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Extraction and purification of β-carotene from filamentous fungus *Mucor azygosporus*

Meenu Thakur and Wamik Azmi

Department of Biotechnology, Himachal Pradesh University, Summer-Hill, Shimla-171005, H.P., India

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Abstract

β-carotene has been used for years as natural food colorants and in cosmetics and healthcare due to their antioxidant property. The production of β-carotene by *Mucor azygosporus* was evaluated, utilizing deproteinized waste whey filtrate. Since pigments are intracellular, their extraction needed after cell cultivation. In this study, different strategies for extraction of β-carotene from *M. azygosporus* MTCC 414 were investigated. The combinations of various cell disruption and extraction methods were employed and maximum β-carotene (985 µg/ml) was extracted when sonicator was used for disruption of cells, followed by extraction with hexane and ethyl acetate (1:1, v/v). The extracted crude pigment was purified by adsorption column chromatography using neutral alumina. The purified β-carotene was, further, analyzed by HPLC before subjecting to NMR and MS.

Key words: β-carotene, *M. azygosporus* MTCC 414, extraction, purification, characterization

Introduction

Epidemiological evidences and experimental results suggest that β-carotene inhibit the onset of many diseases in which free radicals are thought to play a role in initiation such as arteriosclerosis in cardiovascular diseases, cataracts, age related macular degeneration, multiple sclerosis and most importantly cancer due to it's free radical and single oxygen scavenging property (Edge *et al.*, 1997; Shiau *et al.*, 2010; Yurtcu *et al.*, 2011). The industrial production of natural carotenoids through microbial fermentations is expanding (Aksu and Eren, 2005; Batella-Pavia and Rodriguez-conapcion, 2006; Yan *et al.*, 2012; Thakur and Azmi, 2013). Since downstream, processing is a significant contributor to cost of production, efficient recovery of the intarcellular carotenoids, has attracted considerable attention in recent times. Traditionally, disruption of microbial cells for intracellular products was achieved, using mechanically based techniques such as high-pressure homogenization and bead milling. However, such techniques suffer from several drawbacks including the production of fine inclusion bodies, cell debris, high capital investment cost and long processing time. Inclusion bodies are formed when products accumulate in certain compartments of cells or when products aggregate spontaneously inside cells as in case of β-carotene (Roukas and Mantzouridou, 2001). The product inside inclusion bodies can often be recovered by dissolution after permeabilization of the cell wall (Hee *et al.*, 2004). Chemical extraction after cell disruption was attempted for maximum product release (Fontana *et al.*, 1996; Colla *et al.*, 2008). Since carotenoids are soluble in fat, they are commonly extracted with organic solvents such as acetone and petroleum ether. Moreover, the properties of the product strongly depend on the extraction conditions and in some cases the product loses its valuable properties during the extraction process. Most of these studies have focused on carotenoids recovery, using single solvent (Mendes-Pinto *et al.*, 2001; An and Choi, 2003). One area that has received little attention is the investigation of carotenoids release by combinations of solvents. It is plausible that some solvents when used together to disrupt cells may demonstrate synergistic interactions, resulting in enhanced carotenoid yield (Park *et al.*, 2006).

Author for correspondence: Dr. Wamik Azmi
Department of Biotechnology, Himachal Pradesh University, Summer-Hill, Shimla-171005,H.P., India
E-mail: wamikazmi@yahoo.com
Tel.: +91-1772831948, 09418311183, **Fax.:** +91-17728322154

In the present study, attemps have been made to release the β-carotene from cells of *Mucor azygosporus* MTCC 414 and its subsequent extraction with combination of solvents. The extracted pigment was, further, ourified and characterized.

Materials and Methods

Chemicals

All the chemicals used in the experiments were of analytical grade. The media components were procured from HiMedia Laboratories Pvt. Ltd, Mumbai. Analytical reagents were from Merck, Rankem and S.D. Fine Chemicals. The standard β-carotene has been procured from Sigma USA. The solvents used for the HPLC analysis were of HPLC grade and were procured from Rankem. The $CDCl_3$ for NMR analysis was procured from Acros Organics.

Microorganism

The fungus *Mucor azygosporus* MTCC 414 has been procured from Department of Biotechnology, Himachal Pradesh Univeristy, Shimla-5, India. The culture was maintained on YpSS (yeast phosphate soluble starch) agar slants (pH-5.5) at 30°C for 5 days. The sub-culturing of the strain has been carried out periodically at every 30 day.

Mass cultivation was carried out in 14 L laboratory fermenter (BIOFERM-LS2, Scigenics India Pvt. Ltd.) at 6 L working volume to achieve high cell densities for mass production of β-carotene. The deproteinized whey containing 3.5% (w/v) lactose was loaded into the fermenter and sterilization of the medium was carried out at 121°C for 15 min. The sterilized medium was inoculated with 4% (v/v), 12 h old seed culture through the inoculation port by peristaltic pump attached to feed bottle. The temperature was maintained at 30°C for the entire course of fermentation with the help of inbuilt heater and chiller. White light was illuminated with compact fluorescent lamp of 61.33 lumens from central window throughout the course of fermentation.

Analytical methodology

Estimation of biomass

The fungal biomass of *M. azygosporus* MTCC 414 grown in the culture broth and β-carotene content was determined spectrophotometrically with UV-Vis spectrophotometer and HPLC (Azmi *et al*., 2011).

Extraction of β-carotene from dried cells of M. azygosporus MTCC 414

The β-carotene of *M. azygosporus* MTCC 414 was found to be intracellular and fat soluble, hence, various methods were applied for its extraction. The mycelial mass of *M. azygosporus* MTCC 414 was kept for drying at 37°C overnight and following extraction methods described by Valduga *et al*. (2009) were applied with slight modification (Table 1).

Optimization of parameters for maximum extraction of β-carotene

Various parameters *viz.*, number of pulse cycle, cell volume and amplitude of the sonication were optimized for maximum extraction of β-carotene from the cells of *M. azygosporus* MTCC 414. The cell slurry was sonicated (vibra cell, Incarp instruments Pvt. Ltd., India) by keeping the probe (1 inch diameter) above the bottom of vial. The vial was ice-jacketed during the sonication to avoid heat generation.

Purification of β-carotene using adsorption column chromatography

The β-carotene of *M. azygosporus* MTCC 414 was purified from other carotenoids to study its biochemical properties. Adsorption column chromatography using neutral alumina was performed and different coloured fractions obtained were,

Table 1: Methods of disruption and extraction of β-carotene from filamentous fungus M. *azygosporus* MTCC 414

Methods	Disruption method	Solvent used for extraction	β-carotene (µg/ml)
1.	Diatomaceous earth in mortar and pestle	Acetone + Petroleum ether (1:1, v/v)	345
2.	Glass beads	Acetone	234
3.	Glass beads + DMSO (2:1, w/v)	Acetone + Methanol (1:1, v/v)	436
4.	Glass beads	Chloroform + Methanol (1:1, v/v)	364
5.	Glass beads + DMSO	Acetone + Methanol (2:1, v/v)	244
6.	Glass beads + DMSO	Petroleum ether + Methanol (9:1, v/v)	306
7.	Glass beads + DMSO	Tris-HCl buffer (pH 8.0)	121
8.	Glass beads	Sodium phosphate buffer (pH 7.0)	69
9.	Glass beads	Hexane + ethyl acetate (4:2)	389
10.	Bead beater	Hexane + Ethyl acetate (1:1, v/v)	756
11.	Sonicator	Hexane + Ethyl acetate (1:1, v/v)	985

further analysed using HPLC to assess the purity of β-carotene. The 4g of neutral alumina was washed with hexane and packed into a 10ml glass column. The column was equilibrated with excess of hexane and the cell free extract was loaded on the column. Different solvents were used for the elution of different colored components. The β-carotene was eluted with hexane (6 ml) while other water soluble fractions were removed using acetone. All the fractions were analysed, using high performance liquid chromatography (HPLC). Purified fractions were collected and dried under vacuum, using vacuum dryer (Tarson Rocker 300) and dissolved in the minimum amount of hexane and stored at 4°C for further use.

Molecular characterization of β*-carotene*

The molecular characterization of purified β-carotene obtained from *M. azygosporus* MTCC 414 was done, using mass spectroscopy and nuclear magnetic resonance (NMR) at SAIF (sophisticated analytical instrumentation facility), Panjab University, Chandigarh. The spectrum of purified fraction was compared with the spectrum of standard compound using ^{1}H nuclear magnetic resonance spectra as well as mass spectra. The molecular weight and the chemical configuration of the purified sample were determined by mass spectroscopy.

Results and Discussion

Extraction of β-carotene from dried cells of *M. azygosporus* MTCC 414

The results (Table 1) reveal that maximum β-carotene (985 µg/ml) was extracted when sonication was used to disrupt the cells, followed by extraction with combination of hexane and ethyl acetate (1:1, v/v). Although all the solvents tested were able to extract β-carotene, variability in their performance was observed. Extraction of β-carotene ranged from a maximum of 985 µg/ml (disruption with sonicator and extraction with hexane and ethyl acetate, 1:1, v/v) to a minimum of 69 µg/ml (disruption with glass beads and extraction with sodium phosphate buffer) representing 93% difference. The less recovery of β-carotene might be due to the incomplete disintegration of the dried cells of *M. azygosporus* MTCC 414. However, a significant amount of β-carotene (756 µg/ml) was extracted, using bead beater for cell disruption. Similar results were obtained by Lee and Fang (2011) when extraction of carotenoid from *X. dendrorhous* was performed with hexane and ethyl acetate. High pressure homogenization (Park *et al.*, 2006) and ultrasonicator (Gu *et al.*, 1997; Thana *et al.*, 2008) were found to be best for the release of cellular components of fungi. The sonication has been reported as most efficient method for the recovery of the membrane bound products from microbial cells (Singh and Jhamb, 2004). Further, the extraction of pigments from microbial sources has generally been carried out by employing organic solvents.

In the present study, the variability in the extraction of β-carotene from *M. azygosporus* MTCC 414 cells by different methods may be attributed to differences in the ability of a solvent to increase permeability of fungal cell wall and the solubility of β-carotene in that solvent. Moreover, the optimum combination of the two solvents (hexane and ethyl acetate, 1:1, v/v) improved the extent of β-carotene compared with other solvents tested.

Optimization of various parameters for the release of β-carotene

Among various cell disruption methods evaluated, the sonication of the cells of *M. azygosporus* MTCC 414 leads to the maximum release of β-carotene. Therefore, the conditions of sonication, *viz.* cycles (1-12 cycles), volume of solvent (50-70 ml) and amplitude (30, 35 and 39%) were further optimized to maximize the release of β-carotene from *M. azygosporus* MTCC 414. The maximum β-carotene was released after 8th cycle of sonication and with further increase in sonication cycle, the extraction of β-carotene decreased, probably due to the thermal denaturation of the pigment (Figure 1). Whereas, the maximum amount of β-carotene was released when 50 ml of solvent was used for extraction (Table 2). In case of amplitude, most efficient amplitude obtained was 39% for the maximum release of β-carotene from *M. azygosporus* MTCC 414 cells and the lower amplitude of sonication was not found to very effective for this purpose (Table 3). Guerrero (2001) reported the maximum release of β-carotene from *S. cerevisiae* by sonication at 39% amplitude which also supports the present result.

Purification of β-carotene using adsorption column chromatography

Coloured extract obtained after extraction was vacuum dried and dried coloured mass was dissolved in minimum volume (1 ml) of hexane. Neutral alumina (4 g/10 ml) column was packed and the column was equilibrated with 5 ml of n-hexane, prior to loading of 150µg/ml of extract. The β-carotene was eluted from the column with 6ml of n-hexane (flow rate 1 ml/min), while the more polar compounds were collectively eluted with 10 ml of acetone (Masino *et al.*, 2008). Each fractions was dried at room temperature under vacuum and dissolved in a minimum amount of the solvent. These samples were filtered through 0.45 µm membrane filters and analysed with HPLC. The results suggest that β-carotene of *M. azygosporus* MTCC 414 loosely adsorbs on to a neutral alumina as compared to other contaminating carotenoids such as lycopene and xanthophylls and, hence, elutes in the first fraction with hexane.

HPLC analysis of β-carotene

HPLC analysis for the active fraction of adsorption column chromatography revealed single peak corresponding to β-carotene at retention time 5.30 (Figure 2). HPLC has been used as the most common method for detection of carotenoids (Britton *et al.*, 1995). Latha and Jeevaratnam, (2010) used HPLC to establish purity of the fractions obtained during purification of β-carotene from *R. glutinis* DFR-PDY.

Krupa *et al.*, (2010) performed thin layer chromatography for purification of saturated canthaxanthin from *Aspergillus carbonarius*.

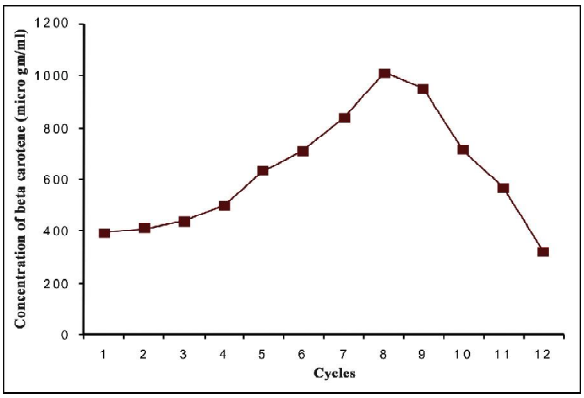


Figure 1: Optimization of cycle of sonication for the release of β-carotene from *M. azygosporus* MTCC 414 cells

Molecular characterization of purified β-carotene using NMR and Mass spectroscopy

The mass spectrum of the purified β-carotene obtained from *M. azygosporus* MTCC 414 was depicted in Figure 3 and compared with the standard β-carotene (Sigma, type I). The mass spectrum of the standard β-carotene and purified β-carotene displayed an $[M^+]$ at m/z 536.1 and 536.4, respectively corresponding with molecular formula of $C_{40}H_{56}$. The peak at m/z 470 has reported to be a characteristic of a carotenoid and the peak at m/z 536 represents baseline peak (Jagannadham *et al.*, 1991).

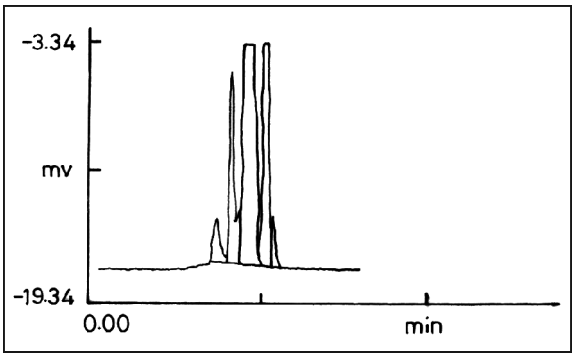
The pattern of NMR spectra were found to be similar in both test as well as standard sample of β-carotene which confirms that purified sample from *M. azygosporus* MTCC 414 is nothing but β-carotene (Figure 4). NMR has been used as the most common method for identification of carotenoids (Feltl *et al.*, 2005). The major carotenoid pigment of a psychotrophic *Micrococcus roseus* strain was characterized by application

Table 2: Optimization of volume of solvent for extraction of β-carotene from *M. azygosporus* MTCC 414 cells

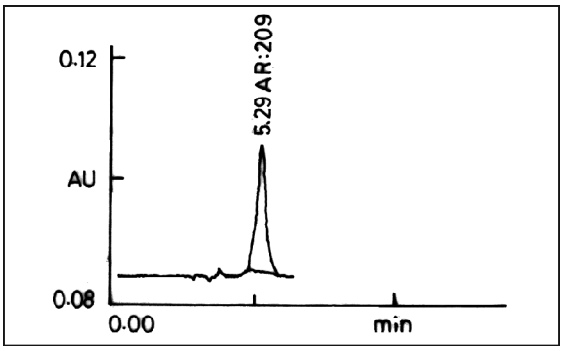
Concentration of β- carotene(µg/ml)			
Cycles	**50ml of hexane and ethyl acetate (1:1, v/v)**	**60ml of hexane and ethyl acetate (1:1, v/v)**	**70ml of hexane and ethyl acetate (1:1, v/v)**
1	398	390	298
2	412	428	309
3	439	501	412
4	502	559	479
5	635	604	514
6	710	680	602
7	840	925	621
8	1010	1045	780

Table 3: Optimization of amplitude of sonication for the release of β-carotene from *M. azygosporus* MTCC 414 cells

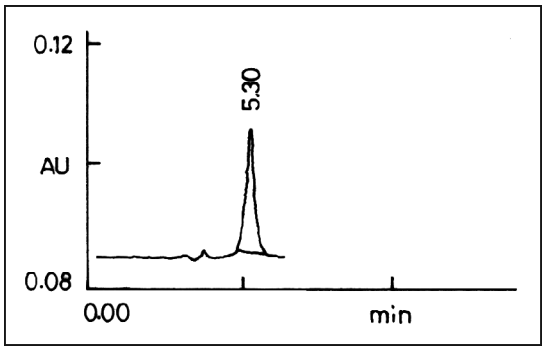
Concentration of β-carotene (µg/ml)			
Cycles	**Amplitude-30%**	**Amplitude-35%**	**Amplitude-39%**
1	308	390	414
2	310	428	478
3	312	501	528
4	314	559	588
5	314	604	625
6	320	680	705
7	322	925	995
8	324	1045	1080



(a)

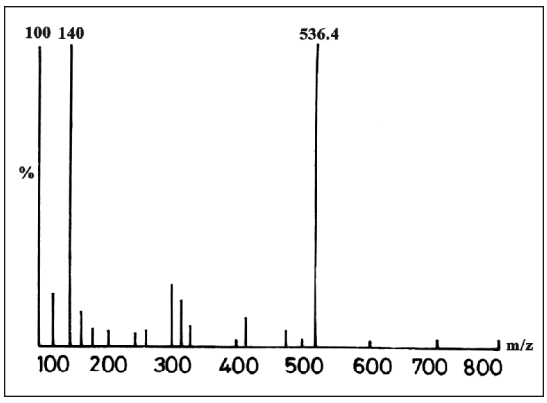


(b)

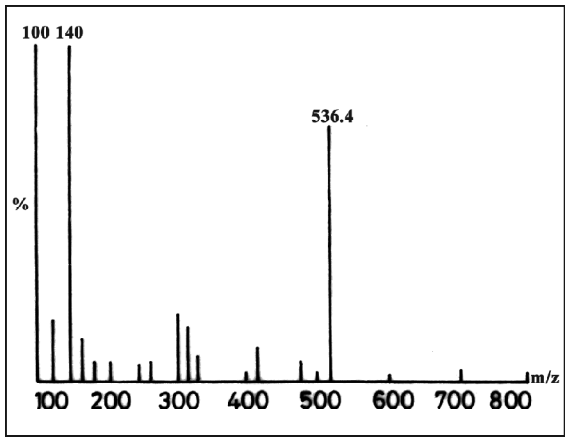


(c)

Figure 2: HPLC chromatogram (a) crude extract (b) purified β-carotene from *M. azygosporus* MTCC 414 (c) standard β-carotene

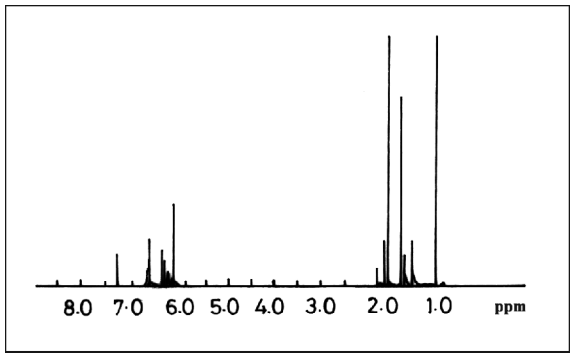


(a)

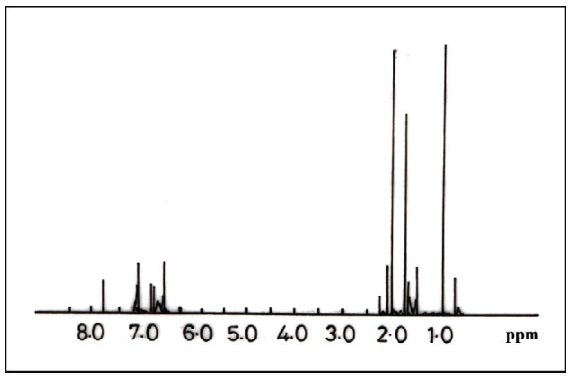


(b)

Figure 3: Mass spectrum (a) standard β-carotene (b) purified β-carotene obtained from *M. azygosporus* MTCC 414



(a)



(b)

Figure 4: Nuclear magnetic resonance spectra of (a) standard β-carotene (b) purified β-carotene obtained from *M. azygosporus* MTCC 414

of proton NMR (Jagannadham *et al.*, 1991). In another study the purified carotenoids obtained from *R. glutinis* were characterized by application of FTIR and NMR (Latha and Jeevaratnam, 2010).

Conclusion

Maximum β-carotene from the cells of *M. azygosporus* MTCC 414 was extracted when sonicator was used for disruption of cells followed by extraction with hexane and ethyl acetate. The recovery of β-carotene was found to be 92%. The mass spectrum of purified β-carotene obtained from *M. azygosporus* MTCC 414 resembles with standard β-carotene. The base peak at (m/z) 536.4 and 536.1 of purified and standard β-carotene, respectively confirms the purity of β-carotene. These findings were also supported by NMR analysis. On the basis of our results it could be concluded that the filamentous fungus *M. azygosporus* MTCC 414 has the potential to produce β-carotene of quality comparable to the commercial preparation.

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Conflict of interest

We declare that we have no conflict of interest.

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