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Purification of Astaxanthin from Mutant of *Phaffia rhodozyma* JH-82 Which Isolated from Forests Trees of Iran

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Abstract: Astaxanthin have been extracted and purified from mutant isolate of *Phaffia rhodozyma* JH-82. Purified astaxanthin was identified by spectrophotometric, TLC and HPLC analysis and were compared with synthetic astaxanthin. Results of TLC analysis indicated that isolate of *P. rhodozyma* JH-82 were able to produce nine different carotenoids and high level of carotenoids was belong to astaxanthin. Results of this study for pure astaxanthin production indicated that mutant of JH-82 of *P. rhodozyma* (230 μg $g^{-1}_{\text{dried yeast}}$) produced more astaxanthin than natural isolate JH-80 (140 μg $g^{-1}_{\text{dried yeast}}$). The HPLC spectrum showed retention time 11 min for both purified and synthetic astaxanthin and solvent was $CDCl_3$.

Key words: Astaxanthin, *Phaffia rhodozyma*, column chromatography, TLC

INTRODUCTION

Astaxanthin (3, 3'-dihydroxy-β, β'-carotene-4, 4'-dione) is a carotenoid pigment found in certain marine animals and plants such as fish, shrimps, algae and fungi (Miki *et al*., 1982; Boussiba *et al*., 1992) and has been widely used as a feed supplement in poultry and aquaculture (Jesús *et al*., 2001). Due to its special structure, astaxanthin is a more powerful scavenger of singlet oxygen and peroxyl radicals than β-carotene, cantaxanthin and zeaxanthin (3, 3'-dihydroxyl-β-carotene); its antioxidant activity is much stronger than all other carotenoids (Goto *et al*., 2001). Astaxanthin exhibits strong free radical scavenging activity and protects against lipid peroxidation and oxidative damage of LDL-cholesterol, cell membranes, cells and tissues. Moreover, astaxanthin has a number of essential biological functions, ranging from protection against oxidation of essential polyunsaturated fatty acids and protection against UV-light effects, supporting good vision, eye health, enhancing immune response, Protective effect of on naproxen-induced gastric and anti-photoaging effect (Tso and Lam, 1996; Terao, 1989; Kurashige *et al*., 1990; Jyonouchi *et al*., 1993, 1994; Cross *et al*., 1987; Arakane, 2002; Chew and Park, 2004; Nishikawa *et al*., 2005; Kim *et al*., 2005). *Phaffia rhodozyma* is carotenoid-producing yeast which synthesizes astaxanthin as its main carotenoid (Andrewes *et al*., 1976) and gives protection against reactive oxygen species (Schroeder and Johnson, 1993, 1995). The Food and Drug Administration of the United States has permitted it for use in the aquacultural industry (Turujman *et al*., 1997). The objective of this study was to extraction and purification of astaxanthin from *P. rhodozyma* JH-82 and JH-80 which isolated from natural forests trees from Iran.

MATERIALS AND METHODS

Micro-organism and culture conditions: A natural isolate of *P. rhodozyma* JH-80 and its NTG mutant, *P. rhodozyma* JH-82 were kindly provided by Iranian Academic Center for Education, Culture and Research, Tehran University Branch They were maintained on slants yeast-malt medium composed of the following (g L^{-1}): glucose, 10; yeast extract, 3; malt extract, 3; bactopeptone (Difco), 5; with 2% agar in the refrigerator, natural and mutants isolates were also stored in 40% glycerol-60% YM broth at -70°C. Proliferation experiments of *P. rhodozyma* were carried out for 8 days at 20°C in shaker incubator (agitation speed 145 rpm) using 1000 mL Erlenmeyer flasks containing 300 mL YMB culture medium. Standard astaxanthin (Sigma) and all other materials were purchased from Merck and/or Sigma with analytical grade.

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Extraction of carotenoid: Astaxanthin were extracted as described by Martin *et al.* (1993).

Purification of Astaxanthin and Analysis

Thin-layer chromatography (TLC) and Column Chromatography: For TLC, With a fine glass capillary, spotted 5 µL aliquots of the *Phaffia* extracts onto a silica TLC plate [25 DC Alufolien (Merck) kieselgel 60F254] and placed the plate in a chromatography tank containing a solvent mixture of hexane/acetone 75:25. When the solvent front has reached the top of the plate, removed the plate from the tank and recorded observations quickly as the carotenoids will gradually fade upon exposure to air and light.

The concentrated filtrate was run through a kieselgel (60G) column chromatography with acetone:n-hexane (12:88). The fractions were collected and compared with standard astaxanthin by Thin Layer Chromatography (TLC) analysis.

Spectrophotometric assay: The fraction which was in accordance with standard astaxanthin was collected from column and characterized by Visible Absorption Spectra (VIS) scanning in acetone by a BioQuest CE2501 spectrophotometer. The concentration of astaxanthin in the petroleum ether extract was estimated by measuring the absorbance at λ_{max} (474 nm). The special extinction coefficient $E^{1\%}_{cm} = 1600$ (Andrewes *et al.*, 1976) and the formula provided by An *et al.* (1989).

HPLC analysis: Purified astaxanthin was analyzed by HPLC with ODS-Spherisob column (5 µm×250 mm×4.6) which astaxanthin was eluted by acetonitrile: methanol (85%:15%) and flow rate 0.8:0.2 mL min^{-1} and analyses with PMT detector (SPD-6AV) at λ_{max} 490 nm We could not obtain a good NMR spectrum because the concentration was not enough.

RESULTS AND DISCUSSION

The majority of carotenoid-containing yeasts belong to the Teliomycetes (Basidiomycotina, or their imperfect counterparts) and their cell wall are thought to be considerably more complex than those of ascomycetous yeasts (2). Because the tough cell wall is believed to be the barrier which prevents the thorough extraction of carotenoids (Simpson *et al.*, 1971), it is necessary to weaken this barrier to allow solvent penetration and consequent carotenoid extraction. This hypothesis is supported by the fact that spheroplasts of yeast or mechanically disrupted cells are amenable to carotenoid extraction (Johnson *at al.*, 1978). In this study for lysis cell wall of *Phaffia* we used grinding with lamp powder, ultrasonic, homogenizer, vortexing with bead and DMSO. Only by grinding with lamp powder and DMSO was broken cell wall of phaffia. After lyses cell wall of yeast with DMSO, the pellet was washed with DDSW and cells were stained by gram staining and observed with microscope. The broken cells appeared pink and the intact cells were purple (Fig. 1).

The total carotenoid and fractions were compared with synthetic astaxanthin by TLC. Results of TLC indicated that in the *Phaffia* extract, the orange-colored astaxanthin forms the major band which migrates slowly because the molecule is polar and has a high affinity for the stationary (silica) phase (Fig. 2). The fraction which was similar to standard astaxanthin was collected from chromatography column.

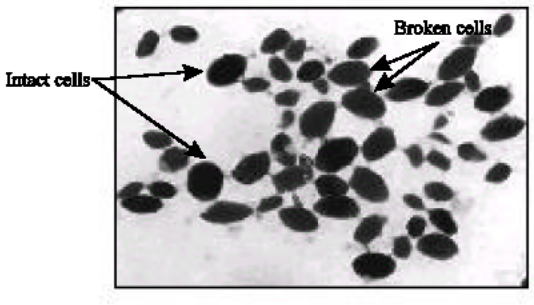

Fig. 1: Microscopic picture of broken and intact cells of *Phaffia rhodozyma* (Magnification 100x)

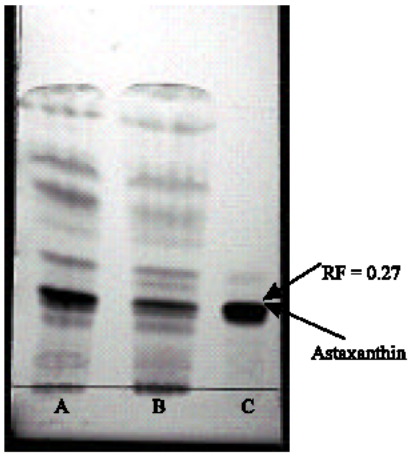

Fig. 2: Thin layer chromatography (TLC) plates of extracts from mutant of *P. rhodozyma* JH-82 (A), Wild type of *P. rhodozyma* JH-80 (B) and standard astaxanthin (C)

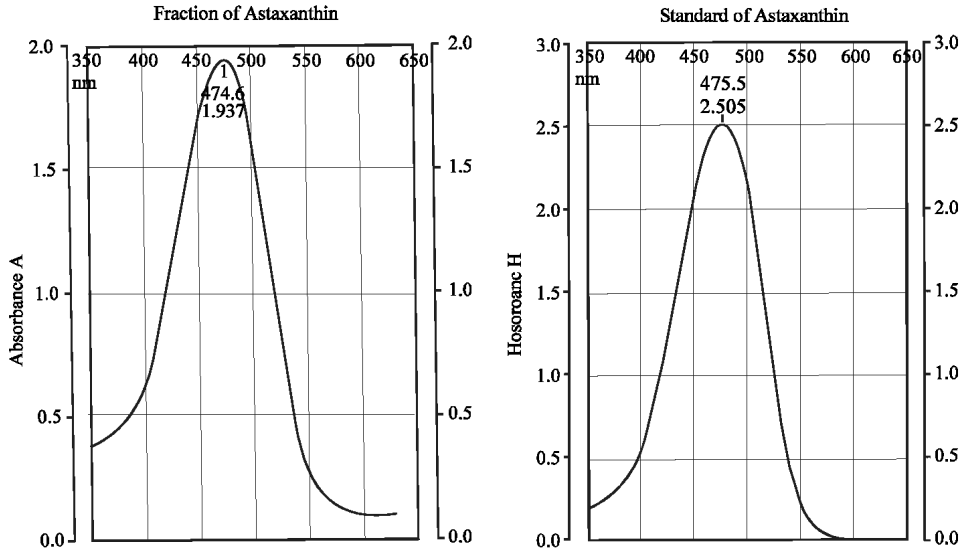

Fig. 3: Scanning spectra of fraction (left) and its comparison with standard astaxanthin (right)

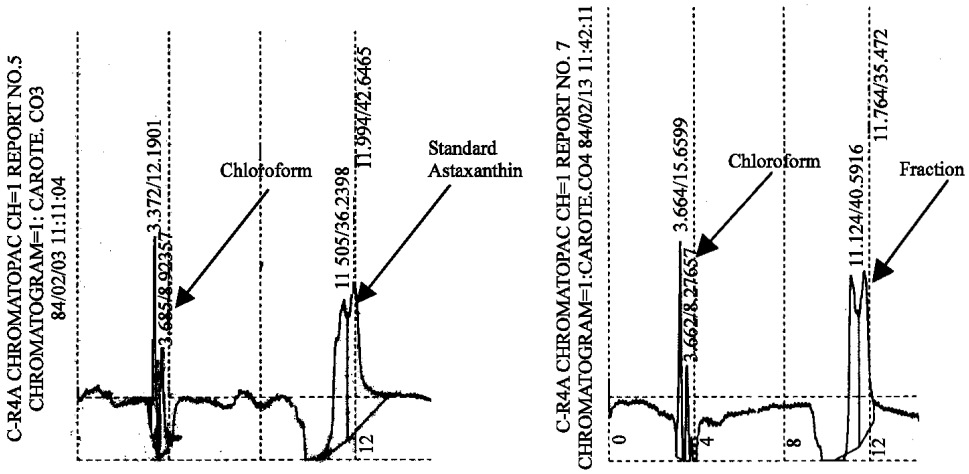

Fig. 4: HPLC chromatogram of standard astaxanthin (left) and fraction (right) RT = 10 min

Astaxanthin concentration was estimated in the petroleum ether by An *et al.* (1989) method. Results of this study for pure astaxanthin production indicated that mutant of JH-82 of *P. rhodozyma* (230 μg g^{-1} $_{dried\ yeast}$) produced more astaxanthin than natural isolate JH-80 (140 μg g^{-1} $_{dried\ yeast}$ Results of TLC analysis indicated that isolate of *P. rhodozyma* JH-82 were able to produce nine different carotenoids and high level of carotenoids was belong to astaxanthin. Results of spectrophotometric assay showed that this fraction has visible absorption spectrum (VIS) in acetone similar to standard astaxanthin and its λ_{max} was 474 nm (Fig. 3).

Results of HPLC indicated that retention time of fraction (RT = 11 min) was identical to standard astaxanthin (Fig. 4).

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