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# *Rhodosporidium* sp. GROWTH IN MOLASSES MEDIUM AND EXTRACTION OF ITS ASTAXANTHIN BY USING HCl

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## ABSTRACT

Astaxanthin is classified as a xanthophyll-carotenoid, which is red-orange colour and powerful antioxidant activity. In this study, astaxanthin was collected from *Rhodosporidum* sp. by pilot culture (10 liters). Molasses medium was investigated with urea ($(NH_4)_2CO$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) and potassium dihydrogen phosphate ($KH_2PO_4$) at different concentrations. Astaxanthin was extracted by using chlorhydric acid (HCl) method.

The highest dried yeast biomass was 8.3682 g/l culture supernatant and astaxathin was 1.932 g/l culture supernatant by molasses medium containing 20 g/l sugar, 0.5 g/l ($(NH_4)_2CO$, 3 g/l $MgSO_4.7H_2O$ and 10 % (v/v) inoculum. HCl extraction method was mixed 10 mg biomass: 1 ml HCl 0.6 N and incubated at 70 $^oC$, 150 minutes.

*Keywords*: astaxanthin, *Rhodosporidium* sp., pilot, molasses medium, HCl extract.

## 1. INTRODUCTION

Carotenoids, tetraterpenes, are organic pigments which play an important role in human health. Astaxanthin, 3,3'-dihydroxy-β, β carotene-4, 4'-dione, is a carotennoid, a red-orange pigment and a high antioxidant activity compound [1, 2]. It contains both the hydroxyl (-OH) and keto (C=O) groups on its ionone ring; therefore it is superior to most of the hydrophobic antioxidants and against UV-light effects, anti-cancer, prevents and reduces the risk of many diseases [3 - 6]. It has been showed pharmaceutical and nutraceutical value to be developed in commercial production. In addition, astaxanthin has been used as natural food supplement for aquaculture and poultry [5, 6].

One of the best sources of natural astaxanthin is *Haematococcus pluvialis*, but it needs large culture area, temperate zone and long time to growing [7, 8]. Besides, the chosen yeast is *Rhodosporidium*, Ustilaginaceae family, has been considered as a good carotenoids producer including β-carotene, torularhodin, torulene [9, 10, 11]. In Viet Nam, *Rhodosporidium* sp. was isolated and identified by Khanh-Bui in 2014 [12]. The primary study revealed that

*Rhodosporidium* sp. had the ability to produce astaxanthin in broth and solid medium, therefore we continue the research on pilot-scale culture of *Rhodosporidium* sp.in molasses medium with mineral addition.

## 2. MATERIALS AND METHODS

### 2.1. Microorganism

The yeast *Rhodosporidium* sp. was isolated and identified by Khanh-Bui in 2014 at Biochemistry Department, Biology Faculty- Biotechnology, University of Science, VNU-HCM City.

### 2.2. Pilot 10 liters system

Pilot 10 liters system was designed including: gas pump brought the air into air-pipe, which prevented dust, some components by waterproof cotton and antibacteria by $KMnO_4$ solution (1/50 N, trapped in an Erlenmeyer flask). The air continuous passed filter 0.2 μm to medium.

### 2.3. Inoculums preparation and fermentation

*Rhodosporidium* sp. was pre-cultured into a 250 ml flask containing 100 ml Hansen medium, pH 6 in a rotary shaker operated at 200 rpm for 24 h at room temperature and daylight.

Molasses medium: pre-treatment molasses, different concentrations of sugar (10 – 35 g/l), $(NH_4)_2CO$ (0 – 2 g/l), $MgSO_4.7H_2O$ (0 – 5 g/l), $KH_2PO_4$ (0 – 5 g/l). The medium was sterilized by autoclaving at 121 $^oC$ for 15 minutes.

For fermentation in pilot, the liquid pre-culture contained 6 – 14 % (v/v) inoculum was added into medium. Biomass was harvested after 96 hours.

Cell growth was determined by the measurement of $OD_{610\ nm}$ by a spectrophotometer.

### 2.4. Harvest of biomass

*Rhodosporidium* sp. cell was centrifuged from supernatant culture at 4000 rpm/10 minutes and rinsed twice with distilled water and then dried until constant weight at 105 $^oC$, yielding the Dry cell weight (DCW).

### 2.5. Extraction of astaxanthin

For investigating cultural conditions, dried cell was disrupted by dimethyl sulphoxide (DMSO) and extracted with acetone [13]. 0.5 g dried yeast was mashed in 3 ml DMSO at 55 $^oC$, the solution containing DMSO was collected by centrifugation (5000 rpm/5 minutes). 5 ml of acetone was added to the rest, stirred well, and again centrifuged at the same speed. The process was repeated 2 – 3 times until the sediment had no pigments left. DMSO extraction and acetone extractions were mixed. And then, petroleum ether (PE) (the ratio of 0.5 PE/ 1 mixture unit) and 10 ml distilled water were added to the mixture, saturated NaCl solution was also added in case of no separation. The PE phase containing pigments was collected, it was rinsed with distilled water (1:1, v/v) until DMSO and acetone eliminated. The crude astaxanthin was dissolved in 10 ml PE and was used to determine content.

Astxanthin extraction by HCl method [14]: the dried biomass was incubated with HCl (0.2 – 2 N, blank by distilled water). Mixing 10 mg dried biomass and 1 ml HCl and incubating at 70 $^{o}$C on the investigated period time (60 – 180 minutes). And then, the mix was separated by centrifuging 5000 rpm/5 minutes at 25 $^{o}$C. The supernatant was collected and pellet was rinsed twice with distilled water. All of extract solution was transformed to pH 7.0 by NaOH 1 N and evaporated. The crude astaxanthin was dissolved in 10 ml PE and was used to determine content.

### 2.6. Analytical procedures

Astaxanthin in extracts was determined by using thin layer chromatography (TLC) and a solvent mixture of n-hexane : acetone (4:1); astaxanthin standard was purchased from Chroma Dex Co. The absorption of pigment extract was measured at $\lambda$ = 468 nm. The astaxanthin content (mg/g) was calculated following Kelly-Harmon [5]:

$$X = A_{\lambda 468\ nm} \times V \times 10^4/(E_{1cm}\% \times G)$$

Where as, $A_{\lambda 468\ nm}$ is the absorbance of pigment extract in PE at $\lambda_{468\ nm}$, 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 experiments were performed in triplication and average results were shown.

## 3. RESULTS AND DISCUSSION

### 3.1. The qualitative of astaxanthin in *Rhodosporidium* sp. in molasses medium

The qualitative astaxanthin from *Rhodosporidium* sp. by wavelength scanning method, we recognized that the maximum absorption of pigment extracts in acetone is the same as that of standard astaxanthin, i.e. $\lambda_{max}$ = 468 nm (Fig. 1 and 2).

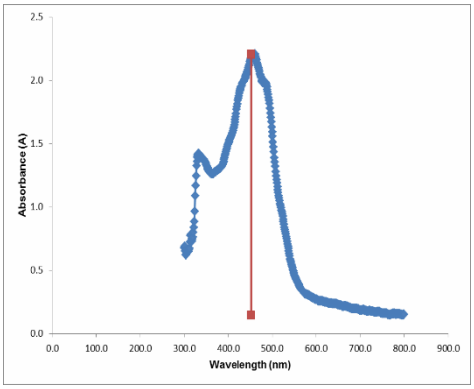


*Figure 1*. Wavelength scanning 468 of astaxanthin in *Rhodosporidium* sp.

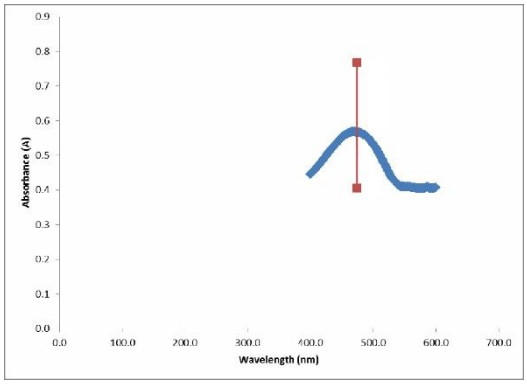


*Figure 2*. Wavelength scanning 468 of standard astaxanthin.

Moreover, the results from TLC method using solvents of n-hexane:acetone (4:1) revealed that the molasses medium including astaxanthin (Rf = 0.21 cm) was conformable to standard astaxanthin (Rf = 0.22 cm) (Figure 3). This means *Rhodosporidium* sp. can produce astaxanthin in molasses medium. The result showed that *Rhodosporidium* sp. can grow and accumulate in molasses medium.

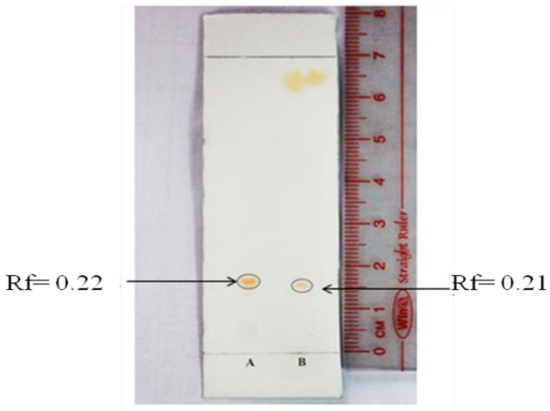


*Figure 3.* Chromatography result of extracted astaxanthin sample A: standard astaxanthin (sigma), B: extracted astaxanthin.

### 3.2. The effect of the additional mineral composition in molasses medium

*3.2.1. The effect of urea content*

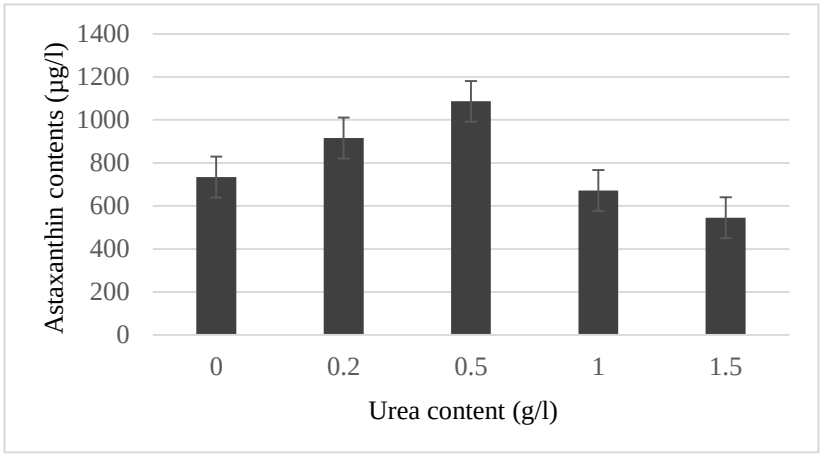


*Figure 4.* Astaxanthin contents taken from various cultures with different urea contents.

We saw that the astaxanthin content per one volume of culture was the highest when the urea content was 0.5 g/l (1086.33 µg/l) (Figure 4). It was 1.48 times higher than the control group in which the culture included molasses medium only, and no urea added (733.92 µg/l). This proves that adding urea to molasses medium increases the astaxanthin content. The reason is probably due to the limited nitrogen content from the additional urea. The molasses medium actually has a certain amount of nitrogen content, so the yeast growth will be hindered if the urea content is too high [15].

*3.2.2. The effect of $MgSO_4.7H_2O$ content*

As shown in the diagram, when the $MgSO_4.7H_2O$ content increased from 0 g/l to 3 g/l, the astaxanthin also gradually augmented and reached its peak at 186.03 µg/g of dry biomass and 849.27 µg/l of culture (Figure 5). This result was 1.44 times higher than control group in which the molasses medium contained no $MgSO_4.7H_2O$ (591.46 µg/l). However, when the

$MgSO_4.7H_2O$ content continued to rise, i.e. 3g/l, 4g/l, or 5g/l, the astaxanthin content gradually decreased. This means the $MgSO_4.7H_2O$ content added in the culture helps boost the astaxanthin. According to Yimyoo et al. [16], the average Mg content in molasses medium is 0.18 ± 0.02 % and when combined with additional $MgSO_4.7H_2O$, it produces the highest content of astaxanthin.

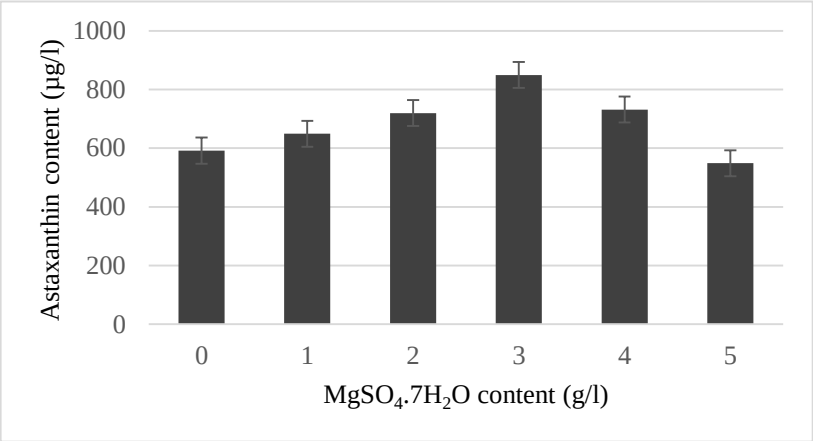


*Figure 5.* Effect of different $MgSO_4.7H_2O$ contents on astaxanthin contents.

*3.2.3. The effect of $KH_2PO_4$ content*

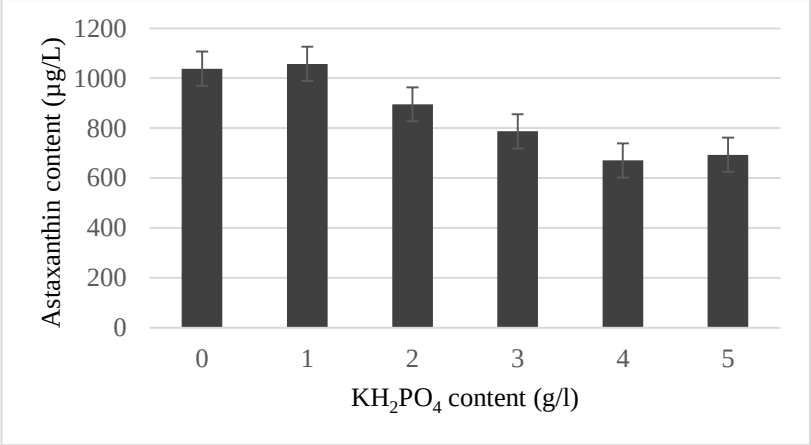


*Figure 6.* Astaxanthin contents taken from various cultures with different $KH_2PO_4$ contents.

As shown in the diagram, the astaxanthin content on the dry biomass and on the culture had the same increase and decrease: the highest results were 0 g/l (187.08 µg/g – 1037.94 µg/l) and 1 g/l (159.75 µg/g – 1057.53 µg/l), and in other cases, astaxanthin dramatically dropped in comparison with that in the control group (Figure 6). The reason for this phenomenon is that high potassium content inhibits the growth of *Rhodosporidium* sp. yeast. The potash included in the molasses medium normally originates from fertilizers used in growing sugar canes [17].

*3.2.4. The effect of the breed rate*

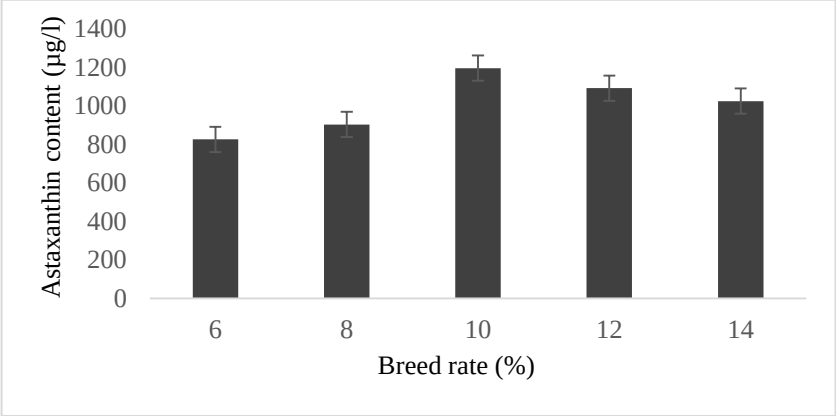


*Figure 7.* Astaxanthin contents with various cultures with different breed rate.

The results showed that astaxanthin content on the dry biomass and on the culture increased. It gradually increased until reaching the highest at 10 % breed rate (189.78µg/g-1197.17µg/l) and started to decrease when the breed rate continued to rise after 10 % (Figure 7). Particularly, when the breed rate was as high as 12 % or 14 %, *Rhodosporidium* sp. yeast used up nutrition in the culture earlier due to higher competitiveness. Consequently, the astaxanthin content was low. Meanwhile, the breed rate of 10 % is the most suitable when producing the highest astaxanthin in comparison with other breed rates.

*3.2.5. The effect of the total sugar content*

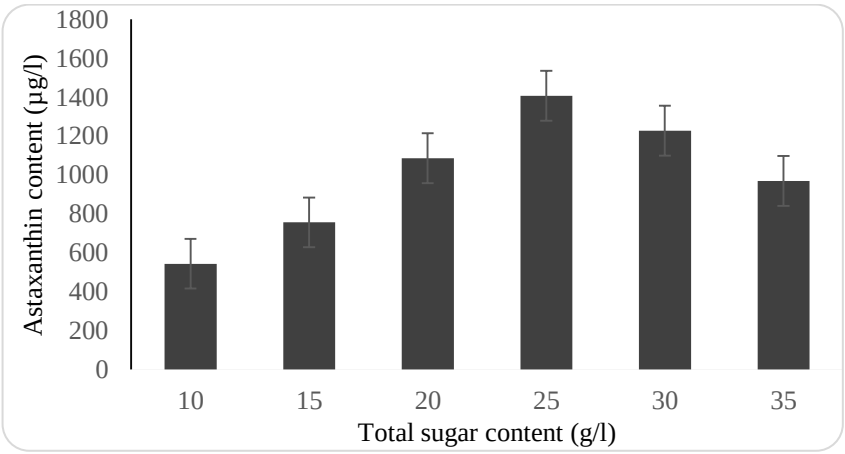


*Figure 8.* The effect of different total sugar contents on astaxanthin contents.

It was shown that the astaxanthin gradually increased when the total sugar content ranged from 10 g/l to 25 g/l, and decreased when it ranged from 30 g/l to 35 g/l. the highest content was 1406.62 µg/l at the concentration of 25 g/l (Figure 8).

**3.3. The growth curve of *Rhodosporidium* sp. in molasses medium with suitable elements**

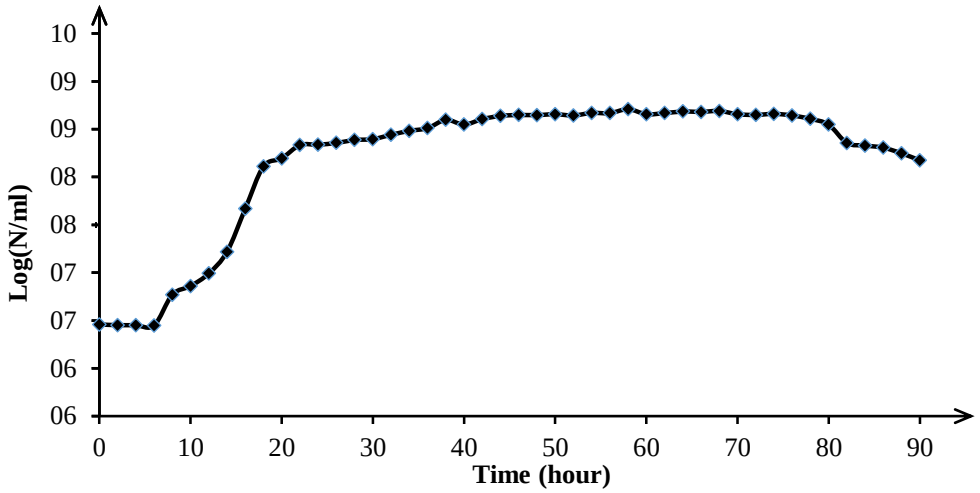


*Figure 9.* The growth of *Rhodosporidium* sp. in molasses medium

The Figure 9 showed that different growth stages: latent phase in the first 6 hours, exponential growth phase from the 8$^{th}$ hour to 30$^{th}$ hour, stable phase from 32$^{th}$ hour to 80$^{th}$ hour, decay phase from the 82$^{th}$ hour.

### 3.4. Extraction astaxanthin from *Rhodosporidium* sp. yeast by using HCl

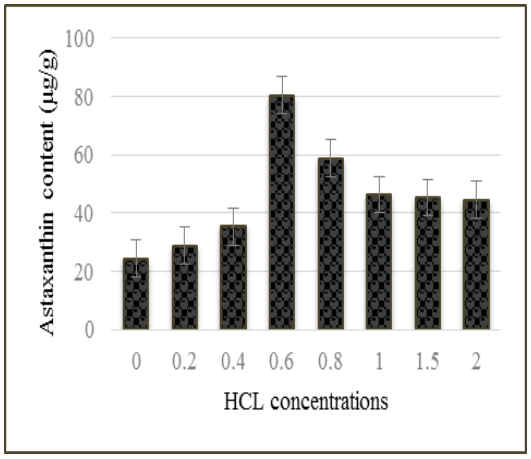


*Figure 10.* Asxatanthin contents (µg/g ) with different HCl concentrations.

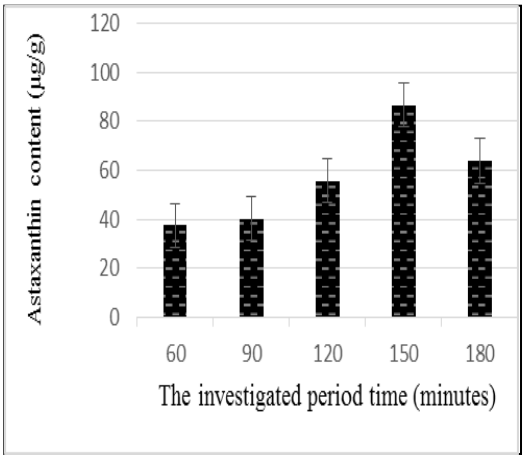


*Figure 11.* Astaxanthin contents (µg/g ) with different the investigated period time.

According to the Figure 10, we saw that astaxanthin content extracted from *Rhodosporidium* sp. cells in HCl 0.2 N and 0.4 N had slight increase. HCl 0.4 N produced the highest astaxanthin (80.48 µg/g in comparison with 24.44 µg/g in the control group 24.44 µg/g, 3.29 times higher). When HCl concentration was higher than 0.6 N, asxatanthin decreased from 0.8 N to 2 N (58.89 > 46.35 > 45.41 > 44.52) (Figure 10). According to Yin et al. [14], astaxanthin extraction reached the highest productivity when *Phaffia Rhodozyma* cells were soaked in HCl 0.4 N. This may be since HCl concentrations suitable for the extraction of two different species are not the same.

According to the Figure 11, we saw the longer investigated period times were (from 60 minutes to 150 minutes), the higher astaxanthin content was. In the period of 150 minutes, the average astaxanthin content reached 86.69 (µg/g dry biomass). However it started to decrease after 180 minutes. Prolonging the heating time conditions HCl to destroy the yeast cell wall, accelerating the extraction effectiveness up to a certain moment when the astaxanthin peaked. However, it also denatures astaxanthin due to the effects of heat and acid; accordingly after 180 minutes, asxatanthin started to decline. Hence, we chose 150 minutes as a proper period to apply for the extraction process to get the highest yield of asxatanthin.

### 3.5. Identifying the mass of dry biomass and astaxanthin content

*Table 1*. The contents of dry biomass and astaxanthin taken from *Rhodosporidium* sp. in molasses medium.

| **Dry biomass content (g/l)** | **Astaxanthin content** | |
|---|---|---|
| | **(µg/g dry biomass)** | **(µg/l culture)** |
| 8.3682 ± 0.1144 | 230.89 ± 6.74 | 1932.21 ± 56.38 |

*Figure 12.* Dry biomass *Rhodosporidium* sp. yeast.

Dry biomass of *Rhodosporidium* sp. is floury, have the light red colour (Figure 12) and contents 230.89 ± 6.74 µg astaxanthin per g dry biomass (Table 1).

### 3.6. Quantifying astaxanthin by HPLC/MS

In this research, the results show that astaxanthin content of *Rhodosporidium* sp. 1.3 times as lower as wild yeast *Phaffia rhodozyma* ATTCC2402 was fermented to produce 303.3 µg/g of astaxanthin content [18] (Figures 13 and 14, Table 2). In the experiment of Tong and Tran [19], yeast *Phaffia rhodozyma* NT5 was grown in liquid culture containing saccharose 20 g/l at pH 5.0 at 22 $^{o}$C in 120 hours, and produced 285.4 µg/g of astaxanthin content, inconsiderably higher than the content taken from *Rhodosporidium* sp. in molasses medium and suitable elements in this study.

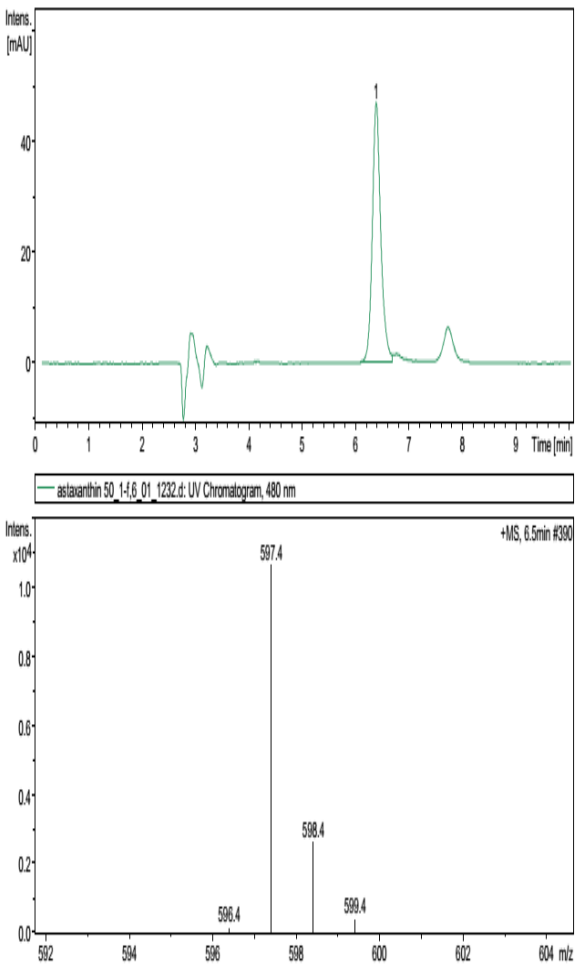


*Figure 13.* Quantifying astaxanthin (sigma) by HPLC/MS

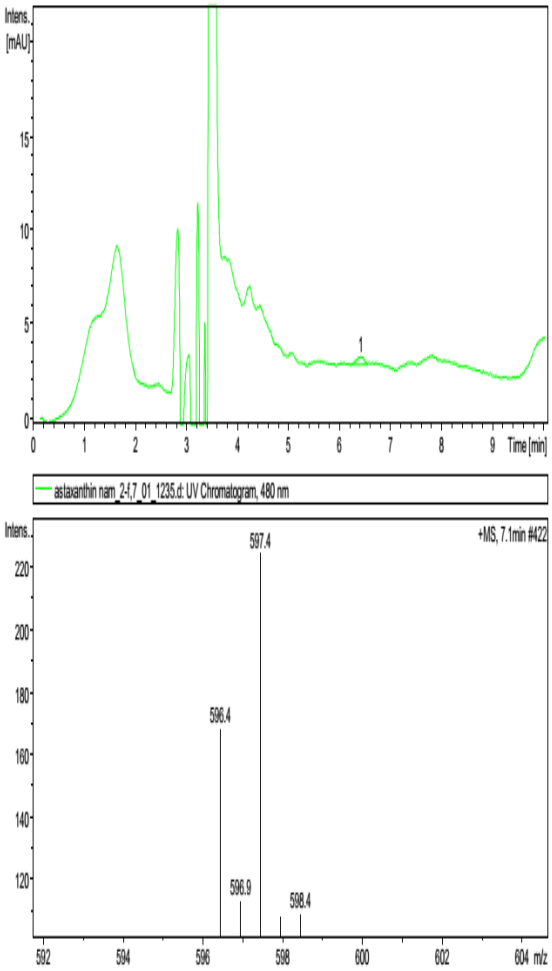


*Figure 14.* Quantifying astaxanthin of *Rhodosporidium* sp. yeast by HPLC/MS

*Table 2.* The results of quantifying astaxanthin of *Rhodosporidium* sp. yeast by HPLC/MS.

| S.No. | Sample | Parameter | Unit | Result | Method |
|---|---|---|---|---|---|
| 01 | *Rhodosporidium* sp. extract | Astaxanthin | µg/10 ml | 0.27 | HPLC-MS |

## 4. CONCLUSION

Our research reveals that *Rhodosporidium* sp. has high potential in producing astaxanthin. The 10 litre culture system was set up in the molasses medium which was added 0.5 g/l of urea, 3 g/l of $MgSO_4.7H_2O$, 0 g/l of $KH_2PO_4$ with 25 g/l of the total sugar content and 10 % of the breed. The final astaxanthin content was 1932.21 µg/l of culture and the ratio of the dry biomass to culture volume was 8.3682 g/l. The extraction process to get the highest astaxanthin content was done in HCl 0.6 N at 70 $^oC$ in 150 minutes. This proves that *Rhodosporidium* sp. can also be

used to extract astanxathin, and the growth of *Rhodosporidium* sp. in molasses medium produces quite high yield and is potential to be applied in industrial production.

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