British Microbiology Research Journal
9(5): 1-7, 2015, Article no.BMRJ.19368
ISSN: 2231-0886

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Investigation of Astaxanthin Production from Yeast *Rhodosporidium* sp.

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Authors' contributions

This work was carried out in collaboration between all authors. Author KTLV designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors HTVT and DNN managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2015/19368
Editor(s):
(1) Raúl Rodríguez-Herrera, Autonomous University of Coahuila, México.
Reviewers:
(1) Klára Kosová, Division of Crop genetics and Breeding, Crop Research Institute, Prague, Czech Republic.
(2) Eugenio Ragazzi, Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Italy.
(3) Anonymous, University of Eastern Finland, Finland.
Complete Peer review History: http://sciencedomain.org/review-history/10269

Original Research Article

Received 5th June 2015
Accepted 10th July 2015
Published 21st July 2015

ABSTRACT

Aims: Astaxanthin, especially natural astaxanthin, is a powerful antioxidant, which is used as a nutraceutical and a common coloring agent in aquaculture. The present study was carried out to investigate the ability of astaxanthin production from the red yeast *Rhodosporidium* sp.
Study Design: *Rhodosporidium* sp. was cultured in medium containing different carbon sources then extracted by various methods. The astaxanthin content (mg/g) was calculated following Kelly-Harmon [1].
Place and Duration of Study: Laboratory of Bio-activate compound, Department of Biochemistry, Faculty of Biology, University of Science, Vietnam National University – Ho Chi Minh City.
Methodology: The result of the wavelength scanning method and Thin-layer chromatography (TLC) showed that *Rhodosporidium* sp. had the ability of astaxanthin accumulation.
Results: Chemical extraction with Dimethyl sulfoxide plus acetone was a possible and economical method to isolate astaxanthin comparing to other methods. To reduce astaxanthin production cost, molasses was found to be the best choice, which supported the highest astaxanthin yield (2.542 g/l).

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Conclusion: *Rhodosporidium* sp. had the promising capability of astaxanthin production, which deserves further research.

Keywords: Rhodosporidium sp.; red yeast; astaxanthin; antioxidant; culture; extract; carbon sources; molasses.

1. INTRODUCTION

Carotenoids are a family of pigmented compounds, which play an important role in human health based on their antioxidant properties and their efficiency in the prevention of many diseases. One of the most important carotenoids is astaxanthin, which is a powerful antioxidant that occurs in a range of living organisms [2,3]. Astaxanthin (3, 3'- Dihydroxy-β, β carotene- 4, 4' – Dione), a red-orange pigment, belongs to xanthophyll class of carotenoids, contains both the hydroxyl (-OH) and keto (C=0) moieties on its ionone ring, therefore it has higher antioxidant activity comparing to other carotenoids [4,5]. Various researches have demonstrated that astaxanthin has considerable promising applications in nutraceuticals and pharmaceuticals, natural feeding supplement for the aquaculture and poultry industry, because it can act as a stronger antioxidant activity, a large number of potential biological functions such as protection against UV- light effects, anti-cancer, preventing or reducing risk of many diseases [6 -8].

One of the best sources of natural astaxanthin is *Haematococcus pluvialis* [9], a green microalga, which can accumulate high astaxanthin content, yet it requires a large area to culture and a longer fermentation time than most of other microorganisms. Whereas, yeast has rapid growth, high density with much less heavy metals found in an algae process and it is easy to culture [10]. Due to its efficiencies, yeast is also used to produce astaxanthin.

Rhodosporidium sp., a heterobasidiomycetes yeast, has been considered as a good carotenoid producer including β-carotene [11,12], torularhodin, torulene [13]. However, it has not been used to produce astaxanthin yet. In Vietnam, *Rhodosporidium* sp. was isolated and identified by Bui in 2011 [14]. Our primary study revealed that it had the ability of astaxanthin accumulation in both the broth medium and semisolid state. Therefore, we carried out this research to investigate the ability of astaxanthin production from *Rhodosporidium* sp. as a new natural source to apply in nutraceuticals, pharmaceuticals and animal feeding.

2. MATERIALS AND METHODS

2.1 Microorganism

The yeast *Rhodosporidium* sp. was obtained by Bui in 2011 [14] in Department of Biochemistry, Faculty of Biology, University of Science, VNU - HCMC. The stock culture was maintained at 4°C on agar and sub-cultured every month. The medium for maintenance of the yeast was Hansen medium (1 L Hansen medium contains 50 g sucrose, 10 g peptone, 3 g KH_2PO_4, 3 g $MgSO_4$).

2.2 Inoculum Preparation and Fermentation

For activating the yeast, the cells were inoculated into a 250 ml Erlenmeyer flask containing 100 ml Hansen medium in a rotary shaking operated at 180 rpm for 24 h, adjusted at pH=6 and sterilized by autoclaving at 121°C, 0.8-1 atm for 15 min.

Yeast malt (YM; 1 L YM contains: 3 g yeast extract, 3 g malt extract, 10 g glucose, 5 g peptone) and Hansen media were employed in this study. The working volume of all culture fermentation was 100 ml. After comparing the two media (YM and HS), the best one would be used for the nutrient medium and glucose or sucrose was replaced by different carbon sources with the same amount of 30 g/l. The medium was sterilized by autoclaving at 121°C for 15 min. For fermentation, the liquid culture contained 1% (v/v) inoculum and was shaken on a rotary shaker at 200 rpm, room temperature and natural light. Biomass was harvested after 96 hours.

2.3 Biomass Collection and Astaxanthin Extraction

To harvest, samples were collected after 96 hours. By centrifugation, the obtained fresh biomass of red yeast was rinsed twice with double distilled water and then dried at 105°C for 1.5 hours to constant weight, yielding the Dry cell weight (DCW).

Approximately 100 mg of dried biomass was used for astaxanthin extraction tested by different methods: 1) Acetone (grinding dried biomass in 0.5 ml acetone, vortexed for about 2 min to vigorously homogenize, followed by centrifugation (5000 rpm/5 min) to collect pellet which was re-extracted with further 5 ml of acetone). 2) Acetone plus glass beads (grinding dried biomass in 0.5 ml acetone, adding 0.1 g of glass beads, vortexed carefully about 2 min then centrifuged (5000 rpm/5 min), the yeast precipitates was re-extracted with further 5ml of acetone). 3) Dimethyl sulfoxide (DMSO) plus acetone (the dried yeast was disrupted by 4 ml DMSO at 55°C for 30 min, then homogenized by vortex for 1 min and centrifuged (5000 rpm/5 min), the pellet continued to be extracted with 5 ml of acetone.

All obtained crude extract was dissolved in 10 ml of Petroleum ether (PE), using sodium chloride solution (NaCl 20%) to remove DMSO and acetone.

2.4 Analytical Methods

To evaluate the ability of astaxanthin production from the yeast *Rhodosporidium* sp., the wavelength scanning method and Thin-layer chromatography (TLC) with standard astaxanthin (in solvent n-Hexane: acetone (4:1)) were performed.

The absorbance of pigment extract was measured at λ=468 nm. The astaxanthin content (mg/g) was calculated following Kelly-Harmon [5]: $X= A_{468} * V * 10^4 / (E_{1cm}\% * G)$. Whereas, A_{468} is the absorbance of pigment extract in PE at λ_{468}, V (ml) is the volume of pigment extract, G (g) is the weight of yeast biomass, $E_{1cm}\%$ is the absorbance of astaxanthin solution 1% in PE (cuvette 1 cm) (E=2100).

All fermentation experiments were performed in triplicate. T-test and one-way analysis of variance (ANOVA) were used to analyze data (IBM SPSS 16.0 software). The obtained results were shown as the average and standard deviation (SD) values.

3. RESULTS AND DISCUSSION

3.1 Evaluating the Ability of Astaxanthin Accumulation of *Rhodosporidium* sp.

Yeast Malt (YM) and Hansen media was used to investigate the possibility of astaxanthin accumulation from *Rhodosporidium* sp. By wavelength scan method, we found that in both media, the maximal absorbance of pigmented extract in acetone was obtained at λ_{max} = 477nm, comparing to the maximal absorbance of standard astaxanthin also at λ_{max} = 477 nm.

Furthermore, the result of Thin-layer chromatography (TLC) using solvent n-Hexane: Acetone (4:1) showed that the pigment bar in both media was homologous and consistent with the astaxanthin standard (R_f = 0.22). However, their color intensity was not similar. The color intensity of the pigment from Hansen medium was darker than the one from YM medium (Fig. 1). It comes from that astaxanthin content in two media is different. Above results proved that *Rhodosporidium* sp. can produce astaxanthin in both YM and Hansen media, but astaxanthin content was not similar. Therefore, we carried out further study to compare astaxanthin yield in Yeast Malt and Hansen medium.

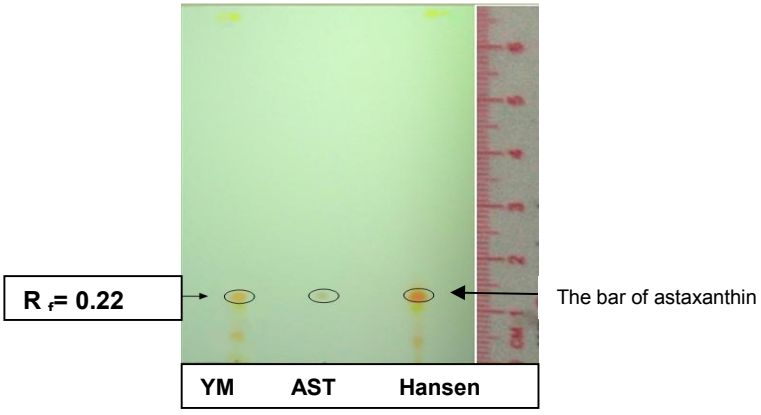

Fig. 1. The pigment bars obtained by Thin-layer chromatography (TLC)
**R_f: retention factor, YM: Yeast Malt medium, AST: standard astaxanthin, HS: Hansen medium.*

The result in Table 1 also showed that *Rhodosporidium* sp. could grow and accumulate astaxanthin in both media, but their dry biomass and astaxanthin yield were rather different. Although, there was no significant difference between the two media according to the results of T-test due to $p > 0.05$ (Appendix), Hansen medium was still better choice for growth and astaxanthin production of *Rhodosporidium* sp., which supported higher astaxanthin yield (3.5-fold higher than YM medium). The higher astaxanthin content could be explained by the presence of two mineral salts KH_2PO_4, $MgSO_4$ in Hansen medium.

3.2 Investigating Methods to Extract Astaxanthin from the Yeast Cell

Astaxanthin is synthesized and remained into the cell, so cell disruption is a vital step to recover this intracellular pigment. The lowest value of acetone was possibly due to less free astaxanthin available, when the cells were not broken. Breaking of the cellular wall, using DMSO as a chemical disruption was more efficient than the physical effect of glass beads (Fig. 2). This finding is in agreement with Wu et al. [15], which found that DMSO supported higher disrupting efficiency than other chemicals. This method could be used to treat a great number of samples, it is efficient and easy to apply comparing to other methods such as sonication or ultrasound used for small samples and requiring long-time disruption. Therefore, chemical extraction with DMSO plus acetone was used to isolate astaxanthin in the present study.

3.3 Studying the Effect of Different Carbon Sources on Astaxanthin Production from the Yeast

Rhodosporidium sp. could utilize various carbon sources to grow and produce, but their biomass concentration and astaxanthin yield were significantly different (Fig. 3 and Table 2). Using sucrose can result in the highest biomass, but in term of astaxanthin content was nearly 1.3-fold lower than that of glycerol and 1.9-fold lower than that of molasses. It indicated that astaxanthin accumulation was not proportional to the biomass. Molasses was found to be the most productive carbon source, which supported the highest astaxanthin yield (2.542 g/l). Like other red yeast, *Rhodosporidium* sp. could also transport and assimilate various carbon sources, but this result was not a similar conclusion drawn by Wu et al. [16] and Yamane et al. [17], which showed that astaxanthin production from the yeast *Xanhthophyllomyces dendrorhous* correlated with cell growth. According to results of other studies, the well-known astaxanthin-producer *X. dendrorhous* or several other species belonging to the genera *Rhodotorula*, *Sporobolomyces* and *Sporidiobolus* are able to grow well on molasses and support satisfactory carotenoid yields [18].

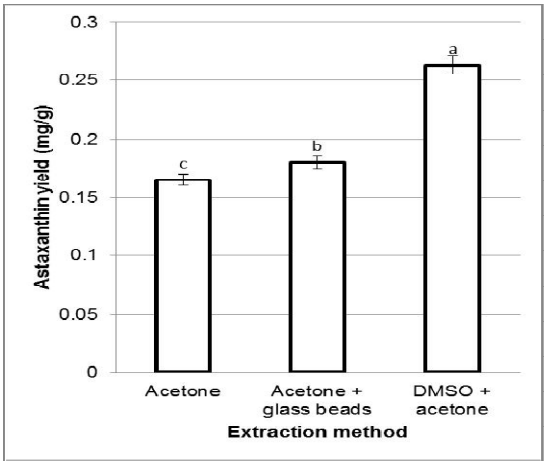

Fig. 2. Astaxanthin production by different extraction methods

**The means difference is significant at the $p \leq .05$. Means followed by the different letters are significantly different.*

Molasses has been considered as a potential carbon source containing high concentration of carbohydrates, besides also containing many mineral sources, vitamin and growth stimulant for the yeast. This is a promising low-cost alternative carbohydrate source to reduce astaxanthin production cost.

Table 1. Astaxanthin production on YM and Hansen media

Medium	Biomass (g/l)	Astaxanthin content (mg/g)	Astaxanthin yield (mg/l)
Yeast malt broth	4.332±0.04	0.132±0.02	0.572±0.03
Hansen	10.307±0.04	0.190±0.02	1.958±0.02

**Means ± SD = Means ± Standard Deviation of three independent experiences.*

Table 2. Effect of different carbon sources on astaxanthin production of *Rhdosporidium* sp.

Carbon sources	Biomass (g/l)	Astaxanthin content (mg/g)	Astaxanthin yield (mg/l)
Glucose	10.325±0.641[b]	0.188±0.02[c]	1.941±0.137[c]
Sucrose	11.586±0.559[a]	0.192±0.01[c]	2.224±0.094[b]
Glycerol	6.531± 0.388[c]	0.243±0.01[b]	1.590±0.099[d]
Molasses	7.213±0.200[c]	0.352±0.006[a]	2.542±0.033[a]

**Means ± SD = Means ± Standard Deviation of three independent experiences*
The mean difference is significant at the p ≤ .05. Means followed by the different letters are significantly different

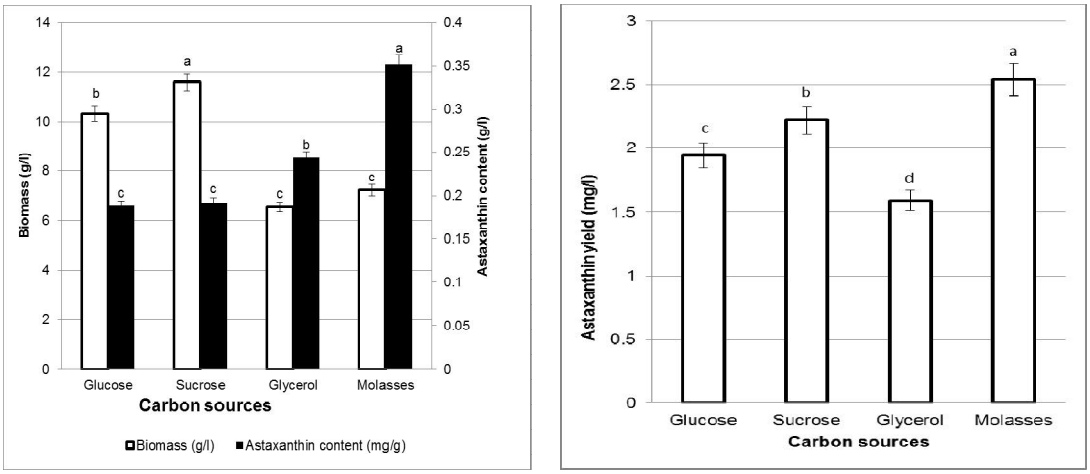

Fig. 3. Effect of different carbon sources on biomass, astaxanthin accumulation and astaxanthin yield

4. CONCLUSION

Our study revealed that *Rhodosporidium* sp. had the great potential capability of astaxanthin production. Using chemical extraction with DMSO, acetone and petroleum ether was possible method to isolate astaxanthin, which supported the highest yield. In terms of economic benefits, molasses was found to be the most productive carbon sources, which supported the highest astaxanthin yield with 2.542 g/l. Further research will optimize culture conditions to reduce the production cost and upgrade for semi-pilot scale.

ACKNOWLEDGEMENTS

The research was supported by Department of Science and Technology Ho Chi Minh City - Vietnam.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

Independent samples test

		Levene's test for equality of variances		t-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference	
									Lower	Upper
Biomass	Equal variances assumed	.026	.880	2.057E3	4	.000	5.97633	.00291	5.96827	5.98440
	Equal variances not assumed			2.057E3	3.997	.000	5.97633	.00291	5.96826	5.98440
ASTcontent	Equal variances assumed	.000	1.000	35.518	4	.000	.058000	.001633	.053466	.062534
	Equal variances not assumed			35.518	4.000	.000	.058000	.001633	.053466	.062534
ASTyield	Equal variances assumed	.308	.609	185.428	4	.000	.386000	.002082	.380220	.391780
	Equal variances not assumed			185.428	3.485	.000	.386000	.002082	.379867	.392133

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