubes were placed in water bath (50°C for 60 min) and the oxidation of emulsion was monitored.

Heat stability test

The heat stability test of carotenoids obtained after partial purification was done by placing the extract at three different temperatures (37°C – incubator, 80°C – hot air oven, 121°C – autoclave) for a period of four hours. Samples were taken out at regular interval of 30 min and extraction of beta carotene was carried out under the optimized conditions. Residual carotene percentage was calculated by measuring the absorbance at 452 nm.

Results and Discussion

Cell disruption and extraction

The beta-carotene pigments of fungi (*Mucor azyosporus*) accumulate in lipid droplet and thus can be extracted with various cell disruption procedures followed by extraction with variety of organic solvent.

Optimization of extraction procedure with best combinatorial solvent sequence for extraction

The procedures for cell disruption and extraction carried out according to Valduga et al. (2009) the results obtained, reveals that a synergetic effect of use of multiple solvent applications has proven a better choice for disruption and extraction of carotenoids. The solvents tested were able of extracting carotenoids and variablities in the performances according to their capabilities were observed. Extraction yields ranging from maximum to a minimum of representing a wide difference was observed. Present studies performed on *Mucor azygosporus* showed that the use of buffers like Tris hcl and sodium phosphate buffer gave very less yields (Tris hcl 94 µg/g and sodium phosphate 67 µg/g) due to the negative effects exerted on beta-carotene extract.

According to Valduga et al. (2009) the correct combination of three solvent (DMSO, acetone and petroleum ether) compared to other solvents in case of *Sporodiobolus salmo-*

Table 2
Methods of extraction and yield of β-carotene

Methods	Solvent used	Carotenoid yield (µg/g/dcw)
Method I	Acetone and petroleum ether	400
Method II	Acetone	138
Method III	Acetone and methanol (1:1) v/v	163
Method IV	Acetone and methanol (7:3)v/v	164
Method V	Acetone + methanol (6:4) v/v	160
Method VI	Petroleum ether and methanol (9:1) v/v	144.2
Method VII	Chloroform and Methanol (1:1) v/v	106
Method VIII	Tris HCL buffer (20mM, 8pH) , Acetone and Methanol	94
Method IX	**Hexane and ethyl acetate (1:1) v/v**	**442.2**
Method X	Hexane and ethyl acetate and sodium phosphate buffer (.1M, 8pH)	67

nicolor of yeast showed spectacular results for extraction, but the present work done on a fungal strain showed maximum disruption and extraction outputs of beta-carotene, when a combinatorial sequence of diatomaceous earth was coupled with a sequence of hexane and ethyl acetate or a sequential use of diatomaceous earth with acetone and petroleum ether (1:1 v/v). The differences in extraction results may be attributed to differences in ability of a solvent to increase the permeability of fungal wall and solubility of carotenoids (Table 2).

Optimization of disruption of cells with sonicator (Sonics vibra microcell)

In the present work the various methods namely physical, chemical and mechanical methods (bead beater and sonicator) for cell disruption were carried out. The attempts were made to find out the suitable method which can give the maximum disruption and extraction of β-carotene. The results obtained showed that the chemical methods were time consuming and resulted in less extraction of β-carotene as well as production of cell debris, whereas the use of bead beater also proved as an ineffective method and gave less outputs of disruption, however sonicator was found to be the best one which registered the maximum cell disruption and extraction of β-carotene (2200µg/g dcw). The present study is in consensus with the studies conducted by Kim et al. (2007) where they obtained maximum β-carotene (3.30 mg/ml) extracted from *Rhodosporidium* using DMSO coupled with sonication.

Optimization of pulse rate

For pulse rate optimization was firstly 12 cycles (1 min on and 1 min off). It is clear from Fig. 1*a* that the initial concentration (0 hr) of beta-carotene in fungal cells was about 240 µg/g dcw, but with the onset of sonication coupled with organic solvent (hexane and ethyl acetate) the disruption was seen to increase after every active cycle. It was seen that the disruption profile increased up to a 8 cycle run, which showed maximum release of pigment, after that it started declining, which indicates that the maximum disruption can be obtained with 8 depicted cycle run which is as a saturation level of disruption, beyond which it starts declining giving less output results.

Optimization of volume

After optimization of pulse rate with 8 cycle run (1 min on and 1 min off), the second parameter was volume. Three different volume of hexane and ethyl acetate (50, 60 and 70 ml) were tested for maximum disruption with 8 cycle sonication. As depicted in Fig. 1*b*, the volume parameter was found to give closely related patterns for disruption and only slight differences were seen in disruption profiles.

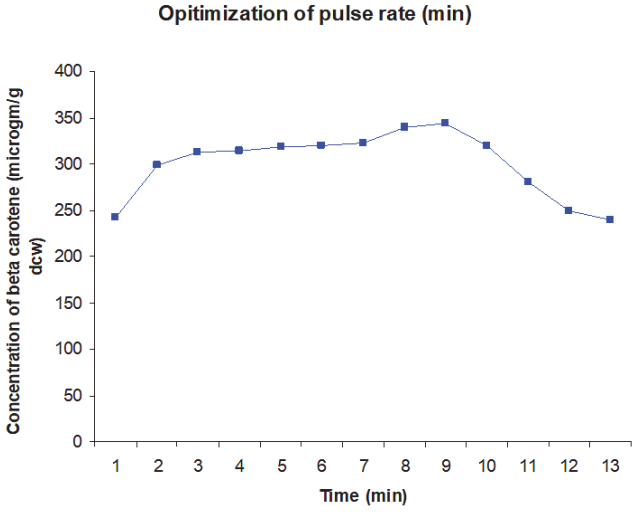

Fig. 1*a*. Optimization of pulse rate (min)

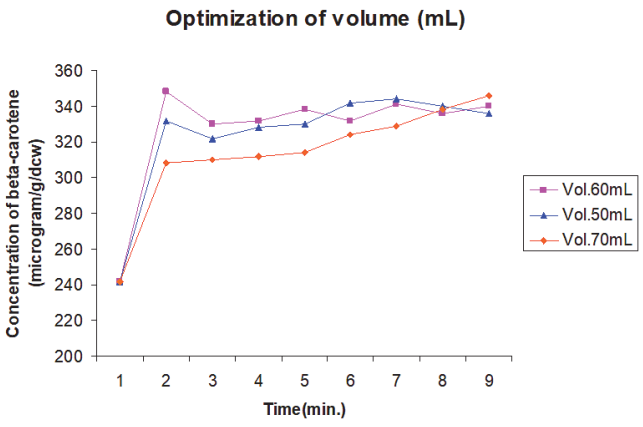

Fig. 1*b*. Optimization of volume (mL)

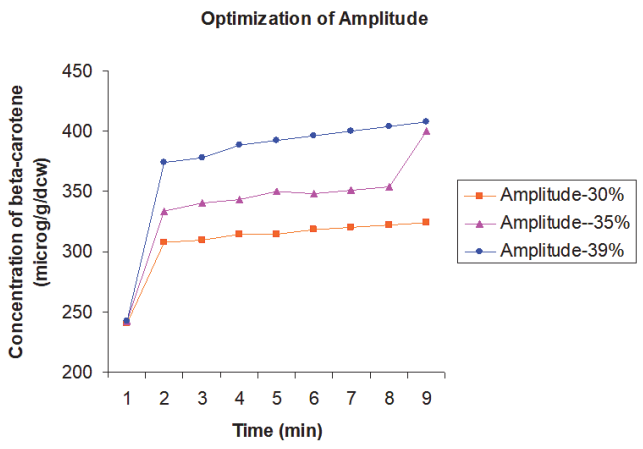

Fig. 1*c*. Optimization of amplitude

Optimization of amplitude

After optimizing the pulse rate and volume parameters the third important parameter was amplitude. Eight cycles were run with 70 ml vol. of hexane at three different amplitudes (30 %, 35 % and 39%). It is clear from Fig. 1*c* that at highest amplitude range, maximum cell disruption was seen. The conclusion emerged that optimization at 39% amplitude proved a good alternative because this is an intermediate range of amplitude and increasing this range to 40% and above did not increased beta-carotene extraction. The disruption patterns at different amplitude are given in Fig. 1*c*.

Antioxidant activity of β-carotene in diluted sample

The antioxidant activity was expressed as inhibition % with reference to control after a 60 min incubation using following equation:

AA (antioxidant activity) = 100 (DRc_DRs) /DRc

where DRc is degradation rate of control, DRs is degradation rate of sample.

The final results were complied before and after 60 min incubation (Tables 3*a* and 3*b*).

Table 3*a*
Antioxidant activity of β-carotene (0 h)

Tubes at 0 hr	O.D. at 470 nm
Blank	0.000
Test	1.708
Control	0.995

Table 3*b*
Antioxidant activity of β-carotene (after 60 min of incubation)

Tubes	O.D. at 470 nm
Blank	0.000
Test	0.767
Control	0.281

The antioxidant activity of β-carotene in diluted sample was found to be 38.09%. It would have been more in case a concentrated sample used which can be further checked.

Heat stability test

A heat stability test was done using β-carotene extract. The extract was kept at three different temperatures: 37°C (incubator), 80°C (hot air oven), 121°C (autoclave) for a period of 3 to 4 hr. Samples were taken after 30 min interval and absorbance was measured spectrophotometrically. The stability was seen to decrease by 84% at the end of 4 hrs at 37°C. At 80°C there was a decrease of 86% after 4 hrs. In third case at 121°C there was 93% decrease after 2 hrs. The maximum decrease in heat stability of beta-carotene was seen at 121°C after 2 hrs interval. It shows that β-carotene exhibits different stability patterns at different temperature. The heat stability patterns are summarized in Table 4.

Conclusions

The toxic and carcinogenic impacts of synthetic pigments have raised a considerable interest towards a safe alternative highlighting the use of natural pigments, which offers great prospects of natural character, safety, and non toxic and gives spectacular yields. Beta-carotene as the natural pigment has great potential both as coloring agent and food and feed additive. The present study has been conducted for optimizations of disruption and extraction of beta-carotene produced from a strain of Mucor (*M. azygosporus*).The major findings of the study conducted were:

Maximum cell disruption was found with macerating cells with DMSO, diatomaceous earth or glass beads in mortar and pestle.

The maximum extraction yield was seen in a method that involved extraction using hexane andethyl acetate (1:1 v/v) after disruption. This method gave the best result due

Table 4
Heat stability of β-carotene at different temperatures

Time (min)	Beta-carotene (μg/g) (37°C)	Residual % of beta-carotene	Beta-carotene (μg/g) (80°C)	Residual % of beta-carotene	Beta-carotene (μg/g) (121°C)	Residual % of beta-carotene
0	250	100	250	100	250	100
30	210	84	176	70	224	89
60	184	74	162	65	152	61
120	170	68	158	63	42	17
150	142	57	132	53	18	7
180	124	50	104	42		
210	101	41	82	32		
240	90	36	58	23		
270	42	17	36	14		

to the highest extraction efficiency within short extraction time.

The antioxidant activity was found to increase with concentrating of extract. The pigment stability was seen to decrease abruptly with temperature.

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