PRODUCTION OF ASTAXANTHIN FROM SAGO FLOUR BY *Phaffia rhodozyma* TISTR 5730

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Abstract

Sago starch from mature sago palm has very low nutritional value and is normally considered as waste. This study focused on a potential conversion of that low-value waste into astaxanthin which has a high commercial value. *Phaffia rhodozyma* was grown on three media, namely: standard media for yeast culture (YM medium), sago starch with essential minerals (SM for Sago medium) and sago medium with addition of essential minerals;- 3 g/L Yeast extract, 0.1 g/L KH_2PO_4, 0.1 g/L NaCl, 0.01 g/L $MgSO_4$ and 0.01 g/L $CaCl_2$ (mSM for modified Sago medium). The initial pH of the mSM medium was adjusted to 5.5 with 1 N HCl. All cultivations were carried out at 25 °C, pH 5.5, 500 lux of illumination by cool white fluorescent lamps and agitation of 200 rpm for a culture period of 96 h. Cell growth was measured by both turbidity (at 660 nm) and dry cell mass after 120 h of fermentation. The target product, astaxanthin, was measured by HPLC with a diode-array detector. The results shown that mSM was the best media for cultivation in comparison to YM and SM, with the highest biomass of 11.56 g/l (10.95 % turbidity) and highest astaxanthin content of 726 μg/g dry yeast. The YM and SM medium had final biomasses at 3.76 (2.58 % turbidity) and 8.77 g/l (6.49% turbidity), respectively. In addition, astaxanthin production is known to be partially growth-associated, astaxanthin content thus followed the similar trend to the biomass yield and reached the maxima value after 84 h of cultivation period. The biomass yield was 726, 445 and 306 μg/g dry cell yeast for mSM, SM and YM, respectively. The results indicated a potential use of sago starch for astaxanthin production by *Phaffia rhodozyma*. However, the astaxanthan concentration obtained from the mSM media is still too low for commercial production and requires further investigation before its potential can be realized.

Keywords: red yeast, *Phaffia rhodozyma*, sago starch, astaxanthin

Introduction

Astaxanthin (3,3′- dihydroxy-β,β-carotene- 4,4′-dione) is the main carotenoid pigment found in aquatic animals, and is presented in many kinds of marine life, including salmon, trout, red seabream, shrimp, lobster and fish eggs. However, recent studies have shifted the focus toward applications of astaxanthin for human health due to its biological functions, including protection against oxidation of essential polyunsaturated fatty acids, and immune responses (Lorenz and Cysewski 2000; Guerin et al. 2003). The global market for astaxanthin in 2007

was about 220 million USD, which has been gradually increasing and is expected to reach 250 million USD by 2019 (März 2008). Although recently, astaxanthin has been synthesized by chemical processes at a lower cost, the percentage of astaxanthin produced by microbial processes has grown steadily, increasing from 16% of total commercial astaxanthin in 1992 to 32% in 1996 (Frengova et al. 1997). It is further expected that the trend will grow as the technology matures. Astaxanthin is important not only as a pigment source in aquaculture but also as a potential antioxidant that may delay aging and degenerative diseases in humans and animals (Johnson and An 1991). The main goal of the work reported here is to explore the capacity of raw, agro-industrial sago flour for astaxanthin production by *Phaffia rhodozyma*, which is interesting microorganisms to be capable of using natural sources of product, as reported by Golubev (1995) and Zhegn et al (2006) for potentials of *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma* yeast) and *Haematococcus pluvialis* (micro alga)) Several reports have shown that media constituents profoundly affect astaxanthin production by *Phaffia rhodozyma* (Johnson and An 1991). The effects of different nutrients on *Phaffia rhodozyma* have been studied using media containing complex sources of nutrients such as sugarcane juice (Florēncio et al. 1998), molasses (Haard 1988), grape juice (Meyer et al. 1994), coconut water (Domínguez-Bocanegra and Torres-Muńuz 2004), prehydrolysed wood (Parajó et al. 1997) and plant extracts (Soo-ki et al. 2007).

Methodology

Yeast strain and inoculums

Phaffia rhodozyma TISTR 5730 was received from the Asian culture collection, Thailand Institute of Scientific and Technology Research (TISTR), Pattum Thani, Thailand. Yeast culture was maintained on slants of yeast-malt medium (YM agar), and stored in 40% glycerol – 60% YM broth at -80 °C. The organism was transferred to a new culture medium before inoculum preparation. The cell inoculum was grown aseptically in a 250 ml-Erlenmeyer flask containing 50 ml of YM medium (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract), at a constant temperature of 25 °C, stirring speed of 200 rpm, and illumination (500 lux) provided by cool white fluorescent lamps for 24 h. Initial pH of the medium was adjusted to 5.5 with 1 N HCl before autoclaving for 15 min at 121 ˚C. Fresh medium was inoculated with the seed culture at 20% volume. The yeast was grown in the same culture medium. The cultures were inoculated with 10 ml YM medium in the exponential phase.

Sago medium preparation

Sago flour was used as the local raw material. The method to obtain fructose from sago flour (Chutinut et al. 2008) was as follows:- Dissolve 1 g of sago flour in 100 ml of water (1). Transfer the solution (1) to a 250 ml-Erlenmeyer flak containing 80 °C water with 2 M of HCl 10 ml, and stir well for 15 min to digest the flour to reducing sugar (2). Pipette the solution (2) and put one drop on a glass plate, or white glazed porcelain, with one drop of iodine, then continue to drop solution on the mixture until no violet color is observed. Normalize remaining solution with 10% NaOH. Test by Benedict's solution, warm in a hot bath for 5 min, and if the starch solution turns brick red then the digested starch solution has been successively turned into reducing sugar. Carry out the experiment in the same way for glucose to check accuracy, as it should give similar results. Concentrate the obtained reducing sugar to 2 ml in volume, and put in sample container. Filter the sample through 0.45 µm filtered paper, then analyzed by HPLC under the following conditions:- (Column:

Carbohydrate Analysis Nova pack with internal diameter 3.9 mm, length 30 mm, particle size 10 µm; Mobile phase: Acetonitrile : H_2O = 80:20, flow rate: 2.0 ml/min; Detester: RI (internal temperature: 40 °C, sample volume: 5 µL, time of analysis: 15 min.). Note that this method gives fructose rather than glucose. The obtained solution is then used for yeast culture, with the ratio of fructose to glucose at 80:20 %.

The medium formulations for yeast culture are as follows:-

Control medium: YM medium;
Sago medium: the assimilated sago starch for monosaccharide by HCL 2 M and heat to 80 °C (SM medium), according to the above mentioned (Chutinut et al. 2008) ; Modified Sago medium: The assimilated sago starch for monosaccharide (Chutinut et al. 2008) 10 g/L and then add 3 g/L Yeast extract, 0.1 g/L KH2PO4, 0.1 g/L NaCl, 0.01 g/L MgSO4 and 0.01 g/L $CaCl_2$. The initial pH was adjusted to 5.5 with 1 N HCl. (mSM medium)

Growth conditions and kinetics;

Cultures were grown aseptically in a 250 ml Erlenmeyer containing 50 ml of mSM, SM and YM medium, incubated using a gyrating shaker at 200 rpm, and illuminated (500 lux) with cool white fluorescent lamps at 25 °C for 5 days (120 h).

Effect of media on yeast growth and astaxanthin production:-anaylical procedures;
Cell growth was measured as turbidity at OD 660 nm and dry cell mass according to Kurane et al. (1994). The cell biomass of *Phaffia rhodozyma* TISTR 5730 was harvested by centrifugation at 7,000 rpm for min, after that the cells were washed twice with deionized water and centrifuged again. The harvested cells were dried in 5 ml test tubes in an oven at 100 ˚ for 18 h. The dry cell weight was calculated by comparing with a standard curve of biomass *Phaffia rhodozyma* TISTR 5730 in YM medium (Kurane et al. 1994). Reducing sugar concentration in the culture medium was determined by the Somogyi-Nelson method. Astaxanthin was measured by HPLC with Diode-Array detection and a stainless steel Nova-Pack C 18 reversed-phase column (4x250nm). The eluting solvent was methanol/water/hexane 95:4:1 by volume with a flow rate set at 0.5 ml/min. Synthetic astaxanthin (Hoffman-la Roche) was used as the external standard. The astaxanthin content was measured 48 h after inoculation until the fermentation was stopped at 4 days (96 h).

Results and Discussion

Effect of substrate on growth of *P. rhodozyma* TISTR 5730 (incubated using a gyrating shaker at 200 rpm)

The growth rate of *P. rhodoayma* TISTR 5730, measured at 660-nm wave length, revealed that the yeast grew well in all media while mSM gave the most promising results (Figure 1(all)). In that case (mSM), the yeast reached its highest mass at 84 h after inoculation where the turbidity reached 10.95% (Figure 1a). The cultures obtained from YM and SM gave maximum turbidities of 2.58% and 8.77%, respectively (Figure 1a) while the dry cell weights are also shown (Figure 1b). In addition, the astaxanthin contents are similar trends to the biomass (Figure 1c).

As shown in Figure 2, the broth obtained from culturing yeast on SM medium exhibited a distinct orange color. Microscopic inspection at 84 h after inoculation revealed that a large

number of yeast cells from YM had became large and the internal pigments were observable (Figure 2a). In SM medium, besides the appearance of a large number of big and mature cells, the cells showed higher concentrations of orange pigments (Figure 2b); Similar result for mSM medium is also provided (Figure 2c). In addition, the analysis of astaxanthin content showed that, after 84 h of cultivation, the astaxanthin content was 726 µg/g dry yeast in mSM medium, whereas YM and SM medium cultures provided astaxanthin contents of 306 and 445 µg/g dry yeast respectively.

Effect of different media on the pH, biomass (dry cell weight), astaxanthin content, maximum specific growth rates and yield coefficients for *Phaffia rhodozyma* TISTR 5730 are summarized in Table 1.

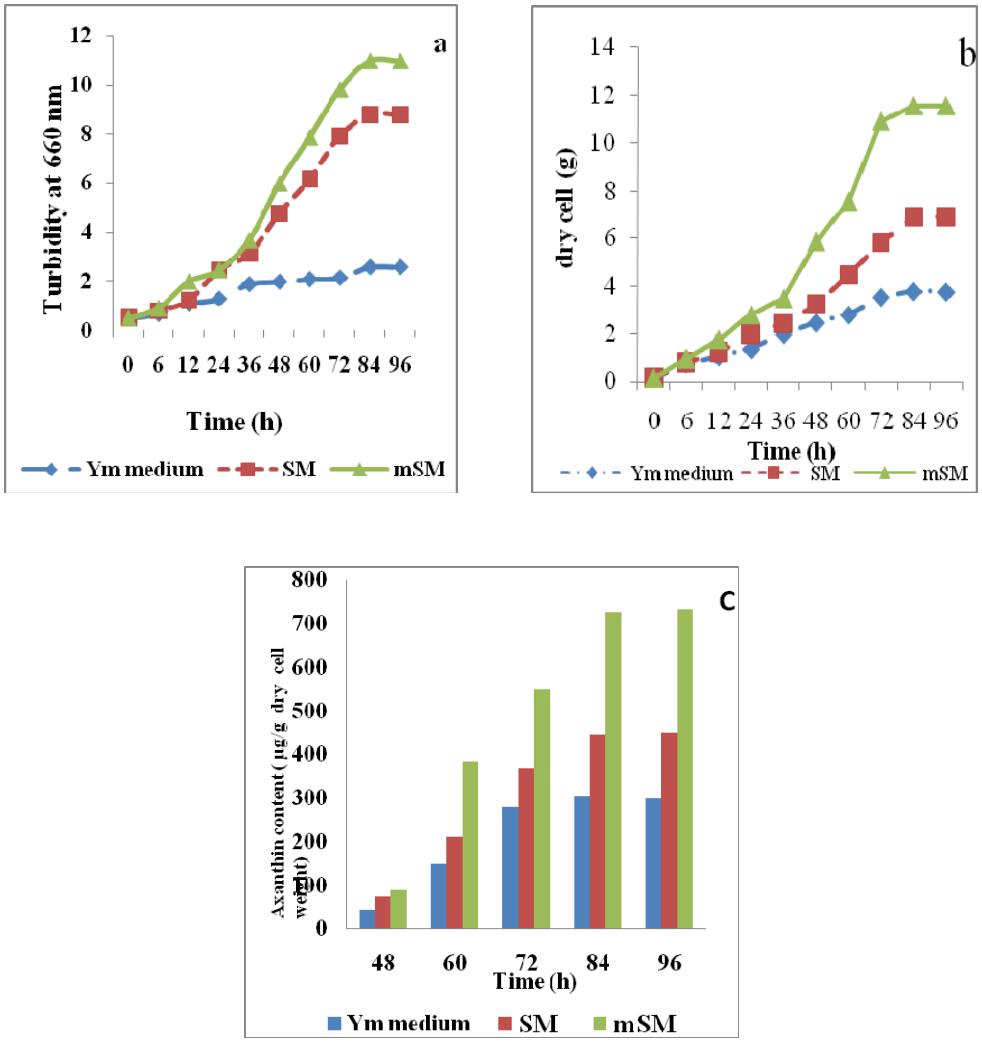
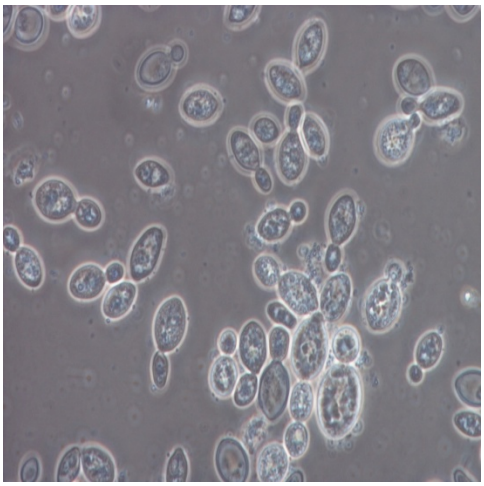
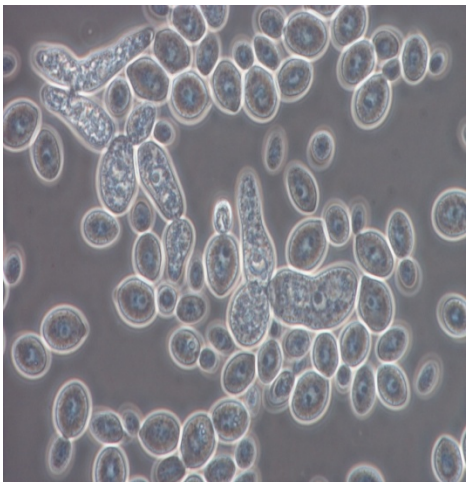


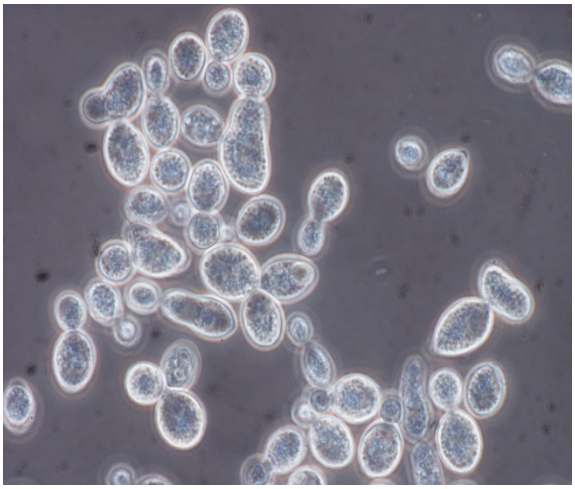
Figure 1. Growth and astaxanthin production of *P. rhodozyma* TISTR 5730 cultured on three different media expressed in term of a) turbidity at 660 nm, b) dry cell mass, and c) astaxanthin content



2a



2b



2c

Figure 2 A comparison of the appearances of different cultures after 84 h .The left (2a), right (2b), and central (2c) images are from broths obtained by cultivation of *P. rhodozyma* in YM, SM and mSM medium, respectively

Table 1 Effect of different media on the pH, biomass, astaxanthin content, maximum specific growth rates and yield coefficients for *Phaffia rhodozyma* TISTR 5730 grown at 25 °C and 200 rpm under a light intensity of 500 lux for 84 h.

Parameters	pH	Biomass (g/L)	Astaxanthin concentration (μg/g dry cell weight)	μ_{max} (h^{-1})	$Y_{x/s}$ (g dry cell weight/ g glucose)
Control (YM)	6.50	3.79	306	0.11	0.23
SM	5.83	6. 89	445	0.12	0.27
mSM	5.78	11.56	726	0.13	0.24

Better growth on mSM indicated that sago starch contained a rich source of essential nutrients, minerals and vitamins which microorganism assimilated readily. In addition, mSM also enhanced the production of astaxanthin due to that reason. Nevertheless, the results indicate that the composition of the three media have a strong effect on both yeast growth and astaxanthin production. By adding a small concentration of carbon into a base media, for example, by adding 10g/l of glucose into mSM, the amount of astaxanthin production changed tremendously. That signals needed for further investigation. Similar result was observed by Meyer and Preez (1993), who cultivated *Phaffia rhdozyma* N9 in YM medium with a supplementation of 100 mL of white grape juice in 2-L batch reactor and it was found that the total cellular pigment and astaxanthin content increased during the stationary growth phase due to a decrease in biomass, reaching final values of 2120 μg/g dry yeast. Although some pioneer work was carried out by other researchers, such as Vazquez and Parajo (1997), more investigation is warranted on the use of different media compositions, carbon and nitrogen sources, C:N ratios, minerals, vitamins, and other factors enhancing growth and astaxanthin production. At this stage, we also suggest that the coconut juice, which has a rich nutritional value, may have potential for use in astaxanthin production. However, it is still in an early phase of research.

Conclusion

To summarize, *Phaffia rhodozyma* TISTR 5730 grown on SM medium reached the highest dry cell mass of 11.56 g/l and a yield coefficient of 0.24 g dry cell weight/g glucose with asthaxanthin content of 726 μg/g dry cell weight at 84 h of cultivation. YM and Sago medium provided dry cell masses of 3.79 and 6.89 g/l and yield coefficients of 0.23 and 0.24 g dry cell weight /g glucose, with asthaxanthin content of 306 and 445 μg/g dry cell weight, respectively. The culture by modified sago medium (mSM) provided the highest astaxanthin content (726 μg/g dry cell weight after 84 h). Based on this work, the results are promising for several reasons. First, although a wild-type strain was used, its productivity was double greater than the highest yield reported in the literature, where sufficient (but not excessive) aeration was applied. Also, sago starch is a cheap raw material and an abundant industrial and domestic waste product that has never been exploited efficiently in many tropical countries.

Finally, we chose to optimize the culture using temperature for tropical regions, where the raw material is inexpensive and thus lowering the costs of production.

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