

Citric acid regulates astaxanthin production by *Phaffia rhodozyma* TISTR 5730 in Thai coconut water

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Abstract: We examined the effects of citric acid (CA) supplementation on astaxanthin production by wild-type *Phaffia rhodozyma* TISTR 5730, grown in coconut water at 25°C, to reduce energy costs for tropical regions. In shaken flask cultures with 3 levels of supplement (0.05%, 0.10%, and 0.15% (w/v)), 0.15% citric acid effected the best growth (13.64 g/L) and highest astaxanthin concentration (1052 µg/g dry cell at 84 h)—3-fold higher than without supplementation (315 µg/g dry cell at 84 h). Grown in a 2-L fermentor at aeration rates of 1, 2, and 3 vvm (540 rpm, 500 lux, 25°C, initial pH 5.5), however, the astaxanthin yields were 3880, 4593, and 753 µg/g dry cell, respectively. At the optimal aeration rate (2 vvm), the astaxanthin yield was approximately 15-fold higher than that of shaken flask cultures that lacked citric acid. Over-aeration (3 vvm), however, caused a severe decline in astaxanthin yield (753 µg/g dry cell at 84 h).

Keywords: astaxanthin, population distribution, yeast fermentation, citric acid, *Phaffia rhodozyma*

Introduction

Astaxanthin (3,3'-dihydroxy- β , β -carotene-4,4'-dione) is an important commercial carotenoid that is used widely as a color induction additive in aquaculture and as a health-enhancing agent in humans due to its biological functions, including protection against oxidation of essential polyunsaturated fatty acids and its effects on the immune response (Lorenz and Cysewski, 2000; Guerin et al., 2003). The global astaxanthin market in 2007 was approximately 220 million USD. The current worldwide market of astaxanthin is approximately 220 million USD and is predicted to increase to over 250 million USD by 2019 (März, 2008).

The current cost of astaxanthin fermentation, however, is not competitive enough to replace the synthetic process (Olaizala, 2003). Commercial fermentation of astaxanthin relies primarily on the growth of microalgae of the genus *Haematococcus* in photobioreactors, which requires a large surface area to receive sufficient light (Olaizola, 2000; Lorenz and Cysewski, 2000).

Recently, *Phaffia rhodozyma*, a yeast in terrestrial plants, was observed to replace the traditional species, because *P. rhodozyma* grows with or without a light

source, thus rendering less efficient photofermentation methods irrelevant (Vazoquez, 2001). A recent study reported comparable astaxanthin production in the wild-type strains of these organisms. Yet, the mutant strain *P. rhodozyma* R1 experienced higher growth rates, and the wild-type *Haematococcus pluvialis* NRRL-144 yielded higher astaxanthin concentrations (2 mg/g dry cell) (Domínguez-Bocanegra et al., 2007).

Several reports have demonstrated that media composition and culture conditions (e.g., initial pH, agitation) influence astaxanthin production in *P. rhodozyma* (Johnson and An, 1991). The effects of complex media constituents on *P. rhodozyma* have been examined for sugarcane juice (Florêncio et al., 1998), molasses (Haard, 1988), grape juice (Meyer et al., 1994), coconut juice (Domínguez-Bocanegra and Torres-Muñuz, 2004), prehydrolyzed wood (Parajó et al., 1997), and plant extracts (Kim et al., 2007). Further, the effects of various precursors have been studied, including acetic acid (Meyer et al., 1993), ethanol (Gu et al., 1997; Yamane et al., 1997), monoterpenes

(Meyer et al., 1994), and mevalonic acid (Calo et al., 1995)—although the latter is too expensive for use in large-scale astaxanthin production.

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One of the most notable low-cost raw materials for astaxanthin production in tropical regions is coconut water—also called coconut milk by Domínguez-Bocanegra and Torres-Muñuz (2004). In recent work on a mutant strain (R1) that was grown in coconut milk (16 g/L total sugar) in an oxygen-limited environment (shaken flask culture), astaxanthin content increased up to 1851 µg/g yeast compared with 1061 µg/g yeast during growth in YM medium. Therefore, coconut milk (or, coconut water) has tremendous potential for use in commercial production due to its low cost in southeast Asia and its ability to support potentially high carotenoid yields.

This work investigated the potential use of coconut water—liquid waste from the local coconut industry—in producing astaxanthin in *Phaffia rhodozyma* TISTR 5730. The traditional approach was combined with population dynamics to determine how citric acid supplement levels affect yeast population dynamics, what factors of fermentation favor astaxanthin production, and which subpopulations mediate high astaxanthin production directly.

Our long-term goal is to identify a set of control strategies, including media formulation, programmed feeding policies, and physicochemical control strategies, to achieve maximum profit from the coconut water that is routinely discarded in tropical regions.

Materials and Methods

Microorganisms

Phaffia rhodozyma TISTR 5730 was obtained from the Asian Culture Collection, Thailand Institute of Scientific and Technology Research (TISTR).

Culture media

Modified basal medium (MB medium), containing glucose (50 g/L), yeast extract (3.0 g/L), KH_2PO_4 (0.1 g/L), NaCl (0.1 g/L), MgSO_4 (0.01 g/L), and CaCl_2 (0.01 g/L) (Sujarit, 2009), was used in the control experiments. Coconut water medium (CW medium) was prepared from a mixture of coconut water from young and ripe coconut fruits (1:1), supplemented with 10 g glucose, 0.1 g KH_2PO_4 , 0.1 g NaCl, 0.01 g MgSO_4 , and 0.01 g CaCl_2 , to ensure that the basic nutrients were present.

The coconut that we used was a local variety that typically is used to extract coconut milk in Nakhon Si Thammarat, Thailand. The pH of the CW media was adjusted to 5.5. Three levels of citric acid were examined: 0.05%, 0.10%, and 0.15% (w/v).

Inoculum preparation

The inoculum was prepared from a 24-h-old culture and the culture was grown aseptically in a 250-mL Erlenmeyer flask, containing 50 mL of YM medium, shaken at 200 rpm, and exposed to 500 lux illumination, provided by cool white fluorescent lamps. This primary inoculum was used directly for the flask culture but was passed twice to attain the volume that was required to inoculate the batch fermentor's 1.2-L working volume. All cultures were inoculated at 20 vol %.

Flask cultures

Cultures were grown aseptically in 250-mL Erlenmeyer flasks that contained 40 mL CW medium and were inoculated with 10 mL yeast culture. The cultures were incubated on a gyrating shaker at 200 rpm and exposed to 500 lux illumination, provided by cool white fluorescent lamps. The temperature was maintained at 25°C for 120 h.

Batch fermentation culture

In the batch culture, the precultured broth (300 mL) was used to inoculate a 2-L stirred fermentor (Bioengineering model Ultramat 23) that contained 1.2 L of modified basal medium. The initial pH was adjusted to 5.5. The dissolved oxygen (DO) concentration was monitored with a DO electrode. The agitation speed was maintained at 540 rpm. Illumination (500 lux) was provided by cool white fluorescent lamps. The cultures were maintained at 25°C for 120 h.

Analytical procedures

Biomass

Cell growth was measured as turbidity at 660 nm and dry cell mass.

Reducing sugars

Reducing sugar (glucose) concentrations in the culture medium were determined by HPLC with a refractive index detector and stainless steel Carbosep CHO-802-CA, particle size 9 µm (7.8 x 300 nm). The eluting solvent was deionized water at a flow rate of 0.5 mL/min (AOAC., 2000; Fabio *et al.*, 2005; Jorge *et al.*, 2004).

The percentage of residual sugar was calculated as (residual sugar concentration) / (initial sugar concentration) x 100. The glucose in the media was used to calibrate the reducing sugar assay.

Astaxanthin content

The cell biomass was harvested by centrifugation at 3000 x g for 15 min at 4°C, washed twice with

deionized water, and recentrifuged. The harvested cells were freeze-dried, and the pellets were resuspended in acetone and homogenized using glass beads and a vortex mixer (Britton *et al.*, 1995; Jian-Ping *et al.*, 1997), after which they were subjected to bath sonication until the solution turned orange.

Astaxanthin was extracted by 2-phase liquid extraction, in which the homogenized acetone cell suspension was mixed with diethyl ether in a separation funnel. The extraction steps were repeated until the cell pellets became colorless. Then, the ether phases were pooled and concentrated on a rotary evaporator under reduced pressure. The resulting material was dissolved in 2 mL nitrogen-purged HPLC eluting solvent (methanol: water: hexane, 95:4:1 v/v) and stored in 2-mL vials, which were also purged of nitrogen gas.

Astaxanthin was measured by HPLC (Waters 2690) on a photodiode array detector and a stainless steel Nova-Pack C-18 reversed-phase column (4 x 250 nm). The flow rate of the eluting solvent was 0.5 ml/min. Synthetic astaxanthin (Hoffman-La Roche) was used as an external standard (Jian-Ping *et al.*, 1997).

The astaxanthin content was measured 48 h after inoculation until the fermentation was stopped at 5 days (120 h).

Morphological characteristics and distribution of population

Total cell number

The total cell number was determined using a hemocytometer (5x5 counting grid) on a phase contrast microscope and a 40x objective lens. Nine of the 25 grids were selected randomly for enumeration.

Population dynamics

The population dynamics of the yeast was investigated using a phase contrast microscope with a wet mount sample. Fifty images per time point were taken to analyze cell volume and shape using the ellipsoid model in Image-Pro Plus, version 5.0 (Media Cybernetics, Inc.). The yeast cells were classified into 5 groups according to size and shape (Figure 1 and Table 1).

Result and Discussion

Cell growth, sugar consumption, and astaxanthin production under limited aeration (shaken flask culture)

In general, in a limited aeration environment, the patterns of cell growth, sugar assimilation, and astaxanthin accumulation by *Phaffia rhodozyma* TISTR 5730 in modified basal medium were similar in all experiments. Cell density was essentially

constant during the first 20 h of fermentation, despite a steady increase in the total cell mass, which was concomitant with a monotonic decrease in sugar, indicating that during the first 20 h, cell growth was attributed primarily to increases in cell size.

After 120 h of culture, the final cell mass for all experiments was approximately 10 g/L, whereas sugar was consumed completely (Table 2 and Figure 2). We infer from the proportion of subpopulations that the first budding period began after 20-35 h of culture and was completed by 45-55 h (Figure 3). Similarly, the second budding cycle started immediately after the first stage completed, ceasing at approximately 84 h. Each budding cycle took approximately 35–45 h in shaken flask cultures.

The majority of astaxanthin was synthesized after 60 h of culture, when the population entered an early stationary phase. Apparently, at least 2 conditions must exist before the first astaxanthin peak develops—the population enters the stationary phase, and the sugar concentration becomes limited but is sufficient to support the ensuing budding cycle.

Astaxanthin content did not undergo a monotonic increase (Figure 3). Instead, the astaxanthin curve was cyclic, indicating that astaxanthin production was not specifically growth-associated. Further, because astaxanthin is an intracellular product and the biomass content in the stationary phase does not change significantly, the generation and consumption of astaxanthin during the budding process imply that astaxanthin regulates the budding cycle and is transformed or consumed when it is no longer required.


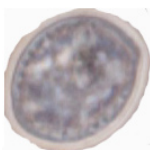
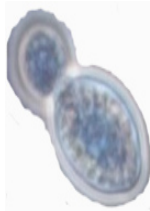


The first astaxanthin peak (corresponding to the second budding cycle) in most shaken flask cultures appeared at approximately 84 h. After bottoming at approximately 100 h, the astaxanthin content climbed. This finding requires verification, however, because the 120 h of total fermentation time was too short to observe whether the next peak actually existed.

Effect of citric acid on shaken flask cultures

On growth in CW medium that was supplemented with 0.05, 0.10, and 0.15% (w/v) citric acid (2.60, 5.20, and 7.80 mM), the cell mass yields were 11.00, 10.91, and 13.25 g dry cell/L, respectively (Figure 2a). At the end of the culture, the CW medium pH ranged between 5.50 and 6.50 (Figure 2b). In these media, the yeast had maximum specific growth rates of 0.17, 0.13, and 0.17 h⁻¹, respectively. Similarly, the yield coefficients were 0.24, 0.27, and 0.27 g dry cell weight/g glucose, respectively.

Citrate enhances astaxanthin synthesis in *Phaffia rhodozyma* NRRL Y-1092 (Flores-Cotera *et al.*,

Table 1. Classification of the yeast population

Group	Size (μm^3)	Budding	shape	Figure
A	0-200	No	Normal	
B	>200	No	Normal	
C	200-400	Yes	Normal	
D	>400	Yes	Normal	
E	NA	NA	Abnormal	

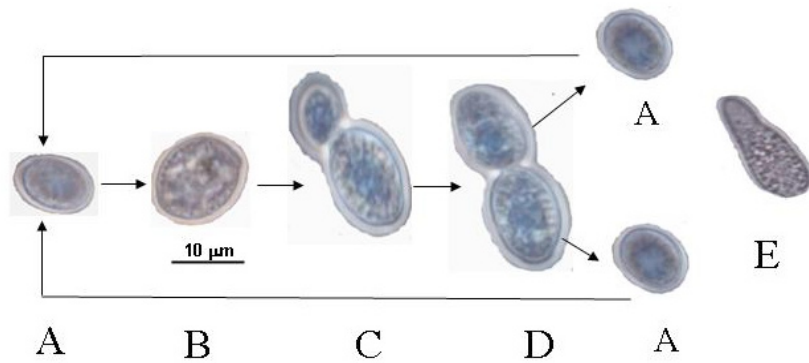
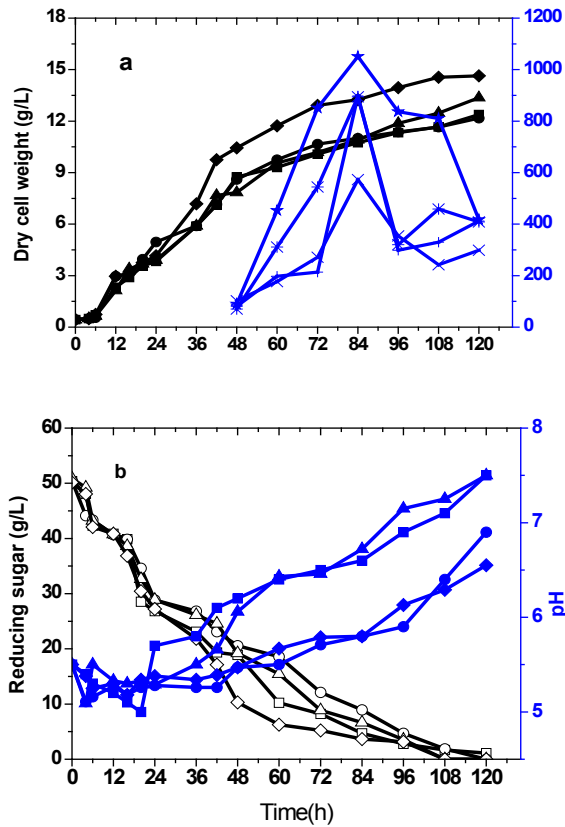


Figure 1. Life cycle of *Phaffia rhodozyma* TISTR 5730.



Control : ■ Biomass □ Reducing sugar × Astaxanthin ■ pH
 Citric acid 0.05% (w/v) : ● Biomass ○ Reducing sugar *Astaxanthin ● pH
 Citric acid 0.10% (w/v) : ▲ Biomass △ Reducing sugar + Astaxanthin ▲ pH
 Citric acid 0.15% (w/v) : ◆ Biomass ◇ Reducing sugar ★ Astaxanthin ◆ pH

Figure 2. Effect of citric acid on growth and astaxanthin production in *Phaffia rhodozyma* TISTR 5730, grown in CW medium at 25°C and 200 rpm under 500 lux of light intensity
 a) growth and astaxanthin production.
 b) sugar consumption and pH

Table 2. Effect of citric acid on biomass, yield coefficient, and astaxanthin content in *Phaffia rhodozyma* TISTR 5730 in CW medium flask and fermentor cultures at 84 h.

Medium	Biomass (g/L)	$Y_{x/s}$ (g astaxanthin/g glucose)	Astaxanthin content ($\mu\text{g/g}$ dry cell weight)
CW medium			
No addition precursor	9.98	0.23	315
Citric acid (% w/v) in flask			
0.05%	11.00	0.24	894
0.10%	10.91	0.27	898
0.15%	13.25	0.27	1051
Citric acid (% w/v) in fermentor			
Air 1 vvm	13.80	0.30	3880
Air 2 vvm	13.75	0.30	4593
Air 3 vvm	12.45	0.26	753

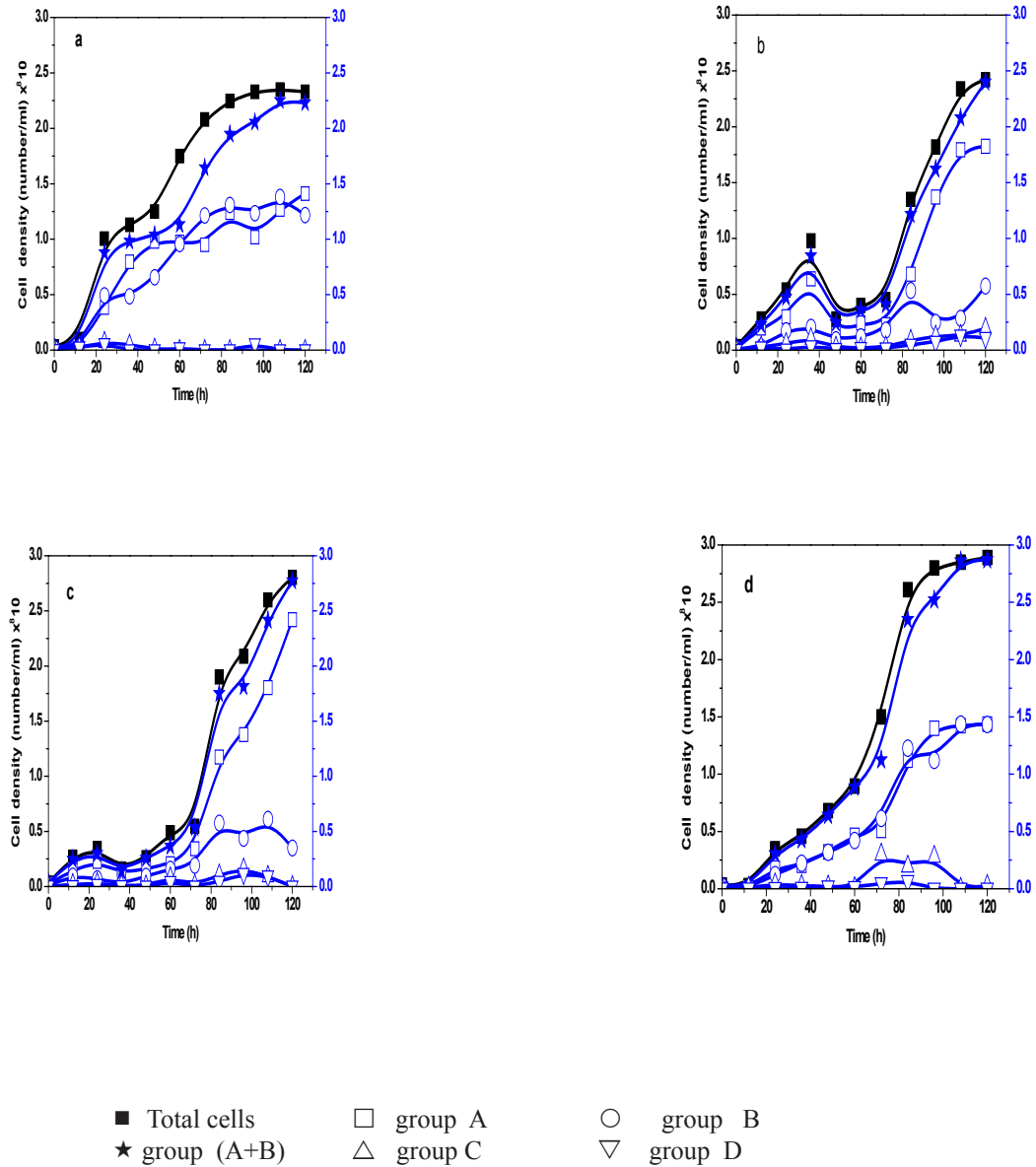


Figure 3. Population dynamics of *Phaffia rhodozyma* TISTR 5730 grown at 25°C and 200 rpm under 500 lux of light intensity in CW medium supplemented with citric acid
 a) without citric acid,
 b) 0.05% (w/v) citric acid,
 c) 0.10% (w/v) citric acid, and d) 0.15% (w/v) citric acid

2001). We observed substantial increases in growth and astaxanthin production by *Phaffia rhodozyma* TISTR 5730, as shown in Figure 2. CW medium with 0.15% (w/v) citric acid effected rapid sugar (glucose 50 g/L) uptake by the yeast, and the final dry cell mass was approximately 13.25 g/L, considerably higher than normal (approximately 10 g/L without citric supplementation).

At 60 h of culture, the 0.15% (w/v) CA batch contained the lowest amounts of residual sugar (approximately 5 g/L), which, nevertheless, was sufficient to support the ensuing budding cycle. As a result of the complex interaction between physicochemical factors, residual sugars, and the yeast's physiological state, the first astaxanthin peak corresponded to 1051 µg/g dry cell weight—more than twice that without the addition of citric acid (Sujarit, 2009 and Figure 2). Further, the reduction of astaxanthin content following the first peak was not substantial as in cultures that were supplemented with lower concentrations of acetic acid, ethanol, and citric acid.

After 60 h, the sugars became limited, the average cell size increased from 202 to 215 µm³, and the astaxanthin content increased sharply from 4593 µg/g dry cell weight at 60 h to 1051 µg/g dry cell weight at 84 h, after which the majority of budding cells initiated daughter-parent separation. After 84 h, the average cell size decreased from 210 µm³ to 178 µm³ at 96 h.

The daughter-parent separation not only reduced the average cell size but also was accompanied by a decrease in astaxanthin content to 836 µg/g dry cell weight at 96 h. The decrease in average cell size from 178 µm³ at 96 h to 137 µm³ at 120 h suggests that the average population began the next budding cycle after 96 h, resulting in a concomitant increase in astaxanthin content to a magnitude similar to the first peak.

Table 1 shows the highest yields of dry cell mass (13.25 g/L) and astaxanthin content (1,051 µg/g dry cell) of the aerated yeast cultures. Astaxanthin synthesis was enhanced in part because citrate diffused into mitochondria and blocked the assimilation of acetyl CoA into the TCA cycle, causing the accumulation of acetyl CoA, which was diverted to other pathways. Acetyl CoA further regulates the TCA cycle or activates carotenoids and thus astaxanthin synthesis.

Acetyl-CoA is generated in the mitochondria primarily from 2 sources—the pyruvate dehydrogenase (PDH) reaction and fatty acid oxidation. During the stage of fermentation in which nutrients were sufficiently available (i.e., the exponential phase), exogenous citrate caused acetyl CoA to accumulate,

which could have been shunted to fatty acid synthesis and astaxanthin synthesis. Thus, in this period, excess citrate not only increased astaxanthin synthesis but also competitively enhanced fatty acid synthesis.

At the onset of the stationary phase, the nutrients were diminished but the energy requirement of the cells remained high, forcing the oxidation of stored fatty acids to fulfill this requirement. Fatty acid oxidation was an additional source of acetyl CoA, which can be converted more readily into carotenoids. This explanation is consistent with the findings of Flores-Cotera *et al.* (2001). Similarly, Lotong *et al.* (1989) reported that coconut water is a better growth substrate than synthetic medium, most likely because coconut water contains many organic and inorganic nutrients, such as glucose, fructose, sucrose, mannitose, vitamin B complexes, and amino acids (Gimwood 1975).

Figure 3 shows the population dynamics for shaken flask cultures over time with regard to the population density for subpopulations and their relative importance to *Phaffia rhodozyma* TISTR 5730 that is grown in coconut water, supplemented with 0.0%, 0.05%, 0.10%, and 0.15% (w/v) citric acid.

Generally, in the first 40 h of fermentation, subpopulations C and D were sparsely populated, but their numbers increased exponentially. The ratio of subpopulations A (small cell size) to B (large cell size: with observable pigment inside) did not change appreciably during this initial period, implying that the budding process was rapid and occurred asynchronously.

When the sugar concentration was limited, however, at approximately 60 h onward, subpopulations C (budding, small cell size) and D (budding, large cells) persisted and fluctuated, and the entire population was relatively more synchronized until the experiment was terminated at 120 h.

The distribution of yeast sizes at 0.15% (w/v) (7.80 mM) citric acid over time also supports this explanation. This condition yielded the most robust enhancement in dry cell weight (13.25 g/L) and astaxanthin production (1015 µg/g dry cell weight) (Figure 9 a-h).

At the start of the culture, the average cell size was small (approx. 140 µm³). As discussed, the reproduction was asynchronous until approximately 60 h, after which residual sugars became limited. Asynchronous budding occurred when each cell in the population could grow independently without interference from other cells, during which the proportions of each subpopulation remained relatively constant while the total population increased. This

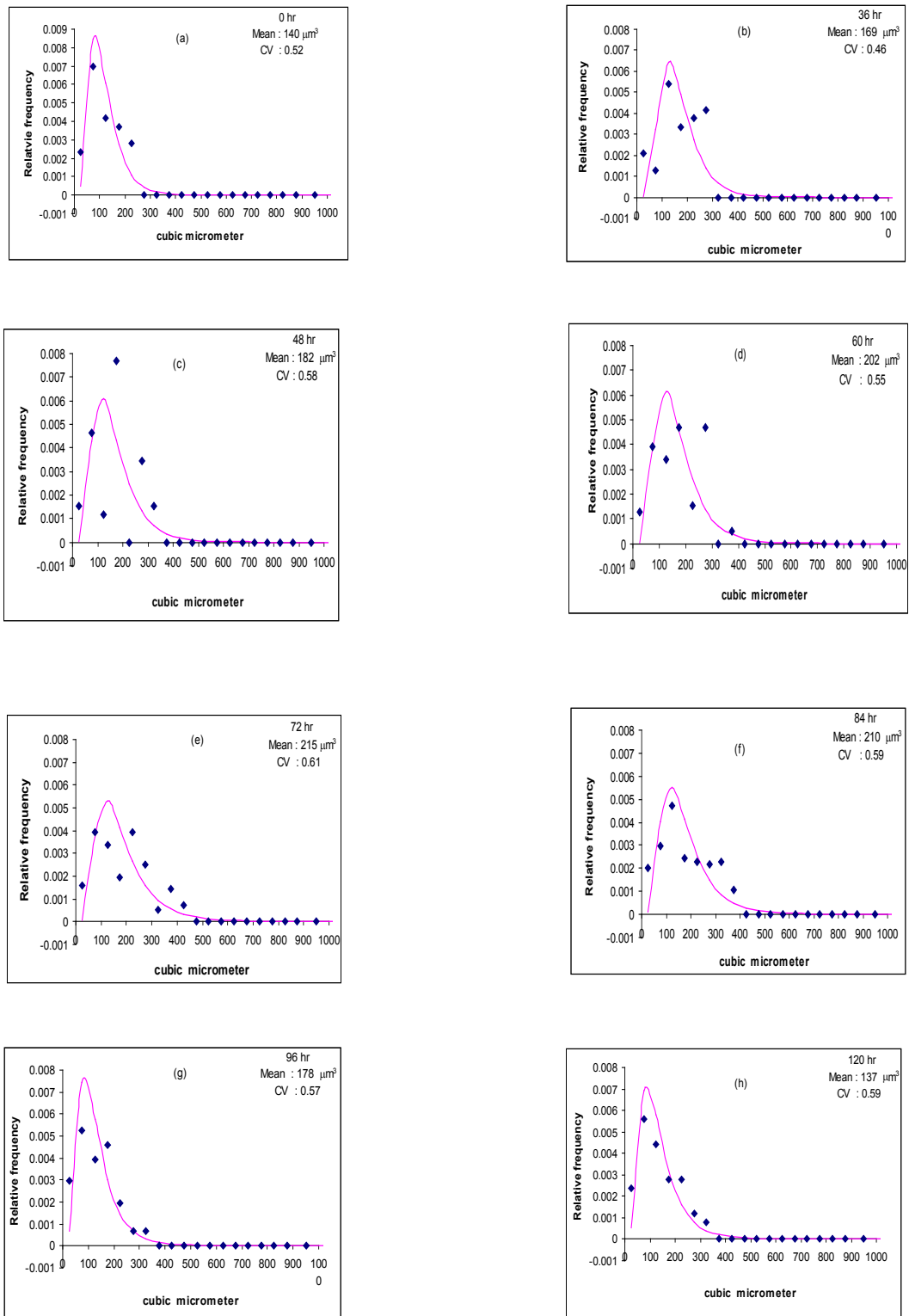


Figure 4. The distribution of mean cell size and coefficients of variation of *Phaffiarhodozyma* TISTR 5730 grown in CW medium with 0.15% (w/v) citric acid at 25°C and 200 rpm under 500 lux.

process was possible only when the nutrient was not limited and when the population was in log-phase.

After 60 h, however, when the population entered the stationary phase and the carbon source was limited, the entire population became relatively synchronized, as indicated by the generation of a log-normal curve. This phenomenon was clearly evident after 84 h of culture (Figure 4 f-h).

In the population dynamics study of *Phaffia rhodozyma* TISTR 5730 in CW medium with citric acid 0.05, 0.10, and 0.15% (w/v) (2.60, 5.20, and 7.80 mM), 7.80 mM citric acid effected the largest average cell size and CV (Figure 5 a and 5 b). For other concentrations of acetic acid, mean size and CV did not differ from each other (Table 1).

Group E was rarely observed, and Groups C and D occupied a small fraction of the entire population at any given time; Groups A and B were detected throughout the growth cycle in large numbers (Sujarit., 2009). Therefore, only the populations in Group A and B were considered significant. The data formed log-normal distributions, as shown in Figure 2.

Effect of citric acid in the fermentor

For the *Phaffia rhodozyma* TISTR 5730 culture in a fermentor that contained CW medium, sufficient aeration increased the initial growth rate and shortened the time to enter the stationary phase. Yet, high aeration rates did not increase the dry cell mass but shortened the time to approach the maximum value (approximately 14 g/L). After 60 h, residual sugars were undetectable, and the biomass level remained constant until the fermentation ceased at 120 h, in contrast to the MB medium, in which the residual sugars were not depleted, even after 84 h of culture, although the ultimate biomass concentration remained essentially the same.

Generally, cell growth is independent of aeration. Aeration, however, influenced astaxanthin production. Higher aeration rates between 1-2 vvm increase astaxanthin production, but an aeration rate of 3 vvm had a negative effect (Figure 6).

Effect of aeration rates on the population dynamics of yeast in a sufficiently aerated environment

In an aerated environment, the entire population tended to assume a log-normal distribution after 24 h compared with limited aeration conditions (Figure 7 a-f). Raising the aeration rate from 1 vvm to 2 vvm increased the cell density from 1.3×10^8 cells/mL to approximately 1.5×10^8 cells/mL, respectively. Excessive aeration at 3 vvm, however, adversely affected growth, decreasing the density to 1.2×10^8

cells/mL.

Subpopulations C and D were not significant at 2 vvm after 40 h of culture, whereas at 1 and 3 vvm, their presence was apparent throughout the entire culture. This finding suggests that at 2 vvm, budding occurred rapidly, leaving a small proportion of budding cells (subpopulations C and D) after 40 h.

Further, the cell densities of various subpopulations, especially A and B, fluctuated which was consistent with the changing stages in budding cycles. After 40 h, when the total cell count and sum of the A and B subpopulations were relatively constant, increases in A and decreases in B occurred simultaneously and vice versa. Thus, the amplitudes of the population densities of subpopulation A or B in the stationary phase and their reproductive cycle periods indicate the strength of budding.

Based on the magnitude of the fluctuation in the subpopulation, it was evident that an aeration rate of 2 vvm yielded the most robust budding, whereas 1 and 3 vvm effected lower and comparable budding, respectively. These findings imply that fluctuations in astaxanthin content in the stationary phase are tightly associated with the average physiological state of the yeast population and the strength of budding.

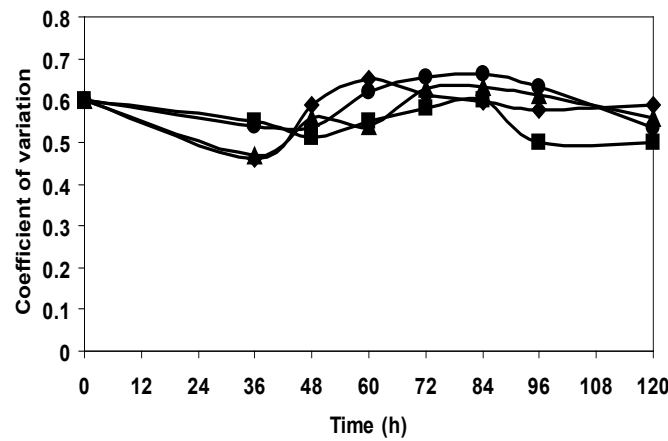
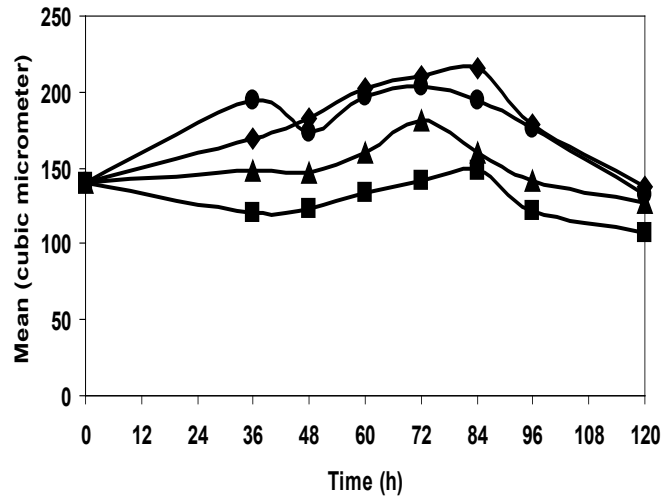
In addition, there was a consistent relationship between the densities of subpopulation B (larger, older cells before budding) and the astaxanthin peaks. At 1 vvm, the first and the second astaxanthin peaks occurred at 72 and 96 h, respectively (Figure 8), corresponding to the 2 successive peaks of subpopulation B at 72 and 96 h. Similar patterns observed at 2 vvm (Figure 8).

Yet, the astaxanthin curve was rather flat at 3 vvm; thus, the relationship between subpopulation B and astaxanthin content was unclear.

Conclusions

Coconut water is an efficient medium for astaxanthin production by the wild-type strain *P. rhodozyma* TISTR 5730. In the range of conditions that we investigated, citric acid was effective in enhancing astaxanthin production, especially in a sufficiently aerated environment.

The highest astaxanthin concentration (4593 $\mu\text{g/g}$ dry cell, or 919 $\mu\text{g/g}$ dry cell per day) was obtained with 0.15% (w/v) (7.80 mM) citric acid and an aeration rate of 2 vvm. This yield was over 2-fold higher than the optimal output (373 $\mu\text{g/g}$ -yeast per day), as reported by Domínguez-Bocanegra *et al.* (2007) for mutant *P. rhodozyma* NRRL-10921 (R1) that was grown on coconut milk. In fact, our value is conservative, because only approximately 60 h (not



■ Control, ● Citric acid 0.05%, ▲ Citric acid 0.10%, ◆ Citric acid 0.15%

Figure 5. Effect of citric acid on mean cell size and coefficient of variation in *Phaffia rhodozyma* TISTR 5730 grown in CW medium at 25°C and 200 rpm 500 lux
a) mean cell size and b) coefficient of variation

120 h) was required to generate the first astaxanthin peak.

These results are promising for several reasons. First, although a wild-type strain was used, its productivity was greater than double the highest yield in the literature, in which sufficient (but not excessive) aeration was applied. Also, coconut water is a cheap raw material and an abundant industrial and domestic waste product in many tropical countries that has never been exploited efficiently. Finally, we chose to optimize culture temperatures for tropical regions where the raw material is inexpensive, thus lowering the costs of production.

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